Theory and applications of geometric scaling of localized calcium release events

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CALCIUM IONS are actively sequestered by the intracellular organelles of almost all cells. The release of these ions into the cytoplasm through channels in the organelle membrane can take on a variety of forms dependent on the channels involved, the stimulus for release, buffering by the cytoplasm and organelle, and many other factors. Localized calcium release (LCR) remains restricted to within a few micrometers of the channels and lasts for a period of milliseconds to a few seconds. Since they were first imaged by Cheng et al. (8), such LCR events have been demonstrated in a large range of cell types, have been shown to be a major physiological mechanism, and have inspired a whole vocabulary, including the most common patterns of LCR—sparks and puffs (7). LCR events are imaged with fluorescence microscopy by loading the cytoplasm with a dye that fluoresces when it binds to calcium. A major concern has been to quantitatively relate this fluorescence signal to the underlying biophysical properties such as channel conductance, channel numbers, and diffusion parameters. Two commonly used metrics for this purpose are the maximum amplitude (MA) and the signal mass (SM). MA, as the name suggests, is simply the peak fluorescence of the LCR event. SM is the sum of LCR fluorescence over space (6, 14, 24, 30–32).

Allometry is the study of the relationship between two metrics across a population of objects similar in nature but of differing size. Specifically, if two metrics a and b have the relationship \( a = mb^n \) (a power law), if \( n = 1 \) the relationship is said to be isometric, but if \( n \neq 1 \) the relationship is allometric. Commonly this relationship is visualized by a double log plot where \( \log(a) = \log(m) + n\log(b) \). Both allometric and isometric relationships are common across the biological sciences and usually reflect underlying physical laws. It was of interest to us to see whether such a relationship applies to LCR events and would be valuable in understanding LCR. We measured LCR events from various cell types with three metrics—the mass above half-maximum amplitude (MHM), the area at half-maximum amplitude (AHM), and MA. Isometric scaling was apparent between these metrics. By simulation of LCR events with an analytic model it was found that this scaling could be used to estimate relative current and understand changes wrought by pharmacological manipulation.

METHODS

Data Collection

LCR events were recorded from three types of isolated cells—guinea pig fundus knurled cells, rabbit portal vein myocytes, and rat middle cerebral artery myocytes—as described previously (12, 13, 21). Gastric fundus muscle was dissected in situ from the stomach of guinea pigs after cervical dislocation and exsanguination. The portal vein upstream of the anastomosis of its right and left branches was removed from New Zealand White rabbits after overdose with pentobarbital. Right and left middle cerebral arteries were removed from Wistar rats after cervical dislocation and exsanguination. All procedures were carried out with personal and project licenses granted according to the United Kingdom (UK) Animals (Scientific Procedures) Act 1986. After muscle was obtained, cells were isolated by enzymatic treatment (collagenase, protease, BSA, trypsin inhibitor) followed by mechanical trituration. Cells were loaded with either fluo-3 AM or fluo-4 AM, and LCR events were recorded in the line scan mode with a Zeiss LSM 510 laser scanning confocal microscope (Jena, Germany).

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typically normalized to the basal cytosolic fluorescence (F/F0). We used a novel algorithm to do this. Briefly, a timeline is segmented at different thresholds (used in order). A timeline is segmented at each threshold the number of segmented regions is counted. If the number of regions at a threshold is less than the number counted at the last current, the algorithm stops and F0 (mean, minimum area). LT, low threshold; HT, high threshold; α, initial arbitrary threshold; β, amplitude resolution; F/F0, fluorescence normalized to basal cytosolic fluorescence; Amin, minimum area; *x resolution (XTR) in Ref. 21. †Typical range of values (depends on temporal and spatial resolution of line scan).

### Computation

All data analysis was carried out with programs written in Java as a plugin for ImageJ image analysis software (http://rsbweb.nih.gov/ij/plugins/metadata/index.html; National Institutes of Health, Bethesda, MD). We utilized Michael Thomas Flanagan’s scientific library (10), Piotr Wendykier’s JTransforms library (28), and code from Burger and Burge (5). All LCR simulations were carried out with programs written in C++.

### Data Processing

Line scan images were first processed by three algorithms, as described below, in order.

**Lee filtering.** The Lee filter is a local statistics filter designed to dampen Poisson noise while conserving signal shape (16). We used a plug in for ImageJ image analysis software (http://rsbweb.nih.gov/ij/plugins/metadata/index.html; National Institutes of Health, Bethesda, MD). We utilized Michael Thomas Flanagan’s scientific library (10), Piotr Wendykier’s JTransforms library (28), and code from Burger and Burge (5). All LCR simulations were carried out with programs written in C++.

**Zero-frequency band removal.** Bands of brightness along the time dimension are common in line scans of calcium fluorophore fluorescence, reflecting loading of organelles. Izu et al. (15) removed these bands by zeroing the zero-frequency component of each timeline’s Fourier transform. We implemented a modification of this, calculating the Fast Fourier Transform (FFT) for the first and last half of each timeline separately and then using the zero-frequency component from each half as interpolation points on a line that was then subtracted from each timeline as a whole. This accommodated for bleaching in some bands.

**Maximum region normalization.** Line scans of LCR events are typically normalized to the basal cytosolic fluorescence (F/F0). We used a novel algorithm to do this. Briefly, a timeline is segmented at different thresholds (T), beginning at the minimum pixel value and then increasing in steps of Δ. At each threshold the number of segmented regions is counted. If the number of regions at a threshold (Tcurrent) is less than the number counted at T = Tcurrent − nΔ, the algorithm stops and F0 = Tcurrent − nΔ. This process is repeated for each timeline, and the whole image is divided through by the average of these F0 values. We used Δ = 0.1 and n = 5.

### Measurement of LCR Events

Previously we described (21) a confinement tree algorithm for identifying and measuring LCR events in line scans. Two sets of variables were used for this study (Table 1) with resultant data identified as either low or high threshold (LT or HT, respectively; see Fig. 2A, ii and iii, for comparison of detected events). For each detected event five metrics were measured—MA, full width at half-maximum amplitude (FWHM), full duration at half-maximum amplitude (FDHM), MHM, and AHM. These are defined in Fig. 1.

### Statistical Analysis

Principal component analysis (PCA) is a common method used to summarize the distribution of linearly correlated data. If a data set consists of paired values \( \{x_0, y_0\}, \{x_1, y_1\}, \ldots, \{x_n, y_n\} \), then

\[
M_{pq} = \sum_{i=1}^{n} x_i^p y_i^q
\]

\[
x_c = M_{10}/M_{00}, \quad y_c = M_{01}/M_{00}
\]

Fig. 1. Metric definitions. All metrics are defined relative to the maximum amplitude (MA), which is defined as the peak localised calcium release (LCR) event amplitude. For simulated events [arbitrary units (au)], half-maximum amplitude (HM) = MA/2. For ratioed images [units of events normalized to basal cytosolic fluorescence (F/F0)] HM = 1 + [(MA − 1)/2]. A: area at half-maximum amplitude (AHM) is the area covered by the LCR at HM. B: mass above half-maximum amplitude (HM) is the integral of (signal − HM) over that area. C: full duration at half-maximum (FDHM) is the length of the time line (t) passing through the MA and bound by AHM. D: full width at half-maximum (FWHM) is the length of the spatial line passing through the MA and bound by AHM.

### Table 1. Input parameters for confinement tree algorithm

<table>
<thead>
<tr>
<th>Set</th>
<th>Cell</th>
<th>α, F/F0</th>
<th>β, F/F0</th>
<th>Amin, μm/s *</th>
<th>Amin, pixels †</th>
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</thead>
<tbody>
<tr>
<td>LT</td>
<td>Fundus</td>
<td>1.0</td>
<td>0.01</td>
<td>0.1</td>
<td>2−5</td>
</tr>
<tr>
<td></td>
<td>Portal/cerebral</td>
<td>1.0</td>
<td>0.01</td>
<td>0.005</td>
<td>10−20</td>
</tr>
<tr>
<td>HT</td>
<td>Fundus</td>
<td>1.1</td>
<td>0.01</td>
<td>5</td>
<td>100−250</td>
</tr>
<tr>
<td></td>
<td>Portal/cerebral</td>
<td>1.1</td>
<td>0.01</td>
<td>0.1</td>
<td>200−400</td>
</tr>
</tbody>
</table>

LT, low threshold; HT, high threshold; α, initial arbitrary threshold; β, amplitude resolution; F/F0, fluorescence normalized to basal cytosolic fluorescence; Amin, minimum area. *x resolution (XTR) in Ref. 21. †Typical range of values (depends on temporal and spatial resolution of line scan).
The formation of a line scan image, the central moment,

\[ \varphi = \left[ \left(1 - \left(\lambda_{\text{major}}/\lambda_{\text{minor}}\right)^{0.5}\right) \right] \tag{10} \]

where \( M_{pq} \) is the moment, \( x_i \) and \( y_i \) are the \( x \) and \( y \) centroids, \( \mu_{pq} \) is the central moment, \( \mu_{pp} \) is the normalized central moment, \( cov \) is the covariance matrix, \( \lambda_{\text{minor}} \) and \( \lambda_{\text{major}} \) are the smaller and larger eigenvalues of the covariance matrix, \( s_{\text{major}} \) and \( s_{\text{minor}} \) are the slopes of the major and minor axes, \( c_{\text{major}} \) is the \( y \)-intercept of the major axis, and \( \varphi \) is the eccentricity.

The centroids mark the center of the distribution. The major axis is the overall direction of the correlation, and the minor axis is orthogonal to this. The eigenvalues associated with each axis are a measure of the variance along those axes. Their ratio is thus a measure of the eccentricity of the distribution: if \( \varphi = 0 \), the distribution is circular, and with larger values (up to 1) the distribution is stretched in the direction of the major axis.

PCA was performed either separately for each cell in a group or for the totality of events from that group. In the former case values are expressed at means \( \pm \) SE, with \( n \) = number of cells. Where paired comparisons are made of cells before a treatment and after, statistical significance was gauged by paired Student’s \( t \)-test.

**Reaction-Diffusion Simulation**

For spherically symmetrical diffusion from a point source of flux, an analytic solution of the reaction-diffusion equation can be found as \((2, 20)\)

\[ c(r, t) = \frac{\alpha}{4\pi Dr} \text{erfc} \left( \frac{r}{4Dt} \right)^{3/2} \tag{11} \]

\[ \alpha = \frac{I_{\text{size}}}{\int F} \tag{12} \]

where \( c(r, t) \) is the concentration of an ion (in this case \( \text{Ca}^{2+} \)) at radius \( r \) from the source and time \( t \), \( \alpha \) is the magnitude of the flux, \( D \) is the diffusion coefficient of the ionic species, \( I_{\text{size}} \) is the flux constant, \( z \) is the charge of the ion, \( F \) is the Faraday constant, and \( \text{erfc} \) is the complementary error function.

By the principle of superposition, \( c(r, t) \) for a flux that ends after a period \( t_{\text{open}} \) can be found by subtracting from \( c(r, t) \) \( c(r, t) \) time-shifted by \( t_{\text{open}} \).

\[ c(r, t) = c(r, t) - c(r, t + t_{\text{open}}) \tag{13} \]

The formation of a line scan image, \( i(x, t) \), of such a process can be described as

\[ i(x, t) = i(x, y = 0, z = 0, t) = F(c(x, y, z, t) - c(x, y, z, t + t_{\text{open}})) \tag{14} \]

where \( F(c) \) describes fluorescence of the fluorophore as a function of ion concentration, \( * \) is the convolution operator, \( G(x, y, z) \) is a Gaussian point-spread function (PSF) of the microscope (see below), and \( c(x, y, z, t) \) is related to \( c(r, t) \) by

\[ r = \frac{(x - x_i)^2 + (y - y_i)^2 + (z - z_i)^2}{2} \tag{15} \]

where \( (x, y, z) = (0,0,0) \) is the center of the line, \( x \) is the axis of the line, \( y \) is the axis orthogonal to the line and coincident with the point source, and \( \delta_x \) and \( \delta_z \) are the \( y \) and \( z \) displacements of the line from the point source [i.e., \( (x, y, z) = (0, \delta_y, \delta_z) \) is the coordinate of the point source].

For efficiency Eq. 14 is computed as

\[ i(x, t) = [F(c(x, y, z, t) + G(x, y, z)) - F(c(x, y, z, t + t_{\text{open}}) + G(x, y, z))] \tag{16} \]

Briefly, \( c(x, y, z, t) \) was calculated for a particular \( t \) and an \( x, y, z \) volume centered around half of the scan line, such that if the length of the scan line is \( L \) and the half-width of the convolution kernel \( G(x, y, z) \) along the relevant axis is \( W \), the boundaries of this volume for \( x \) are \( -(0.5L + W) \) and \( W \) for \( y \) and \( z \) are \( -W \) and \( W \). \( c(x, y, z, t) \) was then converted to \( F(x, y, z, t) \) and convolved with the kernel \( G(x, y, z) \).

This gives the values for \( i(x, y = 0, z = 0, t) \). The whole line was calculated by reflecting the values calculated for half of the line, along the other half. Repeating the above for each \( t \) gives a line scan image \( i(x, t) \). Finally, \( i(x, t) \) shifted by \( t_{\text{open}} \) was subtracted from \( i(x, t) \).

\[ F(c) = kc \tag{17} \]

\[ G(x, y, z) = G_{\text{FWHM}}^{-1} \exp(-x^2/\lambda_{xy}) \cdot \exp(-y^2/\lambda_{yz}) \cdot \exp(-z^2/\lambda_z) \tag{18} \]

where \( k \) is a proportionality constant, \( \lambda_{xy} \) and \( \lambda_{yz} \) are the length constant and FWHM, respectively, of the PSF \( x \) plane, \( \lambda_z \) and \( \eta \) are the length constant and FWHM, respectively, of the axial PSF, and \( G_{\text{FWHM}} \) is the integral of \( G(x, y, z) \) over the dimensions of the convolution kernel \((3 \times \text{relevant } \eta)\).

**RESULTS**

**Theory**

Probably the simplest model of an LCR event is spherical diffusion from a point source of flux (see Methods). This has a well-known and simple analytic solution that introduces only the minimal set of reaction-diffusion parameters—source flux (expressed as source current, \( I_{\text{size}} \)), diffusion coefficient (\( D \)), and flux period (\( t_{\text{open}} \)). We simulated line scan images resulting from a scan line passing through the neighborhood of such a flux source. This introduces two additional parameters—the displacements of the scan line from the flux source along the \( y \)- and \( z \)-axes (\( \delta_y \) and \( \delta_z \)), the \( x \)-axis being defined as the axis of the scan line itself. In addition, we modeled optical convolution by the confocal microscopic, introducing parameters for the microscope’s PSF.

LCR events were simulated with a “base” set of parameter values (Table 2; Fig. 2Di). Five measurements were made for each event (Fig. 1)—MA, FWHM, FDHM, MHM, and AHM. Logarithms of these values are indicated by a “p” prefix (as in \( \text{pH}, \text{pk}, \text{etc.} \)). The effect of varying four of the parameters was examined: \( \delta_y \), \( D \), \( I_{\text{size}} \), and \( t_{\text{open}} \). For each parameter a set of 100 event images was generated, with the parameter in question being varied randomly according to a Gaussian distribution with defined mean and standard deviation (\( \mu \pm \sigma; \delta_y = 1.0 \pm 0.6 \mu \), \( D = 20 \pm 10 \mu \text{m/s}, I_{\text{size}} = 2 \pm 1 \text{pA} \), \( t_{\text{open}} = 20 \pm 10 \text{ms} \)). The distribution of logMHM as a function of logAHM \( [\text{pMHM}(\text{pAHM})] \) of each set was graphed as a scatterplot (Fig. 3, A–D, i). Also, AHM and MHM were plotted against the varying parameter (Fig. 3, A–D, ii and iii, respectively).

AHM increased exponentially with \( \delta_y \) and MHM decreased (Fig. 3A, ii and iii), however, the decrease in MHM was only a twentieth of the increase in AHM, with the result that variation in \( \delta_y \) was apparent as a horizontal displacement in pMHM(pAHM) (Fig. 3Aii). Qualitatively identical results were seen with \( \delta_z \) (not shown). Both AHM and MHM decreased exponentially with \( D \) at approximately the same rate (Fig. 3B, ii and iii), with the result that variation in \( D \) was apparent as a diagonal displacement of unit slope in pMHM(pAHM) (Fig. 3Bi). MHM increased in a linear manner with \( I_{\text{size}} \) (Fig. 3Ciii), but there was no change in AHM (Fig. 3Cii), with the result that variation in \( I_{\text{size}} \) was apparent as a vertical displacement in pMHM(pAHM) (Fig. 3Cii). MHM increased in an approximately linear manner with \( t_{\text{open}} \) (found to be \( \text{MHM} \propto t_{\text{open}}^2 \)) and AHM increased nonlinearly (Fig. 3D, ii and iii), with the result...
Table 2. Parameters used in LCR stimulations

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Unit</th>
<th>Definition</th>
<th>Base Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$</td>
<td>fmol/s</td>
<td>Calcium ion flux at release origin</td>
<td></td>
</tr>
<tr>
<td>$\delta_i$</td>
<td>$\mu$m</td>
<td>$y$ Displacement of release origin from scan center</td>
<td>1</td>
</tr>
<tr>
<td>$\delta_z$</td>
<td>$\mu$m</td>
<td>$z$ Displacement of release origin from scan center</td>
<td>1</td>
</tr>
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<td>$\mu$m</td>
<td>Length constant of microscope PSF in z-axis</td>
<td></td>
</tr>
<tr>
<td>$S$</td>
<td>pC/fmol</td>
<td>Faraday constant</td>
<td>96.485</td>
</tr>
<tr>
<td>$c$</td>
<td>fmol/($\mu$m)$^3$ (M)</td>
<td>Concentration of calcium ion</td>
<td></td>
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<tr>
<td>$D$</td>
<td>$\mu$m$^2$/s</td>
<td>Apparent diffusion coefficient of calcium ion</td>
<td>20</td>
</tr>
<tr>
<td>$F$</td>
<td>au</td>
<td>Fluorescence intensity</td>
<td></td>
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<td>$G$</td>
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<td>$G_T$</td>
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<tr>
<td>$i$</td>
<td>au</td>
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<td>Release current</td>
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<td>$k$</td>
<td>au ($\mu$m)$^{-1}$ ($\text{fmol})^{-1}$</td>
<td>Calcium-fluorescence proportionality constant</td>
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<td>$L$</td>
<td>$\mu$m</td>
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LCR, localized calcium release; FWHM, full width at half-maximum amplitude; PSF, point-spread function; au, arbitrary units. *F = 1 au corresponds to $c = 1 \mu$M.

that variation in $t_{open}$ was apparent as a vertical displacement in pMHM(pAHM) at low $t_{open}$, followed by a diagonal displacement at greater $t_{open}$ (Fig. 3Di).

The maximum amplitude (MA) is a commonly used metric for LCR events. Therefore it was of interest to look at the relationship among AHM, MHM, and MA. Plotting the distribution of log[MA/MA] as a function of log MA (p[MA/MA]) (Fig. 4A) for all the simulated events in Fig. 3 gave a linear relationship of unit slope and $y$-intercept of $0.73$ arbitrary units (au) (by regression). This suggests that increasing MA corresponds to an increase in the pMHM(pAHM) intercept. This, together with the parameter trends illustrated in Fig. 3, is schematized in Fig. 4B.

**Application: Relative Current of Two LCR Populations**

It has previously been demonstrated for LCR events in myocytes of the rabbit portal vein that the MA distribution is bimodal, indicating the presence of two populations with differing current (11). We also found two modes in the MA distribution of 14 portal vein myocytes, for events of large area (Fig. 5, Ai and Bi). Also, two LCR populations could clearly be distinguished in the pMHM(pAHM) distribution (Fig. 5, Aii and Biit). According to our model (see Theory), this could possibly be accounted for by two populations with differing displacement from the scan line (resulting in displacement along the AHM axis) or two populations with differing $t_{size}$ (resulting in displacement along the MHM axis). The former might be reasonable if the data were from one cell with only two LCR sources in separate focal planes. However, this is not the case (Fig. 2A), so the latter would seem a better explanation. As MHM is linearly related to release current (Fig. 3Cii), the relative current of the two populations can be estimated simply from the difference in MHM intercepts of lines of unit slope fitted to each population (Fig. 5D). The pMHM intercepts of the two populations were $-1.2$ and $-1.5$, corresponding to a 10($^{1.2-1.5}$) or 2-fold difference in MHM and therefore release current. Although the two populations are clearest at large AHM, they were still apparent at low AHM (Fig. 5C).

As a check against the possibility that the distribution of portal vein events was somehow spurious we looked at the distribution of events from middle cerebral artery cells, which generate LCR events of spatiotemporal magnitude similar to those in the portal vein (13). Middle cerebral artery LCR events did not have a two-population distribution (Fig. 5E).

**Application: Pharmacological Effects**

Pharmacology is commonly used to functionally characterize LCR events. We were curious to see whether pharmacological effects could be detected in the LCR distribution. We looked at the effect of ryanodine on LCR events recorded from knurled cells in the guinea pig gastric fundus (21). These events are much larger and longer than the sparks in portal vein and middle cerebral artery (Fig. 2C). Similar events in fibroblasts have been termed flickers by Cheng and colleagues (27). It was shown previously that flickers in knurled cells were sensitive to ryanodine (21), which binds to the ryanodine receptor (RYR), one of the two main sarcoplasmic reticulum (SR) channels that are the origin of LCR flux [the other being the inositol trisphosphate receptor (IP$_3$R)].

LCR events were measured for two groups of knurled cells before (control) and 10 min after ryanodine (either 10 or 50 $\mu$M) was applied to the bath. In both groups (10 and 50 $\mu$M ryanodine) the change in distribution was remarkably consistent (Fig. 6). Under control conditions the pMHM(pAHM)
distributions were quite stretched out along the major axis (Fig. 6, Ai and Bi). With ryanodine the distributions contracted along their major axis (Fig. 6, Aii and Bii). These changes, which were apparent by eye, were confirmed statistically with PCA (Fig. 6 C and Table 3; see Statistical Analysis). Both x and y centroids, major axis eigenvalue, and eccentricity decreased significantly with ryanodine. This shortening along the major axis would suggest either a decrease in $t_{open}$ or an increase in $D$ (Figs. 3, 4 B). Ryanodine also decreased the major axis slope and at 50 $\mu$M decreased the major axis intercept. These effects fit with a decrease in $I_{size}$, both being more significant at 50 $\mu$M.

Invariance of Gross LCR Geometry

The model suggests that the slope and y-intercept of the $p(MHM/AHM)/(p(MA - 1))$ distribution are independent of reaction-diffusion parameters (Fig. 4A). Instead, this distribution would seem to reflect the invariant geometry of LCRs as objects with a common physical basis. For instance, if LCRs were cubes, the slope would be 1.0 with an intercept of $-0.3$ [log(0.5)]. The LCR model predicts a slope of 1.00 (unitless) and y-intercept of $-0.73$ au (see above). These values are in good agreement with the LCR data from portal vein myocytes, middle cerebral artery myocytes, and fundus knurled cells (Fig. 7). It might be possible that filtering of the line scan images would introduce spurious scaling into the data. This was found not to be the case for line scans that had not been Lee filtered or had removal of the zero-frequency band (Fig. 7 D). It should be noted that the values of $p(MHM/AHM)$ and MA are higher than for the filtered data (Fig. 7 C), because the unfiltered data are much noisier and therefore a lower $F_0$ is calculated by the maximum region normalization algorithm.

DISCUSSION

Metrics of Simulated LCR Events

The model chosen to simulate LCR events imaged by line scan was kept extremely minimal. The point was that any results of the model would be direct outcomes of fundamental physical law (the reaction-diffusion equation) and generally applicable, rather than reflecting parameters of particular models of LCR events of particular cell types under particular
conditions. Also, the authors are not overly familiar with numerical modeling. This minimalism means that the model does not account for several variables that may apply to real LCR. The most gaping of these neglected variables is nonconstant current due to stochastic opening of multiple channels or subconductance states. Also, the model assumes a single apparent diffusion coefficient, which is only valid where the “rapid buffer approximation” applies (19). The model does not

Fig. 3. Effects of parameter variation on LCR metrics. For each parameter 100 LCR events were simulated with the parameter varying randomly as a Gaussian distribution of mean ± SD (base set of parameters given in Table 1). A: offset of release source from line ($\delta_0 = 1.0 \pm 0.6 \mu m$). B: apparent diffusion coefficient ($D = 20 \pm 10 \mu m^2/s$). C: release current ($I_{size} = 2 \pm 1 pA$). D: release period ($\tau_{open} = 20 \pm 10 ms$). i: pMHM(pAHM). ii: AHM plotted against varying parameter. iii: MHM plotted against varying parameter.

Fig. 4. Scaling of MA with MHM and AHM. A: p[MHM/AHM](pMA) for all the simulated LCR events in Fig. 3 (400 in total). Line was fitted by regression to give a slope of 1.00 and y-intercept of $-0.73$ au. B: schematic of the effect of different model parameters on the pMHM(pAHM) distribution and their correlation with MA.
account for nonconstant SR uptake or anomalous/anisotropic diffusion. The results presented here will have to be compared with numerical models in which these variables can be taken into consideration. However, despite the simplification of our model much of the theoretical results are unsurprising given past theoretical studies. MHM, as for the analogous SM, is linearly related to current (6, 14, 24, 32). AHM and MHM, in analogy to FWHM (7), decrease with $D$. We have made attempts to mathematically derive these relationships based on Gaussian geometry but have had only partial success.

Two LCR Populations

A CRU or “calcium release unit” is a cluster of calcium channels (IP3R or RyR) on the sarcoplasmic/endoplasmic reticulum that open to give an LCR event (7). CRUs have been
observed directly by electron and light microscopy (see discussion of Ref. 1 for review). Theoretically, the number of channels in a CRU should affect LCR activation and termination mechanism, likelihood of propagation (initiation of calcium waves), and interchannel cooperativity. Therefore, as Cheng and Lederer (7) have put it, “delineating the number of RyRs activated in a spark is a matter of fundamental importance to sparkology.” However, determining this number from line scans has proved difficult. The main difficulty has been that in most cells there are several CRUs distributed randomly through the cell, so that a scan line will pick up LCR from several CRUs each at a different displacement (δy or δz). This

Fig. 6. Effects of ryanodine on scaling of LCR events of fundus knurled cells. A and B: pHMH(pAHM) distributions (LT set) for 2 groups of cells (A = 16 cells, B = 16 different cells) before (i) and 10 min after (ii) ryanodine was applied to the bath (10 μM ryanodine in A, 50 μM in B). C: principal component analysis of the distributions in A and B. i: x and y centroids (xc and yc). ii: Slope of major axis (s_major). iii: y-Intercept of major axis (c_major). iv and v: Eigenvalues of major and minor axes (λ_minor and λ_major), respectively. vi: Eccentricity (φ). Open bars (labeled “C”), control; gray bars, with ryanodine (labeled “10” or “50” for 10 μM or 50 μM ryanodine, respectively). Bracketed column pairs indicate significant difference by paired t-test (*P < 0.05, **P < 0.01, ***P < 0.001).
variable displacement should smear out any peaks in the distribution of LCR metrics that could relate to channel number, such as MA, SM, and rate of rise. Typically a monotonically decaying distribution will be produced (see Ref. 9 for theoretical consideration of this matter; Refs. 15, 22).

There have been a couple of practical solutions to this problem: 1) recording from a single CRU per scan, with the scan line passing through the center of the CRU by adjusting xy and z position of the line until the LCR appears brightest or “in focus” (see, e.g., Refs. 6, 11, 14, 23, 24, 26), or 2) recording LCRs with two-dimensional, nonconfocal imaging (see, e.g., Refs. 30–32).

However, neither solution is entirely satisfactory. The first solution is a little clumsy. It may take some time to focus the LCR events, during which time bleaching and photodamage can occur. Also, it limits the preparations that can be studied and the number of LCRs that can be recorded per scan. The second solution is undoubtedly the most ideal, but with current technology, sampling rate and duration are very much limited compared with line scanning and computation is more severe with three-dimensional data sets. In a few years this may no longer be the case, and this issue will become largely redundant but for a few sparkologists who might stick with line scans for their aesthetic qualities.

Gordienko and Bolton (11) applied the single-in-focus CRU solution to measuring LCRs in rabbit portal vein myocytes. They found bimodal distributions for both MA and FWHM. Our measurements of LCR events from portal vein myocytes also showed a bimodal MA distribution for events above a minimum AHM. However, we did not actively apply the single-in-focus CRU solution. This suggests that either 1) the single-in-focus solution occurred “naturally” (of the several CRUs in each of the scan lines of several cells, all were roughly in the same focal plane) or 2) theory must be adjusted in some way. A rather ad hoc, mixed explanation could be a relatively large difference in population current, with a limited inter-CRU displacement due to cell geometry (if the cells are quite flat or SR is configured in a peculiar way).

The pMHM(pAHM) distribution for portal vein LCR events also indicated two populations. Unlike with the MA distribution, the populations could be distinguished even for LCR events with very small area, events that might previously have been considered noise. This increase in resolution illustrates one advantage of the pMHM(pAHM) plot, that it graphically separates the effects of scan line displacement from current size so that modes in the latter can be better resolved. At least one study (17) has actively tried to analyze very small LCR events or “calcium noise.” pMHM(pAHM) plots may prove advantageous in the study of such events. Another advantage of the pMHM(pAHM) plot is that because of the linear relationship between MHM and pMHM(pAHM) plots may prove advantageous in the study of such events. Another advantage of the pMHM(pAHM) plot is that because of the linear relationship between MHM and pMHM(pAHM) (see Metrics of Simulated LCR Events), in particular the assumption of constant current, we do not wish to make any sweeping statements about the significance of this number. However, it might prove insightful given that the smaller population is purely RyR dependent, while the larger population is generated by coupling between RyR and IP3R (11).

### Pharmacology

Beyond the fundamental quantities of the CRU—channel numbers and current size—biophysical mechanisms must be studied by experimental manipulation. Typically any such manipulation will be pleiotropic. For instance, adjusting cytosolic calcium buffering with exogenous chelators will not only adjust diffusion coefficients but also dampen calcium-induced calcium release, affecting CRU cooperativity and activation/inactivation, and cause passive store depletion, affecting driving force and gating of CRU channels. Therefore it is probably
unrealistic to expect that any one manipulation would change a single aspect of the LCR distribution, relatable to one reaction-diffusion variable. However, the effects of ryanodine on LCRs of knurled fundus cells suggest that changes in the pMHM-(pAHM) distribution can be broadly interpreted in terms of mechanism. The contraction of the pMHM(pAHM) distribution along the major axis was the major effect seen with ryanodine. This would suggest a decrease in channel open time. At submicromolar concentrations ryanodine increases $t_{\text{open}}$ while locking the RyR in a subconductance state. How-

Fig. 7. Invariant LCR geometry. p[MHM/AHM](p[MA - 1]) distributions (LT set) for portal vein myocytes ($N_{\text{cell}} = 14, N_{\text{event}} = 32,179; A$), middle cerebral artery myocytes ($N_{\text{cell}} = 18, N_{\text{event}} = 104,928; B$), fundus knurled cells ($N_{\text{cell}} = 95, N_{\text{event}} = 138,572; C$), and a set of fundus knurled cells without Lee filtering or zero-frequency band removal ($N_{\text{cell}} = 35, N_{\text{event}} = 224,950; D$). i: p[MHM/AHM]/(p[MA - 1]) distributions. ii: y-intercept, given a unit slope, as a function of p[MA - 1]. In both i and ii gray lines indicate the function $p[MHM/AHM] = -0.73 + p[MA - 1]$ fit to the model data (Fig. 4A).
ever, at the concentrations used here it has been shown that ryanodine decreases open time with little alteration of conductance (3, 4, 18, 25, 29). However, it must be emphasized that strict comparisons between duration of a LCR event and a channel open time cannot be made because the former reflects the activity of a group of interdependent channels. In the particular case of knurled fundus cells this is further complicated by the fact that, as for portal vein myocytes, LCR events are both RyR- and IP3, RyR dependent (21).

Summary

SM and MA have previously been used to quantify and understand LCR. Without any study it is apparent that both MMH and AHM are related to these metrics by simple geometry. In this sense much of the results of this paper are not surprising and could have been predicted from previous work [e.g., the bimodal pHM(pAHM) distribution in portal vein myocytes]. However, it was the exact nature of these geometric relationships that intrigued us and is the novel result of this paper. The power law relationships demonstrated by both theory and experimental data appear to be universal in that they reflect the fundamental physical law underlying LCR, Fickian diffusion, and are observed across different cell types with differing patterns of LCR. Whether SM, MA, MMH or AHM is employed, the graphing of these relationships allows for a better understanding of LCR, or at least gives more information, than looking at the distribution of individual metrics alone. It just happens that certain relationships among MA, MMH, and AHM are power laws and so are perhaps easiest to comprehend. This paper is one step toward this comprehension.

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