Identification and Spatiotemporal Characterization of Spontaneous Ca\(^{2+}\) Sparks and Global Ca\(^{2+}\) Oscillations in Retinal Arteriolar Smooth Muscle Cells

Tim M. Curtis, James Tumelty, Jennine Dawicki, C. Norman Scholfield, and J. Graham McGeown

PURPOSE. To identify spontaneous Ca\(^{2+}\) sparks and global Ca\(^{2+}\) oscillations in microvascular smooth muscle (MVSM) cells within intact retinal arterioles and to characterize their spatiotemporal properties and physiological functions.

METHODS. Retinal arterioles were mechanically dispersed from freshly isolated rat retinas and loaded with Fluo-4, a Ca\(^{2+}\)-sensitive dye. Changes in [Ca\(^{2+}\)]\(_i\) were imaged in MVSM cells in situ by confocal scanning laser microscopy in x-y mode or line-scan mode.

RESULTS. The x-y scans revealed discretely localized, spontaneous Ca\(^{2+}\) events resembling Ca\(^{2+}\) sparks and more global and prolonged Ca\(^{2+}\) transients, which sometimes led to cell contraction. In line scans, Ca\(^{2+}\) sparks were similar to those previously described in other types of smooth muscle, with an amplitude (ΔF/F\(_0\)) of 0.81 ± 0.04 (mean ± SE), full duration at half maximum (FDHM) of 23.62 ± 1.15 ms, full width at half maximum (FWHM) of 1.25 ± 0.05 μm, and frequency of 0.56 ± 0.06 seconds\(^{-1}\). Approximately 35% of sparks had a prolonged tail (>80 ms), similar to the Ca\(^{2+}\)-embers described in skeletal muscle. Sparks often summed to generate global and prolonged Ca\(^{2+}\) elevations on which Ca\(^{2+}\) sparks were superimposed. These sparks occurred more frequently (2.86 ± 0.25 seconds\(^{-1}\)) and spread farther across the cell (FWHM = 1.67 ± 0.08 μm), but were smaller (ΔF/F\(_0\) = 0.69 ± 0.04).

CONCLUSIONS. Retinal arterioles generate Ca\(^{2+}\) sparks with characteristics that vary during different phases of the spontaneous Ca\(^{2+}\)-signaling cycle. Sparks summate to produce sustained Ca\(^{2+}\) transients associated with contraction and thus may play an important excitatory role in initiating vessel constriction. This deserves further study, not least because Ca\(^{2+}\) sparks appear to inhibit contraction in many other smooth muscle cells. (Invest Ophthalmol Vis Sci. 2004;45:4409–4414) DOI: 10.1167/iovs.04-0719

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The goal of the present study was to determine the spatio-temporal characteristics and functional relevance of spontaneous Ca\(^{2+}\)-transients in retinal MVSM by obtaining the first images from retinal arterioles of both localized and generalized Ca\(^{2+}\) signaling events. Furthermore, we show that Ca\(^{2+}\) sparks can summate to produce sustained global Ca\(^{2+}\) oscillations, some of which are followed by cell contraction. This is consistent with a model in which local Ca\(^{2+}\) release events play an excitatory, rather than an inhibitory, role in the retinal vasculature, and thus runs counter to current paradigms concerning the functional significance of Ca\(^{2+}\) sparks in vascular smooth muscle.\(^7,8\) We also demonstrate the existence of two distinct populations of Ca\(^{2+}\) sparks for the same release sites during different phases of spontaneous signaling, presumably reflecting changes in localized Ca\(^{2+}\)-release with different levels of cytoplasmic and SR-Ca\(^{2+}\).

**METHODS**

**Retinal Microvessel Preparation**

Male Sprague-Dawley rats (200–300 g) were anesthetized with CO\(_2\) and killed by cervical dislocation. Animal use conformed to the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and UK Home Office Regulations. Retinas were rapidly removed and arterioles devoid of surrounding neuropile isolated as previously described.\(^7,6\) In brief, retinal quadrants were lightly triturated using a fire-polished Pasteur pipette (internal tip diameter, 0.3 mm) in a low-Ca\(^{2+}\) Hanks’ solution. Homogenates were centrifuged at 3000 rpm for 1 minute, the supernatant aspirated off, and the tissue washed again with low-Ca\(^{2+}\) medium. The remaining fragments were incubated at 21°C in 1 mL of low-Ca\(^{2+}\) Hanks’ solution containing 10 \(\mu\)M of Fluo-4 AM, a Ca\(^{2+}\)-sensitive dye (Molecular Probes, Eugene, OR) and the suspension agitated every 15 minutes for 2 hours. This prolonged incubation was necessary to facilitate adequate loading of the retinal MVSM cells with Fluo-4.

Homogenates were diluted with 10 volumes of low-Ca\(^{2+}\) medium and the mixture vigorously triturated. Of this mixture, 1 mL was pipetted into a rotatable circular glass-bottomed recording bath on the stage of an inverted microscope (Eclipse TE300; Nikon, Tokyo, Japan). Microvessels were anchored with tungsten wire (50 \(\mu\)m diameter, 2 mm length) and superfused with normal Hanks’ solution at 37°C. The recording bath was rotated so that the long axis of the arteriolar wall was parallel to the \(x\)-axis of the microscope. Drug solutions were delivered via a five-way micromanifold with an exchange time of \(-1\) second, as measured by switching to a dye solution.

**Solutions**

The bath solution had the following composition (in mM): 140, NaCl; 5, KCl; 5, d-glucose; 2, CaCl\(_2\); 1.3, MgCl\(_2\); 10, HEPES (pH 7.4) with NaOH. Low-Ca\(^{2+}\) medium differed only in that it contained 0.1 mM CaCl\(_2\).

**Ca\(^{2+}\) Imaging and Data Analysis**

Changes in [Ca\(^{2+}\)]\(_i\) were imaged in MVSM cell arrays with a confocal scanning laser microscope (MRA1; Bio-Rad, Richmond, CA) used in \(xy\) mode at a rate of 1 image per 1.2 seconds and in line scan mode at a rate of 500 scans per second.\(^4,14\) Confocally imaged microvessels were excited at 488 nm, and emitted light was filtered through a 530- to 560-nm band-pass filter. Data acquisition was controlled by computer (Timecourse software; Lasersharp; Bio-Rad) and images were processed and analyzed on computer (Image J; available by ftp at zippy.nimh.nih.gov/ or at http://rsb.info.nih.gov/nih-image); developed by Wayne Rasband, National Institutes of Health, Bethesda, MD). Confocal fluorescence data (\(F\)) were normalized to the average resting fluorescence (\(F_0\)) for periods that exhibited no spontaneous elevations in [Ca\(^{2+}\)]\(_i\).

**RESULTS**

**Identification of Ca\(^{2+}\) Sparks and Global Ca\(^{2+}\) Oscillations in Retinal MVSM Cells**

To explore whether distinct subcellular Ca\(^{2+}\) transients exist in rat retinal MVSM cells, we monitored changes in fluorescence intensity by laser scanning confocal microscopy in myocytes still embedded within their parent arterioles and loaded with the Ca\(^{2+}\) indicator dye Fluo-4 (Molecular Probes). For the purposes of this study, recordings were confined to retinal arteriole segments that were 35 to 40 \(\mu\)m in diameter, which represent the main trunk arterioles that emanate from the optic disc.\(^6\) The wall of isolated retinal arterioles consisted of a monolayer of MVSM cells surrounding an intact endothelium (Fig. 1). Under resting conditions, vascular myocytes within
well as more prolonged global Ca

13 Likewise, we observed that spontaneous global Ca
tion was associated with a 19% reduction in MVSM cell area. Although it is apparent, therefore, that a prolonged global increase in [Ca\(^{2+}\)]

i, can trigger retinal MVSM contraction, this was relatively uncommon. In six vessels (82 cells), only 18 of 103 global Ca\(^{2+}\) oscillations were followed by a decrease in cell area. The amplitude of the [Ca\(^{2+}\)]
i increases was no higher in cells that contracted than in those that did not (P = 0.55), and so this variability probably reflects differences in the sensitivity of the individual cells to [Ca\(^{2+}\)]
i. Mechanical contraction was never observed in response to individual Ca\(^{2+}\) sparks (6 vessels, 82 cells, 163 sparks).

**Ca\(^{2+}\) Sparks and Global Ca\(^{2+}\) Oscillations**

Although spontaneous [Ca\(^{2+}\)]
i transients were visualized in retinal MVSM cells by using x-y scan imaging, this approach did not allow adequate temporal resolution of rapid, localized [Ca\(^{2+}\)]
i changes within a cell. Consequently, it appears from Figure 2B that both Ca\(^{2+}\) sparks and global Ca\(^{2+}\) oscillations have a similar time course. To improve temporal resolution, we used the line-scan mode of confocal imaging. Analysis was limited to vessel segments exhibiting no frame-to-frame movement of the cells. A typical example of an image obtained by scanning a line oriented at right angles to the long axis of a cell is shown in Figure 4A. A brief, localized Ca\(^{2+}\) spark can be clearly seen in the image. This spontaneously localized Ca\(^{2+}\) event had a peak amplitude ($\Delta F/F_0$) of 0.95 and an FWHM of 14 ms and was restricted to a relatively small area (FWHM of 1.57 \mu m). Some Ca\(^{2+}\) sparks (\approx 35% of recorded sparks in seven cells) were associated with a prolonged tail (>80 ms) similar to that in the example in Figure 4A. These events resemble the Ca\(^{2+}\) embers or “gloves” recently described in skeletal muscle cells.\(^15\) Ca\(^{2+}\) embers were site dependent and, in some sites, practically all Ca\(^{2+}\) sparks displayed prolonged tails.

It has been demonstrated in ileal myocytes that spatiotemporal recruitment of “elementary” Ca\(^{2+}\) sparks may give rise to cell-wide elevations in [Ca\(^{2+}\)].\(^16\) Using line-scan imaging, we were able to visualize the initiation site of some global Ca\(^{2+}\) oscillations in retinal MVSM cells. In the example shown in Figure 4B it is evident that a global Ca\(^{2+}\) oscillation was initiated from a site where spontaneous Ca\(^{2+}\) sparks were also observed. The temporal profile of the global Ca\(^{2+}\) oscillation consisted of a series of step-like increases in fluorescence resulting from the summation of consecutive Ca\(^{2+}\) sparks. It is also striking that even after the global Ca\(^{2+}\) oscillation reached its maximum, Ca\(^{2+}\) sparks persisted throughout both the plateau and declining phases.

**Two Populations of Ca\(^{2+}\) Sparks**

The spatiotemporal properties of Ca\(^{2+}\) sparks and global Ca\(^{2+}\) oscillations in retinal MVSM cells were characterized from line-scan images of 60 cells in 6 vessels, and the data are summarized in Table 1. Ca\(^{2+}\) sparks were separated into two groups: “basal” sparks that arose from resting fluorescence values ($F/F_0$ of 0.95–1.05) and those that were superimposed on global Ca\(^{2+}\) oscillations (Ca\(^{2+}\) sparks on oscillations). At 0.81, the mean spark amplitude under basal conditions was nearly six times the SD of the background signal noise (noise SD = 0.138). Global Ca\(^{2+}\) oscillations were similar in amplitude to basal Ca\(^{2+}\) sparks, but were nearly 100-times longer in duration (as measured from the FDHM) and occurred less frequently (Table 1). Differences in the spatiotemporal properties of basal Ca\(^{2+}\) sparks and Ca\(^{2+}\) sparks overlying Ca\(^{2+}\) oscillations were also observed. The latter were smaller in amplitude, increased in width, and increased in frequency (Table 1).

**Global Ca\(^{2+}\) Oscillations and Retinal MVSM Contraction**

In arterial smooth muscle, global Ca\(^{2+}\) oscillations have been recognized as the main driving force underlying vasoconstriction.\(^13\) Likewise, we observed that spontaneous global Ca\(^{2+}\) oscillations in retinal MVSM cells triggered contractile responses. In the example shown (Fig. 3), a global Ca\(^{2+}\) oscilla-

![Image](https://example.com/image.png)
DISCUSSION

In this study, we describe the first visualization of spontaneous subcellular \( \text{Ca}^{2+} \) transients in retinal arteriolar smooth muscle cells. Two distinct \( \text{Ca}^{2+} \) signaling events were seen, discretely localized: spontaneous near-membrane \( \text{Ca}^{2+} \) sparks and more global and prolonged \( \text{Ca}^{2+} \) oscillations. In most studies on elementary \( \text{Ca}^{2+} \)-signaling events in arterial smooth muscle, investigators have used single, isolated myocytes, even though the ultimate goal is to understand how function is regulated in intact vessels.\(^{10,17}\) A major advantage of the technique described herein is the use of intact arteriole segments in which the physiological relationships between the retinal MVSM cells, basal lamina, and endothelium are preserved. Our technique also allowed simultaneous imaging of subcellular \( \text{Ca}^{2+} \) signals in several MVSM cells, thus allowing cell-to-cell variation to be assessed while increasing the amount of data that can be collected in a single experiment.

\( \text{Ca}^{2+} \) sparks are thought to result from transient local release of \( \text{Ca}^{2+} \) from intracellular stores and have been described in cardiac,\(^{18}\) skeletal,\(^{19}\) and several smooth muscle cell preparations,\(^{12}\) including arteriolar smooth muscle cells.\(^{20}\) In smooth muscle cells, \( \text{Ca}^{2+} \) spark amplitudes are known to be quite variable, with the average peak increase in \([\text{Ca}^{2+}]_i\) ranging from 50 to 200 nM.\(^{12}\) In retinal MVSM cells, the average spark amplitude (\(\Delta F/F_0\) of 0.81; Table 1) equates to an elevation in \([\text{Ca}^{2+}]_i\) of \(\approx 80\) nM, as determined using the pseudoratiometric calculation of Cheng et al.,\(^{18}\) assuming an in situ dissociation constant for Fluo-4 of 1000 nM\(^2\) and a resting \(\text{Ca}^{2+}\) level in retinal arterioles of 66 nM.\(^7\) The average frequency (0.56 second\(^{-1}\) ), duration (23.6 ms FDHM), and spatial spread (1.25 \(\mu\) m FWHM) of \(\text{Ca}^{2+}\) sparks in retinal MVSM are all similar to those...
in other smooth muscle cells, with reported values for these parameters ranging from 0.5 to 1 second \(^{-1}\), 30 to 65 ms, 22 and 1.2 to 2.3 \(\mu m\), 23,24 respectively. There are, however, some reports of more prolonged \(Ca^{2+}\) sparks (100–600 ms) in tracheal 25 and urinary bladder smooth muscle cells, 26 whereas the events observed in human cerebral arterial smooth muscle cells appeared to spread further, with an average FWHM of 8.2 \(\mu m\). 27

Our records provide clear evidence that \(Ca^{2+}\) sparks in retinal MVSM cells fuse to produce cell-wide global \(Ca^{2+}\) oscillations that can lead to cell contraction. These findings are of particular interest, because they imply that \(Ca^{2+}\) sparks in retinal arterioles are primarily excitatory in nature, whereas it has been proposed that \(Ca^{2+}\) sparks exert a predominantly inhibitory effect in vascular smooth muscle, providing a negative feedback mechanism that favors decreased \(Ca^{2+}\) influx and vasodilatation. 1,2 Only a small proportion of global \(Ca^{2+}\) oscillations actually led to retinal MVSM cell contraction. \(Ca^{2+}\) ions regulate nearly every cell function, and subcellular \(Ca^{2+}\) transients are known to cause a pulsatile activation of \(Ca^{2+}\)-dependent enzymes, 28 and to drive changes in gene expression. 29 Consequently, those \(Ca^{2+}\) oscillations that failed to initiate excitation–contraction coupling are still likely to be physiologically relevant.

Detailed analysis revealed two distinct populations of \(Ca^{2+}\) sparks, with those superimposed on global \(Ca^{2+}\) oscillations displaying an increased frequency and spread, but reduced amplitude, when compared with sparks originating at the same release sites but from basal [\(Ca^{2+}\)] levels. \(Ca^{2+}\) sparks are thought to be generated by the opening of ryanodine receptor-linked channels (RyRs) on the SR, 12 and elevations in cytosolic \(Ca^{2+}\) are known to increase RyR open probability. 30 This may well explain the increased spark frequency during global \(Ca^{2+}\) oscillations. Likewise, increased spatial spread may also be accounted for by an overall increase in the open probability of RyRs, since this would favor recruitment of release sites. Because global \(Ca^{2+}\) oscillations in smooth muscle cells are known to involve \(Ca^{2+}\) store release, 13 the smaller amplitudes of the \(Ca^{2+}\) sparks during such oscillations in retinal MVSM cells may reflect a reduction in SR \(Ca^{2+}\) content. Clearly, further studies are now needed to unravel the precise mechanisms through which localized \(Ca^{2+}\) release is modified during \(Ca^{2+}\) oscillations, as well as to determine the functional implications of such modifications.

Under basal conditions, many sparks in retinal MVSM cells had protracted tails similar to the \(Ca^{2+}\) embers of skeletal muscle cells. 15 We have described prolonged, spontaneous \(Ca^{2+}\) release events in isolated smooth muscle cells during store-overload, 14 but no such events have been reported in any intact tissue. In skeletal muscle cells, embers are thought to reflect direct RyR opening by voltage sensors 16 but, because the RyRs are not believed to be under direct voltage control in smooth muscle, it is unclear what mechanism generates \(Ca^{2+}\) embers in retinal MVSM cells. It seems likely, however, that events in which \(Ca^{2+}\) release is prolonged well beyond the average channel-open time associated with RyRs in lipid bilayer experiments 31 may have important consequences, perhaps increasing the likelihood of spark summation and the initiation of global \(Ca^{2+}\) oscillations. These findings also underline the fact that the study of intact tissues may reveal subtleties of signaling behavior not apparent in isolated cells or molecules.

A possible limitation of our current model is the use of nonpressurized retinal arterioles. Passive stretching of the retinal vessel wall during increases in intraluminal pressure may lead to an elongation of the MVSM cells and thereby provoke changes in the spatiotemporal properties of the spontaneous \(Ca^{2+}\) signals. Increases in retinal MVSM cell length could modulate spontaneous \(Ca^{2+}\) sparks and global oscillations through the activation of stretch-activated currents 32 or via stretch-induced gating of RyRs. 33 In rat cerebral arterioles, pressurization increases the frequency of \(Ca^{2+}\) sparks and global \(Ca^{2+}\) oscillations, but other spatiotemporal features such as amplitudes and rise times are similar to those in nonpressurized vessels. 34 In summary, the application of high-resolution imaging to intact retinal arterioles has allowed us to visualize subcellular \(Ca^{2+}\) signaling events in retinal MVSM cells. These cells are the primary effectors of retinal arteriolar tone, and the data from the present study takes us a step closer to elucidating the basic mechanisms involved in the regulation of local blood flow in the retina. Understanding how such control is achieved will be fundamental to the development of novel therapeutic strategies designed to restore adequate blood flow in disease states, such as diabetic retinopathy 35 and glaucoma. 36

### References


### Table 1. Basic Properties of \(Ca^{2+}\) Sparks and Global \(Ca^{2+}\) Oscillations in Retinal MVSM Cells

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<tr>
<th>(Ca^{2+}) Sparks</th>
<th>(Ca^{2+}) Oscillations</th>
</tr>
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<tbody>
<tr>
<td>(\Delta F/F_c)</td>
<td>0.81 ± 0.04</td>
</tr>
<tr>
<td>Spread (FWHM)</td>
<td>1.25 ± 0.05 (\mu m)</td>
</tr>
<tr>
<td>Duration (FDHM)</td>
<td>25.6 ± 1.15 ms</td>
</tr>
<tr>
<td>Frequency</td>
<td>0.56 ± 0.06 s(^{-1})</td>
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<tr>
<th>(Ca^{2+}) Sparks</th>
<th>(Ca^{2+}) Oscillations</th>
</tr>
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<tbody>
<tr>
<td>(n)</td>
<td>102</td>
</tr>
<tr>
<td>(P)</td>
<td>*</td>
</tr>
<tr>
<td>Oscillations</td>
<td>n/a</td>
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</tbody>
</table>

It was generally not possible to measure the FWHM for oscillations because most of these events spread across the full cell width. With the exception of frequency data, \(n\) represents the number of \(Ca^{2+}\) events analyzed from a minimum of six vessels. The statistical significance for comparisons between sparks from baseline and sparks superimposed on oscillations as follows: NS, \(P > 0.05\); * \(P < 0.05\); *** \(P < 0.001\).


30. Saftenku E, Williams AJ, Sitsapesan R. Markovian models of low and high activity levels of cardiac ryanodine receptors. *Biophys J.* 2001;80:2727–2741.


