UltraRapid Communication

Spatiotemporal Characteristics of Junctional and Nonjunctional Focal Ca\(^{2+}\) Release in Rat Atrial Myocytes

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Abstract—Atrial myocytes have two functionally separate Ca\(^{2+}\) release sites: those in peripheral sarcoplasmic reticulum (SR) adjacent to the Ca\(^{2+}\) channels of surface membrane and those in central SR not associated with Ca\(^{2+}\) channels. Recently, we have reported on the gating of these two different Ca\(^{2+}\) release sites by Ca\(^{2+}\) current. In the present study, we report on the spatiotemporal properties of focal Ca\(^{2+}\) releases (sparks) occurring spontaneously in central and peripheral sites of voltage-clamped atrial myocytes, using rapid 2-dimensional (2-D) confocal Ca\(^{2+}\) imaging. Peripheral and central sparks were similar in size and release time (≈300 000 Ca\(^{2+}\) ions for ≈12 ms), but significantly larger and longer than ventricular sparks. Both sites were resistant to Cd\(^{2+}\) and inhibited by ryanodine. Peripheral sparks were brighter and flattened against surface membrane, had ≈5-fold higher frequency, ≈2 times faster diffusion coefficient, and dissipated abruptly. Central sparks, in contrast, occurred less frequently, were elongated along the cellular longitudinal axis, and dissipated slowly. Compound sparks (composed of 2 to 5 unitary focal releases) aligned longitudinally and occurred more frequently at the center. The diversity of peripheral and central sparks with respect to shape, frequency, and speed of spatial development and decay is consistent with regional ultrastructural heterogeneity of SR. The retarded dissipation of central atrial sparks, and high prevalence of compound sparks in cell center may be critical in facilitating the propagation of Ca\(^{2+}\) waves in atrial myocytes lacking t-tubular system and provide the atrial myocytes with functional Ca\(^{2+}\) signaling diversity. The full text of this article is available at http://www.circresaha.org. (Circ Res. 2003;92:e1-e11.)

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Contraction of mammalian ventricular myocytes is controlled by a sequence of events (E-C coupling) that includes Ca\(^{2+}\) current (\(I_{Ca}\))-gated opening of Ca\(^{2+}\) release channels (RyRs) and the release of Ca\(^{2+}\) from the sarcoplasmic reticulum (SR).\(^{1-5}\) The close (≈12 nm) proximity of the RyRs and Ca\(^{2+}\) channels (DHPRs) in dyadic junctions of transverse (t-) tubules provides for the privileged Ca\(^{2+}\) cross signaling between the two sets of proteins.\(^{8,9}\) Confocal imaging of ventricular myocytes reveals the presence of focal Ca\(^{2+}\) transients (Ca\(^{2+}\) sparks)\(^{10}\) that are triggered either spontaneously or by \(I_{Ca}\).\(^{10-13}\) The unitary property of Ca\(^{2+}\) sparks, but not their frequency, appears to be independent of \(I_{Ca}\) and voltage, indicating that they may represent the elementary event underlying cardiac E-C coupling.\(^{5,10-15}\)

In cell types lacking t-tubules (atrial cells, Purkinje cells, avian ventricular cells) RyRs are found not only as peripheral couplings, but also in nonjunctional or corbular SR throughout the central regions of the cell\(^{16-20}\) with no direct association with DHPRs.\(^{16}\) The mode of gating and the contribution of this set of RyRs to cytosolic rise of Ca\(^{2+}\) remains uncertain. It has been suggested that non-junctional RyRs might be activated by diffusion of Ca\(^{2+}\) from neighboring Ca\(^{2+}\) release sites.\(^{20-24}\) Such a mechanism is likely to be slow and regenerative,\(^{25}\) because it would lack the features that are associated with peripheral or dyadic junctions and the associated negative feedback mechanism.\(^{5,8,26}\) Using 2-dimensional (2-D) confocal imaging in voltage-clamped rat atrial myocytes dialyzed with different concentration of Ca\(^{2+}\) buffer (Fluo-3, EGTA), we have recently shown that the peripheral focal Ca\(^{2+}\)-release was directly activated by \(I_{Ca}\), while the magnitude of Ca\(^{2+}\) release from the central SR was a cooperative function of peripheral Ca\(^{2+}\) release and dependent on a sequential salutary Ca\(^{2+}\) propagating mechanism.\(^{24}\) Although central and peripheral sparks have been previously described,\(^{19,20,23,24,27,28}\) little is known of their spatiotemporal properties or comparative characteristics. In the present study, we quantify and characterize atrial Ca\(^{2+}\) sparks originating from RyRs associated with DHPRs and those not in close proximity of the L-type Ca\(^{2+}\) channels using rapid (240 Hz) 2-D confocal imaging. Our study shows that peripheral sparks occur more frequently, develop with higher rate of spatial growth, and dissipate faster, but have similar average Ca\(^{2+}\) release flux as those of the central sparks. The central sparks were significantly retarded in their spatial decline, which may facilitate the Ca\(^{2+}\) diffu-
sion–dependent Ca\textsuperscript{2+} release mechanism, in the absence of close proximity of Ca\textsuperscript{2+} channels.

Materials and Methods

Whole-Cell Patch Clamp

The surgical removal of hearts of Wistar WKY rats (250 to 280 g, 32 animals; Charles River Laboratories, Wilmington, Mass) was performed in accordance with institutional and national ethical guidelines. Atrial myocytes were dispersed enzymatically\textsuperscript{24} and电压–clamped in the whole-cell configuration\textsuperscript{29} using glass micropipets (tip resistance \( \approx 3 \text{ M}\Omega \)) containing (in mmol/L) 110 CsCl, 110 Aspartic acid, 5 NaCl, 20 TEA-Cl, 20 HEPES, 0.2 Ca\textsuperscript{2+} cAMP, 1 K-fluo-3 (Molecular Probes Inc.), and 2 EGTA, with the pH adjusted to 7.2 with CsOH. Membrane currents were meaured as described previously.\textsuperscript{24} cAMP (200 \( \mu \text{M} \)) was used to prevent rundown of \( I_o \) during long periods of cell dialysis and to accomplish Ca\textsuperscript{2+} loading of the SR in spite of the added Ca\textsuperscript{2+} buffers.\textsuperscript{8,9} Large concentrations of the fluorescent Ca\textsuperscript{2+} indicator fluo-3 in combination with EGTA were used to sharpen the images of focal Ca\textsuperscript{2+} releases by limiting the diffusion distance of free Ca\textsuperscript{2+}.\textsuperscript{10} Hence, so that spatial and temporal resolutions were determined primarily by the less-mobile Ca\textsuperscript{2+}–bound fluo-3.\textsuperscript{31} The external solution contained (in mmol/L) 137 NaCl, 5.4 KCl, 10 CaCl\textsubscript{2}, 10 HEPES, 1 MgCl\textsubscript{2}, 10 Glucose, buffered to pH 7.4 with NaOH. Alternate switching between various external solutions was accomplished rapidly (\( <50 \text{ ms} \)) using an electronically controlled multibarrel puffing system.\textsuperscript{4} All experiments were performed at room temperature (22 to 24°C).

Two-Dimensional Confocal Ca\textsuperscript{2+} Imaging

Cells were loaded with the Ca\textsuperscript{2+} indicator dye fluo-3 via the patch pipette (see previous section) and were imaged using a Noran Odyssey XL 2-D laser scanning confocal microscopy system (Noran Instruments) as previously described.\textsuperscript{11,12,14,32} Images were scanned at 30 Hz (Figures 1 and 3) or 240 Hz (Figures 2, 4, 5, 6, and 7) depending on the experimental objectives. Data were acquired by the Intervision program in a workstation computer (IRIX-operating system, Indy, Silicon Graphics). The measured point-spread function of the confocal microscope was approximately a truncated cylinder 0.3 \( \mu \text{m} \) in radius and 0.8 \( \mu \text{m} \) in length. Fluorescence measurement was performed following 6 to 7 minutes after rupture of the membrane with the patch pipette.\textsuperscript{32} Recording of spontaneous sparks was preceded by a train of ten 100-ms conditioning pulses applied from \(-90 \) to \(-10 \text{ mV} \) at 0.1 Hz in 5 mmol/L Ca\textsuperscript{2+}–containing external solution. This procedure produced Ca\textsuperscript{2+}–gated Ca\textsuperscript{2+} transients of similar magnitude and kinetics, indicating stable Ca\textsuperscript{2+} loading of the SR in the presence of Ca\textsuperscript{2+} buffers. We confirmed that this approach maintained the Ca\textsuperscript{2+} load of the SR by measuring caffeine-triggered Ca\textsuperscript{2+} release (see Figure 2).

Image Analysis

Focal Ca\textsuperscript{2+} releases were identified by a computerized algorithm. This algorithm\textsuperscript{32} screened records based on their signal-to-noise ratio and identified local maxima in the interior of cell by means of a center–minus-surround detection kernel (inset in Figure 7E), which consists of pixels (0.207 \( \mu \text{m} \) spacing) approximating a central positively weighted disc (radius 0.6 \( \mu \text{m} \)) and a concentric negatively weighted ring (radius 1 to 1.4 \( \mu \text{m} \)). To detect local maxima underneath the cell membrane, the detection kernel was modified by excluding the extracellular space from the negatively weighted ring (inset in Figure 7B), and then aligned parallel with the direction of the cell edge. This procedure allowed to resolve sparks positioned close in time and produce records with local maxima that were identified as focal Ca\textsuperscript{2+} releases if they could be followed from frame to frame (at 240 Hz; often increasing in intensity, then spreading and fading). The focal Ca\textsuperscript{2+} releases that had one stationary center for their growth and decay were then subjected to Gaussian approximations,\textsuperscript{32} which allowed detailed morphometry and routine measurements of the amplitude, width, and equivalent area of sparks originating from the peripheral and central regions. Individual focal Ca\textsuperscript{2+} release signal in single frames were approximated in a restricted area (30x30 pixels) by a Gaussian function defined in space\textsuperscript{4,25,27} by its center (\( x_o, y_o \)), the central increase in fluorescence (\( \Delta F \)), and its standard deviation (\( \sigma \)), giving a total fluorescence:

\[
F = F_o + \Delta F \exp\left(-\frac{(x-x_o)^2+(y-y_o)^2}{2\sigma^2}\right),
\]

where \( F_o \) is the background fluorescence measured in the restricted area. The fitted values of \( \sigma \) were converted to full-width at half maximum amplitude (FWHM=2.3548\( \sigma \)). The size of the spark calculated as the equivalent area of background fluorescence, \( A \), was derived from the following equation:

\[
A = \frac{1}{2} \pi \sigma^2 \times (\Delta F/F_o).
\]

For peripheral Ca\textsuperscript{2+} sparks, the Gaussian distribution was approximated by the data obtained within a straight line defining the edge of the cell (Figure 4Ad), and the effective area was calculated accordingly (\( A_{\text{ew}} \)) using the error function (\( Erf \)) to compensate for the tail of the Gaussian distribution extending outside of the edge of the cell:

\[
A_{\text{ew}} = A \times \frac{1+\text{Erf}(\frac{\sigma}{\sqrt{2}})}{2},
\]

where \( l \) is the distance of the center of the Gaussian distribution to the edge. Central sparks were elongated (see Figure 4Ba) and could be approximated better by the following expression:

\[
F = F_o + \Delta F \exp\left(-\frac{X^2}{2\sigma^2} - \frac{Y^2}{2\sigma_1^2}\right),
\]

where the local coordinates are rotated by the angle \( \theta \) (\( X = \text{cos}(\theta) \times Y - \text{sin}(\theta) \), \( Y = \text{sin}(\theta) \times X + \text{cos}(\theta) \)) to be aligned with long and short axes where the standard deviations (\( \sigma \) and \( \sigma_1 \)) are, respectively, expanded and compressed by the factor \( d \) (eccentricity, \( d=1 \text{-} d \)).\textsuperscript{38} We measured the angle of the longitudinal direction of the spark, \( \theta \), relative to the longitudinal axis of the cell, \( \theta_{\text{ece}} \), in such a way that the absolute difference, \( |\theta - \theta_{\text{ece}}| \), is in the rage from 0 to 90°. This means that 0° corresponds to the exact alignment of the long axis of sparks in the direction of the cell, 90° corresponds to the exact alignment in the transverse direction of the cell, and 45° corresponds to random orientation. The expressions for the FWHM and area (Equation 2) of the spark remain unchanged in terms of the mean standard deviation \( \sigma \) (\( \sqrt{d \times (\sigma_1 \times \sigma)} \)).

We have routinely measured the quality of the fit by calculating the standard deviation (SD, root-mean-square) between the raw data and the fitted Gaussian distribution. We compared these SD values to an estimate of the noise (individual pixels as compared with the mean their 8 nearest neighbors) and found that they agree within \( \pm 20\% \) in \( \pm 85\% \) of analyzed sparks.

To calculate frequency of sparks, central and peripheral areas were estimated by considering the major part of cell image excluding both ends of the cell, recorded at 30 Hz (see Figures 1A, 3A, and 3C), to be a square. The area up to 1.5 \( \mu \text{m} \) immediately underneath the cell membrane was denoted as the peripheral domain (peripheral area=2x1.5 \( \mu \text{m} \times \text{image length} \), \( \mu \text{m} \)). The central area was then measured as a difference between the whole image area and the peripheral area.

Only cells with little leak current and clear edges were included in the final analysis of the results. Statistical comparisons were performed using Student’s \( t \) test. Differences were considered to be statistically significant to a level of \( P<0.05 \). Numerical results are given as mean±SEM.

Results

Spontaneous Focal Ca\textsuperscript{2+} Releases in the Periphery and Center

Figure 1A shows 2-dimensional confocal images of spontaneous Ca\textsuperscript{2+} sparks in a rat atrial myocyte voltage clamped at \( -80 \text{ mV} \). Imaging in extended periods (2 seconds) at 30 frames/s were used to monitor the major part of the cell and compensate for the
sparsity of spontaneously occurring sparks. Spontaneous Ca\textsuperscript{2+} sparks (arrowheads) were detected in both the periphery (images 45 and 46) and center (images 47 and 49) of myocyte. At imaging speeds of 30 Hz each, Ca\textsuperscript{2+} spark was clearly seen for only a single frame. In 22 cells (12 hearts) examined, we found spontaneous sparks occurrence at rates of 2.5±0.9/s in the peripheral (220±19 μm\textsuperscript{2}) and 2.6±0.6/s in the central regions (1008±261 μm\textsuperscript{2}). Thus, the frequency of occurrence of sparks, normalized for the myocyte area (sparks/[10\textsuperscript{-5} μm\textsuperscript{2} second]), was significantly higher (≈5 times; \( P<0.01 \)) in the periphery (11.2±1.25) compared with the center (2.44±0.48) (Figure 1C).

To examine if the regional spark frequency differences was caused by differential Ca\textsuperscript{2+} loading of the SR,\textsuperscript{2,33} local Ca\textsuperscript{2+} transients were measured in response to “puffs” of caffeine (10 mmol/L) after the conditioning pulses (see Materials and Methods). Confocal images recorded at 240 Hz show that the shape (rise and decay times) and magnitude of caffeine-induced Ca\textsuperscript{2+} release was not significantly different in the periphery and center of the cell (Figures 2A, 2C, and 2D). Quantification of Ca\textsuperscript{2+} release in the periphery and center suggests an increase in fluorescence (ΔF/F\textsubscript{o}) of 3.49±0.39 in the periphery and 3.32±0.43 in the center (n=19, \( P>0.05 \); Figure 2D), and a velocity of release (time-to-peak) of 216±25 ms in the periphery versus 220±31 ms in the center (n=19, \( P>0.05 \)). The rise in Ca\textsuperscript{2+} concentrations, as expected, activated only a brief and small Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange current (\( V_{h}=-80 \) mV),\textsuperscript{34} in part because cytosolic Ca\textsuperscript{2+} is well buffered by 2 mmol/L EGTA and 1 mmol/L fluo-3 (Figure 2B).\textsuperscript{9} The data suggest that SR Ca\textsuperscript{2+} loading is uniform in different regions of the myocytes.

To examine if the higher spark frequency in the cell periphery was related to the activity of Ca\textsuperscript{2+} channels, preferentially associated with junctional release sites,\textsuperscript{35} cadmium (Cd\textsuperscript{2+}) was used to block the Ca\textsuperscript{2+} channels. Figure 3A compares sequential images of a myocyte clamped at –80 mV before and after exposure to 50 μmol/L Cd\textsuperscript{2+}. Full suppression of \( I_{Ca} \) (activated at 0 mV) failed to alter the spontaneous Ca\textsuperscript{2+} spark frequency in the periphery or center of the cells (Figure 3A and 3B). Spark frequency (events/[10\textsuperscript{3} μm\textsuperscript{2} second]) was 11.8±1.2 in the periphery and 2.4±0.2 in the center in control, and 13.2±1.6 in the periphery and 2.8±0.2 in the center in the presence of Cd\textsuperscript{2+} (\( P>0.05 \), n=6). On the other hand, 10 μmol/L ryanodine (known to block the RyRs by causing long-lasting subconductance states)\textsuperscript{36} completely suppressed spontaneous sparks occurrences in ≥1 minute (n=9). At times <30 seconds after the application of ryanodine, when a submaximal inhibitory effect was achieved, the regional ratio of spark occurrences of peripheral and central release remained constant (data not shown). These results are consistent with previous reports that the spontaneous Ca\textsuperscript{2+} sparks in resting cardiac cells are generated by RyRs independent of Ca\textsuperscript{2+} channel activity.\textsuperscript{10}

2-D Spatiotemporal Properties of Peripheral and Central Spark

Figure 4 compares the unitary properties of representative spontaneous Ca\textsuperscript{2+} sparks measured at 240 Hz at the periphery (Figure 4A) and center (Figure 4B) of an atrial cell. As expected, peripheral sparks were flattened against the surface membrane (Figure 4Aa, dashed line). Such releases were first analyzed by measuring the fluorescence intensity at the spark center versus the surrounding areas with increasing distances from the center, excluding the space outside of the membrane (Figure 4Ab, inset). Compared with the normalized peak fluorescence at the center of the mass (red, Figure 4Ab, \( \Delta F/F_{o}=2.8 \)), the more distant signals from the center were reduced and delayed in amplitude (Figure 4Ab, gray and light gray), consistent with Ca\textsuperscript{2+} diffusion. Figure 4Ac shows the difference between the normalized signals of the center of the

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**Figure 1.** Spontaneous Ca\textsuperscript{2+} sparks in the periphery and center of rat atrial myocytes. A, Sequential 2-D confocal Ca\textsuperscript{2+} images measured at 30 Hz (at \( V_{h}=-80 \) mV) during periods indicated by box in B. Image \( F_{ref} \) shows average fluorescence, revealing the clear outline of the myocyte. Numbered images, 45 to 52, are sequential frames measured differentially as the increase in fluorescence (ΔF) relative to the average fluorescence. In the differential measurements of fluorescence, the outline of the cell is seen as only variations in the noise, but Ca\textsuperscript{2+} sparks appear clearly (arrowheads). B, Time course of the occurrences of peripheral (PERI) and central (CEN) sparks in cell shown partly in A. C, Average frequency of peripheral and central sparks measured in 22 atrial cells. **\( P<0.01 \), significantly different from values measured in the periphery.
mass and the area immediately adjacent to it (inner surround). The duration of Ca\(^{2+}\) spark was measured as the time at half-maximal amplitude (FDHM, \(\approx 15\) ms) of the normalized center-minus-surround signal, with temporal resolution of \(\approx 2\) ms. Figure 4B shows the spatial and temporal profiles of a Ca\(^{2+}\) spark monitored in the cell interior. Ca\(^{2+}\) signal intensity \((F/F_o)\) was highest at the center of the spark and decreased gradually with distance in the surrounding rings (Figure 4Bb) in a manner similar to peripheral sparks (Figure 4Ab). The FDHM of central spark, estimated by the similar method (center-minus-surround, Figure 4Ac), was \(\approx 22\) ms (Figure 4Bc).

2-D spark images were also analyzed in greater detail by approximating the individual focal Ca\(^{2+}\) release signal in single frames by a spatial Gaussian function\(^{32,37}\) (see Materials and Methods). For peripheral Ca\(^{2+}\) sparks, the Gaussian distribution was approximated by the data obtained within a straight line defining the edge of the cell (arrowheads, Figure 4Ad). We used this method (Equation 3) rather than eccentricity (Equation 4) to account for the flattening of the spark against the membrane. Figure 4Ae illustrates the time courses of normalized amplitude \((\Delta F/F_o)\), width (FWHM), and equivalent area \((\text{Area})\) of the peripheral Ca\(^{2+}\) spark measured using Gaussian analysis. The amplitude was measured as peak fluorescence of Gaussian curve \((\Delta F)\) normalized relative to average resting background fluorescence \((F_o)\) in the restricted area \((\approx 30 \times 30\) pixels; Figures 4Ad and 4Bd). Spark amplitude reached peak value of 1.75 within 8 ms and then decayed to 50% of its peak value in \(\approx 20\) ms. The peak amplitudes of sparks \((\Delta F/F_o)\) in the cell periphery measured by the Gaussian approximations were usually somewhat smaller than the peak Ca\(^{2+}\) signals obtained using pixel measurement at the center of release (compare Figures 4Ab and 4Ae). This difference arises primarily because the \(F_o\) used for normalization of the pixel measurements is so close to the edge that it is somewhat reduced as compared with the value that pertains to the Gaussian measured over an area extending away from the edge of the cell. The time course of spark width revealed a gradual enlargement over 4 to 12 ms that succeeds an initial rapid expansion phase \((<4\) ms) and is then followed by a maintained constant phase \((<12\) ms). The effective spark area \((A_{\text{corr}}, \mu m^2)\) increased progressively to a peak value of 5.3 \(\mu m^2\) in 12 ms, where the spark width was 1.75 \(\mu m\). This rising phase appeared to be terminated abruptly and followed by a rapid decline.

Central sparks often appeared elongated with roughly elliptical isofluorescence curves (Equation 4; Figures 4Ba and 4Bd). The eccentricity \((d/r-1)\),\(^8\) the degree of deviation of the spark from circular symmetry, measured in 36 central sparks, showed eccentricity \(>0.5\) (0.5 to 2) in 23, and of 0.25
Figure 3. Effects of Cd2+ and ryanodine on the Ca2+ spark occurrence. Top images of atrial myocytes in A and C show the average fluorescence intensity \( I \) obtained from 60 frames recorded at 30 Hz in cells voltage clamped at a holding potential \(-80 \text{ mV}\). Bottom images in A and C show four consecutive frames recorded at the times indicated by the dotted box in B and D that represent the time courses of spark occurrences in the periphery (PERI) and center (CEN). Left and right images in A and C show frames in the absence and presence of drug interventions, respectively. Notice that the Ca2+ sparks are brief so that at 30 Hz they are seen clearly only in single frames (arrowheads, A and C) and have, at most, a faint afterglow in the following frame.

Figure 4. Comparison of atrial myocytes in the presence of drug interventions, respectively. Notice that the Ca2+ sparks are brief so that at 30 Hz they are seen clearly only in single frames (arrowheads, A and C) and have, at most, a faint afterglow in the following frame.

Time Courses of Peripheral and Central Sparks

Figure 6 compares the mean time courses of amplitude, area, and width of 75 central and 42 peripheral sparks (29 cells), which showed single focal Ca2+ release site (see Materials and Methods) for \( \sim 30 \text{ ms} \) after their activation. The time to peak of central ("CEN") and peripheral ("PERI") Ca2+ spark amplitudes were similar (\( \sim 8 \text{ ms} \); Figure 6A). There was no difference in the decay time constant (\( \tau \)) of the spark amplitude between peripheral (12.0 \( \pm \) 1.1 ms) and central sparks (14.1 \( \pm \) 1.2 ms; \( P > 0.05 \); Figure 6A), but there was significant difference in the time course of their area (Figure 6B). Peripheral sparks dissipate immediately after reaching their peak with a clear break point. In sharp contrast, the area of central sparks reached its peak gradually and was maintained for 16 ms at its peak value, before its dissipation (Ca2+ removal in space). Efficacy of spark dissipation was evaluated as the slope (\( \mu \text{m}^2/\text{ms} \)) of the declining phase of the area (Figure 6B, inset). The mean declining slope of the peripheral sparks (-0.106 \( \pm \) 0.006) was significantly steeper than that of central sparks (-0.033 \( \pm \) 0.009; \( P < 0.001 \)).

To investigate whether the difference in the regional spark dissipation was caused by Ca2+ diffusion during the focal Ca2+ release, we evaluated diffusion coefficients of focal Ca2+ release using the signal averaged time courses of spark width in the cell periphery and center (Figure 6C). As predicted from Fick’s diffusion law, FWHM was expected to increase proportional with the square root of time, as shown.
by the linear fit of a straight line when plotting FWHM against time (Figure 6D). The dotted curves in Figure 6C were obtained by back-calculating the linear functions in Figure 6D. The slopes of the linear fits (periphery, 0.50 ± 0.022, n = 42; center, 0.27 ± 0.012, n = 75; P < 0.001; Figure 6D) could then be used to estimate the apparent diffusion coefficient, $D = \frac{C}{C_0} = \exp(-r^2/4Dt)$, where $C/C_0$ is the Ca$^{2+}$ concentration at a given time normalized to the Ca$^{2+}$ concentration at the center of the spark at the same time. Then, $D$ can be derived from, $D = \text{slope} \times (16\ln(C/C_0))$. In this approximation (dotted curve in Figure 6C), the FWHM at 0 ms gives a rough estimate of lateral point-spread-function (optical blurring of confocal microscopy, 0.7 to 1 μm) of the spark. Apparent diffusion coefficient ($D$, μm$^2$/s) for the fluo-3-bound Ca$^{2+}$ during spatial growth of spark was 25 at center and 45 at periphery, suggesting 1.8 times faster diffusion coefficients of the peripheral focal Ca$^{2+}$ releases. The spatial spread of peripheral sparks terminated at 12 ms as the spark width reached a value of approximately 2.5 μm (Figure 6C, top panel), whereas the central sparks progressively expanded for 25 ms as the FWHM reached about 2.7 μm (Figure 6C, top panel). These results suggest that the peripheral sparks initially grow significantly faster in space, but that diffusion alone is not sufficient to account for their time course.

Quantification of Focal Ca$^{2+}$ Releases

The size of Ca$^{2+}$ spark was estimated in terms of the number of Ca$^{2+}$ ions required to produce a spark. The following assumptions were made in this estimation: (1) the dissocia-
tion constant \((K_d)\) of the dye is substantially larger than the background \(Ca^{2+}\) concentration, \([Ca^{2+}]_{bg}\), (2) the total concentration of the fluo-3 within the cell, \([fluo]_t\), is the same as that of pipette solution, and (3) the point-spread-function in the vertical direction, \(h\), is large enough to cover most of the spark. Considering these assumptions the amount of \(Ca^{2+}\) \((S, \text{mole})\) associated with a spark can be calculated from the following:

\[
S = A \times h \times \frac{[fluo]}{K_d} \times [Ca^{2+}]_{bg}
\]

where \(A\) (or \(A_{cor}\) for peripheral sparks), the mean area of sparks \((\mu m^2)\), and \(h\) \((-0.8 \mu m)\) together determine the equivalent volume, \([fluo]/K_d\) is the buffering capacity \((1 \text{ mmol/L})/[0.4 \text{ mmol/L}] \approx 2500\), and \([Ca^{2+}]_{bg}\) is \(\approx 60 \text{ mmol/L}\). Computer simulations of \(Ca^{2+}\) binding by fluo-3 and EGTA suggested that, in these experiments, the time resolution of measurements of \(Ca^{2+}\) release depends mainly on diffusion of \(Ca^{2+}\) bound fluo-3; \(^{3,13}\) Thus, mean amounts of focal \(Ca^{2+}\) releases were similar in the periphery, \(336 \pm 300 \text{ 000 Ca}^{2+}\) ions, and in the center, \(318 \pm 300 \text{ 000 Ca}^{2+}\) ions, for \(\approx 12 \text{ ms release time. Therefore, the flux of Ca}^{2+}\)
release associated with a spark was \( \approx 4.5 \times 10^{-17} \) moles/s, corresponding to ionic currents of 8.7 pA.

**Compound Ca\(^{2+}\) Spark**

We occasionally found localized Ca\(^{2+}\) release composed of two to five unitary events occurring more or less synchronously, “compound spark,” occupying normally more than two sarcomeres. A total of 19 compound sparks were detected in 8 myocytes out of 33 cells, 14 of which were observed in the cell interior and 5 in the periphery. Considering the \( \approx 5 \) times large central region the occurrence of compound sparks normalized for area was \( \approx 2 \) times higher in the cell interior. Figure 7A and 7D display the morphology of representative compound sparks monitored in the periphery and center of the same atrial myocyte. Figure 7B and 7E show contrast-enhanced images of the 16-ms images in Figure 7A and 7D, respectively, using center-minus-surround kernel (inset), representing well-resolved focal maxima of the Ca\(^{2+}\) releases (numbered sites). The compound sparks occurring at the periphery were mostly composed of well-defined two or three smaller unitary release events (Figure 7B), whereas the central compound sparks had two to five unitary events (Figure 7E) distributed in a longitudinal direction of the cell (\( \approx 80\% \); see Figure 7F). The center-to-center separations between the subunits of peripheral and central compound sparks were 1.4 to 2 \( \mu \)m. The time courses of fluorescence, measured from the centers of each unitary release composing a compound spark, revealed similar rise and decay of the signals (Figure 7C and 7G).

**Discussion**

The rapid 2-D confocal imaging and analysis of Ca\(^{2+}\) sparks in atrial myocytes provides the first 2-dimensional comparison of peripheral and central Ca\(^{2+}\) sparks. We report three rather novel findings: (1) although the frequency of spontaneous unitary sparks was significantly higher in the periphery, the compound sparks appeared more prevalent in cell center; (2) peripheral and central sparks were similar in size and release time (\( \approx 300 \) 000 Ca\(^{2+}\) ions in \( \approx 12 \) ms), but were significantly larger than ventricular sparks (\( \approx 100 \) 000 Ca\(^{2+}\)).
ions in \(\approx 7\) ms);\(^{13,31}\) and (3) peripheral sparks were flattened against the surface membrane, grew faster in space, and dissipated abruptly and rapidly, as compared with central sparks, which were elongated along the cellular longitudinal axis and grew slower (\(\approx 2\) times) in space and were retarded in their dissipation. The diversity in unitary functional characteristics of atrial myocytes appears to be consistent with their ultrastructural regional differences\(^{16,17}\) and may provide the atrial myocyte with its unique E-C coupling characteristics.

**Faster Expansion and Dissipation of Peripheral Spark**

The dominant process (\(\approx 75\%)\) that determines the decline of \(\text{Ca}^{2+}\) spark is thought to be \(\text{Ca}^{2+}\) diffusion.\(^{40}\) In the present study, we found that the diffusion of released \(\text{Ca}^{2+}\) was about 2 times faster in the periphery than the center (\(D=45\) versus 25 \(\mu\)m\(^2\)/s). The faster \(\text{Ca}^{2+}\) diffusion in the peripheral sites may arise from the proximity of these sites to the cell membrane acting as a diffusion barrier, providing also for the flattening of the spark on one side (Figure 4A). Because the amount of \(\text{Ca}^{2+}\) released is similar in both the peripheral and central sparks (\(\approx 300\) 000 ions), the limited diffusion space in the peripheral junctions can account for the apparent faster \(\text{Ca}^{2+}\) diffusion constant. In fact, the surface membrane may serve as a reflective boundary to diffusion, which would effectively double the strength of the \(\text{Ca}^{2+}\) source, thereby increasing their apparent peak amplitudes and their rapid spatial expansion and dissipation. The exogenous \(\text{Ca}^{2+}\) buffers introduced in the myocytes are not likely to be responsible for the differential regulation of the time courses of peripheral and central sparks as \(\text{Ca}^{2+}\) buffering would be equally effective throughout the cytosol.\(^{13,31,32}\)

The enhancement of the activity of SR \(\text{Ca}^{2+}\)-ATPase has been reported to accelerate the decay of spark amplitude and reduce its width.\(^{40,41}\) It is possible therefore that the larger peak local concentration of \(\text{Ca}^{2+}\) at the peripheral sites could have activated more rapid \(\text{Ca}^{2+}\) removal by SR \(\text{Ca}^{2+}\)-ATPase in its close vicinity. In our study, however, the decay time constant of spark amplitude was not found to be significantly different between the two regions (Figure 6A). It is likely that the routine use of 200 \(\mu\)mol/L cAMP in the patch pipette masks such a mechanism. We also considered the contribution of \(\text{Na}^{+}\)-\(\text{Ca}^{2+}\) exchanger to the rapid dissipation of the peripheral sparks, as high levels of exchanger appear to be expressed in the interdyadic junctional membranes,\(^{30,42}\) making the exchanger favor its \(\text{Ca}^{2+}\) efflux mode following the release of \(\text{Ca}^{2+}\). Nevertheless, the role of \(\text{Na}^{+}\)-\(\text{Ca}^{2+}\) exchanger in the decay kinetics of \(\text{Ca}^{2+}\) spark amplitude remains somewhat controversial as inhibition of the exchanger in 0 \(\text{Na}^{+}\) and 0 \(\text{Ca}^{2+}\)-external solutions failed to alter the kinetics of decay of spark amplitude.\(^{40,43}\) We also failed to find a significant difference in the decay time constant of amplitude of sparks at cell periphery and center, thus minimizing the role of \(\text{Na}^{+}\)-\(\text{Ca}^{2+}\) exchanger in determining the kinetics of the spark amplitude (Figure 6A).

**Eccentricity of the Central Sparks**

We often found that central sparks were elongated in longitudinal direction (Figure 4Ba). The eccentricity of spark has been previously reported for ventricular myocytes (18\% longer longitudinally)\(^{44}\) and skeletal muscle.\(^{38,45}\) In skeletal muscle the eccentricity of spark appears to be aligned with the Z planes,\(^{38}\) especially when caffeine was used to sensitize the RyRs. We find that eccentricity in spark appearance is also present during the growing phase of the central spark (Figure 4Ba). In addition, the elongated sparks were somewhat symmetric in the Gaussian approximation, and the eccentricities were constant over time and not correlated with amplitude or width (data not shown). These observations suggest that the eccentricity is not caused by anisotropy in the diffusion or binding properties of \(\text{Ca}^{2+}\) pump or SR membranes, etc.\(^{41,44}\) but rather by the \(\text{Ca}^{2+}\) release from a spatially extended source, such as corbular SR with multiple channels.\(^{38}\) The elongation of central sparks in the longitudinal direction may be critical in often observed longitudinal orientation of spontaneous \(\text{Ca}^{2+}\) propagation waves in the cell, also seen for the central compound sparks (Figure 7D).

**Size and Frequency of Atrial \(\text{Ca}^{2+}\) Spark**

The amount of \(\text{Ca}^{2+}\) released during activation of peripheral and central sparks was similar (\(\approx 300\) 000 \(\text{Ca}^{2+}\) ions for \(\approx 12\) ms; equivalent to 8.7 pA). There were, however, significant variations in the duration (FDHM, Figure 5D) and area of sparks, with prominent variations mostly in the peak area of the central sparks compared with peripheral sparks (Figure 5C). This is consistent with the existing structural evidence on variation in the number of RyRs (feet) in the junctional and nonjunctional domains of the SR.\(^{7}\) In addition, both our data and the structural data support the idea that \(\text{Ca}^{2+}\) sparks result from the opening of multiple \(\text{Ca}^{2+}\) release channels clustered within discrete SR domains. Interestingly, the amount of the focal \(\text{Ca}^{2+}\) release in rat atrial myocytes was significantly larger than that measured in rat ventricular cells under similar conditions (\(\approx 100\) 000 \(\text{Ca}^{2+}\) ions for 7 ms, \(\approx 4.6\) pA).\(^{13,31}\) Although the larger \(\text{Ca}^{2+}\) spark finding is consistent with the atrial ultrastructural data,\(^{16,17}\) the higher extracellular \(\text{Ca}^{2+}\) (5 mmol/L) used in the present study could have contributed to the larger \(\text{Ca}^{2+}\) release. This possibility was evaluated in some experiment by using the same [\(\text{Ca}^{2+}\)]\(_o\). Consistently, we found atrial sparks to be significantly larger in duration and size than ventricular sparks in the rat heart (unpublished data, 2001).

The frequency of occurrence of spontaneous spark was significantly higher in the cell periphery than in its center (Figure 1C), as also reported for cat atrial myocytes.\(^{23}\) Because the \(\text{Ca}^{2+}\) content of the SR in peripheral and central domains is similar (Figure 2), and both sites are resistant to \(\text{Cd}^{2+}\) but sensitive to ryanodine (Figure 3), one possible explanation for the higher frequency of spontaneous sparks is that there is a higher density of dyadic couplings in the periphery as suggested from ultrastructural studies in rat, rabbit, and mouse.\(^{16,46,47}\) It has been also recently suggested that the higher peripheral spark frequency arises from the spatial differences in the RyR sensitivity to \(\text{Ca}^{2+}\). Such “eager sites,”\(^{719}\) found primarily in
subsarcolemmal regions, were suggested to have the highest frequency of spontaneous activity, not associated with SR Ca\(^{2+}\) load or Ca\(^{2+}\) influx.\(^{19}\)

Interestingly, in sharp contrast to the frequency of unitary sparks, the compound sparks appear to be more frequent in the cell interior. It is possible that the retarded dissipation of unitary central focal Ca\(^{2+}\) releases may facilitate the activation of neighboring release sites, leading to recruitment of the larger number of unitary events associated with the compound sparks (Figure 7D).

**Physiological Implication**

The present findings suggest that junctional and nonjuncti-
onal atrial Ca\(^{2+}\) sparks differ significantly with respect to their spatial and temporal characteristics and frequency. Highly frequent peripheral sparks may be related to large number of dyadic couplings or the “eager sites,” reported in rat atrial myocytes.\(^{19}\) The \(\approx 2\) times faster diffusion and larger amplitude of focal Ca\(^{2+}\) releases in the peripheral junctions may be critical in activation of central release sites not associated with the Ca\(^{2+}\) channels. It is likely that the focal Ca\(^{2+}\) releases in the periphery may efficiently regulate Ca\(^{2+}\)-dependent membrane conductance,\(^{3,14,16}\) and/or inositol 1,4,5-trisphosphate receptors associated with peripheral SR in response to hormonal interventions.\(^{28}\) The diversity of peripheral and central sparks with respect to shape, frequency, and speed of spatial development and decay is consistent with regional ultrastructural differences of SR. The significantly larger and longer atrial sparks, the retarded dissipation of central atrial sparks, and high prevalence of compound sparks in cell center maybe critical in facilitating the propagation of Ca\(^{2+}\) waves in atrial myocytes lacking t-tubular system. Such Ca\(^{2+}\) propagation waves in the cell interior have been observed during Ca\(^{2+}\) current activation of atrial myocytes lacking t-tubules.\(^{19,20,24}\)

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**References**


33. Györke I, Györke S. Regulation of the cardiac ryanodine receptor channel by luminal Ca\(^{2+}\)/H\(^+\) involves luminal Ca\(^{2+}\)/H\(^+\) sensing sites. *Biophys J*. 1998;75:2801–2810.


Spatiotemporal Characteristics of Junctional and Nonjunctional Focal Ca$^{2+}$ Release in Rat Atrial Myocytes
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