Ca2+ syntillas, miniature Ca2+ release events in terminals of hypothalamic neurons, are increased in frequency by depolarization in the absence of Ca2+ influx

Valerie De Crescenzo
University of Massachusetts Medical School

Let us know how access to this document benefits you.
Follow this and additional works at: https://escholarship.umassmed.edu/oapubs

Part of the Medicine and Health Sciences Commons, and the Neuroscience and Neurobiology Commons

Repository Citation

This material is brought to you by eScholarship@UMassChan. It has been accepted for inclusion in Open Access Publications by UMass Chan Authors by an authorized administrator of eScholarship@UMassChan. For more information, please contact Lisa.Palmer@umassmed.edu.
Cellular/Molecular

Ca\(^{2+}\) Syntillas, Miniature Ca\(^{2+}\) Release Events in Terminals of Hypothalamic Neurons, Are Increased in Frequency by Depolarization in the Absence of Ca\(^{2+}\) Influx

Valérie De Crescenzo, Ronghua ZhuGe, Cristina Velázquez-Marrero, Lawrence M. Lifshitz, Edward Custer, Jeffrey Carmichael, F. Anthony Lai, Richard A. Tuft, Kevin E. Fogarty, José R. Lemos, and John V. Walsh Jr

1Department of Physiology, University of Massachusetts Medical School, Worcester, Massachusetts 01605, 2Biomedical Imaging Group, University of Massachusetts Medical School, Worcester, Massachusetts 01605, and 3 Wales Heart Research Institute, University of Wales College of Medicine, Cardiff CF14 4XN, United Kingdom

Localized, brief Ca\(^{2+}\) transients (Ca\(^{2+}\) syntillas) caused by release from intracellular stores were found in isolated nerve terminals from magnocellular hypothalamic neurons and examined quantitatively using a signal mass approach to Ca\(^{2+}\) imaging. Ca\(^{2+}\) syntillas (scintilla, L., spark, from a synaptic structure, a nerve terminal) are caused by release of \(~250,000\) Ca ions on average by a Ca\(^{2+}\) flux lasting on the order of tens of milliseconds and occur spontaneously at a membrane potential of \(~80\) mV. Syntillas are unaffected by removal of extracellular Ca\(^{2+}\), are mediated by ryanodine receptors (RyRs) and designated "Ca\(^{2+}\) syntillas" by Parker and Yao (1991) and then found in myocytes, where they were mediated by ryanodine receptors (RyRs) and designated "Ca\(^{2+}\) sparks" (Cheng et al., 1993). Ca\(^{2+}\) syntillas, resembling puffs and sparks in some respects, have been observed in cultured hippocampal preparations (Cozzi et al., 1999), and three recent studies have provided indications of spontaneous transients in nerve terminals. First, Melamed-Book et al. (1999) found outliers in the fluorescence noise of Ca\(^{2+}\) images in cultured hippocampal somata and neuron terminals at the lizard neuromuscular junction, which they compared to spontaneous Ca\(^{2+}\) sparks in muscle. Importantly, in the cell bodies of hippocampal cultures, Melamed-Book et al. (1999) observed the noise outliers in Ca\(^{2+}\)-free medium, whereas in the nerve terminals the Ca\(^{2+}\) transients were not studied in Ca\(^{2+}\)-free medium, leaving their intracellular origin in doubt. Second, Llano et al. (2000) in their landmark study on "maximinis" observed long duration Ca\(^{2+}\) transients lasting seconds in presumed nerve terminals. Third, Emptage et al. (2001) illustrated several instances of spontaneous Ca\(^{2+}\) transients in axon-like processes and synaptic boutons. However, neither of these latter two studies demonstrated spontaneous transients in Ca\(^{2+}\)-free medium or blockade by ryanodine (see Discussion). Moreover in none of the preceding studies were the effects of membrane potential on the observed Ca\(^{2+}\) transients examined.

Key words: calcium imaging; calcium spark; intracellular calcium; neurosecretion; presynaptic; ryanodine receptor

Introduction

From the time that the central role of Ca\(^{2+}\) in neurotransmitter release was first recognized, the study of nerve terminal function and exocytosis has focused intensively on Ca\(^{2+}\) influx (Berridge, 1998). In contrast, Ca\(^{2+}\) release from internal stores in nerve terminals has received less attention, and its role is controversial (Meldolesi, 2001; Carter et al., 2002). A major problem in this field is that release of Ca\(^{2+}\) from presynaptic stores has often been inferred from effects on postsynaptic currents rather than examined directly. Nevertheless, a number of studies using this indirect approach have proved highly informative (Bers, 1999; Llano et al., 2000; Emptage et al., 2001). In addition, direct examination of global changes in neuronal cytosolic [Ca\(^{2+}\)] have shown release from intracellular stores via a calcium-induced Ca\(^{2+}\) release (CICR) mechanism after Ca\(^{2+}\) influx (Narita et al., 1998; Krizaj et al., 1999), but there has been no demonstration of a mechanism wherein depolarization, in the absence of Ca\(^{2+}\) influx, causes Ca\(^{2+}\) release from stores in terminals or in any other neuronal structure.

The direct observation and analysis of miniature Ca\(^{2+}\) release events in non-neuronal cells has contributed greatly to our understanding of the role and regulation of Ca\(^{2+}\) stores (Bers, 2002). These miniature events were first observed in response to IP\(_3\) in oocytes and designated "Ca\(^{2+}\) puffs" by Parker and Yao (1991) and then found in myocytes, where they were mediated by ryanodine receptors (RyRs) and designated "Ca\(^{2+}\) sparks" (Cheng et al., 1993). Ca\(^{2+}\) transients, resembling puffs and sparks in some respects, have been observed in cultured hippocampal preparations (Kozumi et al., 1999), and three recent studies have provided indications of spontaneous transients in nerve terminals. First, Melamed-Book et al. (1999) found outliers in the fluorescence noise of Ca\(^{2+}\) images in cultured hippocampal somata and neuron terminals at the lizard neuromuscular junction, which they compared to spontaneous Ca\(^{2+}\) sparks in muscle. Importantly, in the cell bodies of hippocampal cultures, Melamed-Book et al. (1999) observed the noise outliers in Ca\(^{2+}\)-free medium, whereas in the nerve terminals the Ca\(^{2+}\) transients were not studied in Ca\(^{2+}\)-free medium, leaving their intracellular origin in doubt. Second, Llano et al. (2000) in their landmark study on "maximinis" observed long duration Ca\(^{2+}\) transients lasting seconds in presumed nerve terminals. Third, Emptage et al. (2001) illustrated several instances of spontaneous Ca\(^{2+}\) transients in axon-like processes and synaptic boutons. However, neither of these latter two studies demonstrated spontaneous transients in Ca\(^{2+}\)-free medium or blockade by ryanodine (see Discussion). Moreover in none of the preceding studies were the effects of membrane potential on the observed Ca\(^{2+}\) transients examined.
In the present study we observed spontaneous, miniature, highly localized, short-lived Ca$^{2+}$ transients, which arise from intracellular Ca$^{2+}$ stores in single isolated nerve terminals from hypothalamic neurons. Because they resemble Ca$^{2+}$ sparks in muscle in some, but not all respects, they were designated Ca$^{2+}$ syntillas (scintilla, L., spark, from a synaptic structure, a nerve terminal). We also quantified the transients by using the same signal mass approach previously used in studies of Ca$^{2+}$ sparks (ZhuGe et al., 1999, 2000, 2002). Surprisingly, physiological levels of depolarization, in the absence of Ca$^{2+}$ influx, increased syntilla frequency without significant effect on the amplitude of individual syntillas. To the best of our knowledge, this is the first direct demonstration of release of Ca$^{2+}$ from intraneuronal stores elicited by depolarization in the absence Ca$^{2+}$ influx. By virtue of their regulation by membrane potential, Ca$^{2+}$ syntillas provide a new functional link between neuronal activity and cytosolic [Ca$^{2+}$] in nerve terminals. A preliminary report of this work has been published previously (De Crescenzo et al., 2003).

Materials and Methods

Whole-terminal patching

Tight seal, “whole terminal” recording on nerve terminals (Lemos and Nowycky, 1989) freshly prepared from adult Swiss Webster mice (Nordmann et al., 1987) was done with an Axopatch-1D amplifier. Pipette solution (in mM): 0.05 K$_5$fluoro-3 (Molecular Probes, Eugene, OR), 135 KCl, 2 MgCl$_2$, 30 HEPES, 4 MgATP, and 0.3 Na-GTP, pH 7.2. Bath solution: 135 NaCl, 5 KCl, 10 HEPES, 10 glucose, 1 MgCl$_2$, and 2.2 CaCl$_2$, pH 7.2. Ca$^{2+}$-free bath solution: 135 NaCl, 5 KCl, 10 HEPES, 10 glucose, 0.2 EGTA, and 1 MgCl$_2$, pH 7.2. We added 100 $\mu$M Ca$^{2+}$ and 100 $\mu$M Cd$^{2+}$ to the latter bath solution in the absence of EGTA for experiments with these ions. To prevent depletion of Ca$^{2+}$ from internal stores, the terminals remained in normal Locke’s solution (2.2 mM Ca$^{2+}$) until the beginning of the experiment, and the duration of the Ca$^{2+}$-free protocol was ~15 min (ZhuGe et al., 1999). Resting global cytosolic [Ca$^{2+}$] was determined with the ratiometric indicator fura-2 (50 $\mu$M in the patch pipette) under the same conditions (but fluo-3 absent) was performed according to the method of Grynkiewicz et al. (1985) with an assumed Ca$^{2+}$-fura 2 $K_D$ of 200 nM, as previously described (Becker et al., 1989). Resting values for global cytosolic [Ca$^{2+}$] in the presence and absence of extracellular Ca$^{2+}$ were 68.5 ± 22.3 nM (n = 7) and 46.2 ± 7.5 nM (n = 8), respectively, and these values were not significantly different (p = 0.4). These values agree well with previous estimates in these terminals (Troadeck et al., 1998). Reagents (from Sigma, St. Louis, MO, unless otherwise noted) were bath perfused or delivered by a picospritzer (Parker, Hollis, NH). In the latter case, 170 ± 36 msec (n = 5) was required for the solution to reach the nerve terminal, and an additional 426 ± 126 msec to reach an equilibrium around the terminal, as determined by a “puff” of 100 nM KCl (replacing NaCl) to induce an inward current. Terminals were suspended from the tip of the patch pipette out of contact with the floor of the chamber.

Image acquisition and data analysis

General. Fluorescence images using fluo-3 as a calcium indicator were obtained using a custom-built wide-field digital imaging system (ZhuGe et al., 1999). Rapid imaging at 200 Hz (exposure, 5 msec) or 50 Hz (exposure, 10 msec) was made possible by equipping the system with a cooled high-sensitivity, charge-coupled device camera developed in conjunction with the Massachusetts Institute of Technology Lincoln Laboratory (Lexington, MA) (ZhuGe et al., 1999) (Fig. 1). The camera was interfaced to a custom-made inverted microscope. The terminals were imaged using a 100× Nikon 1.4 NA oil immersion objective, giving a pixel size of 133 nm at the specimen. A laser shutter controlled the exposure duration. The 488 nm line of an argon-ion laser (Coherent) provided fluorescence excitation, and emission of the Ca$^{2+}$ indicator was monitored at wavelengths >500 nm. Subsequent image processing and analysis was performed off-line using a custom-designed software package, running on either a Silicon Graphics or Linux/PC workstation. Two quantitative measures of Ca$^{2+}$ were used: one to assess the properties of transient, focal increases in Ca$^{2+}$, i.e., Ca$^{2+}$ syntillas, and another to assess global increases in Ca$^{2+}$.

Signal mass. To assess the properties of Ca$^{2+}$ syntillas and do so quantitatively, the signal mass approach was used, as conceptualized by Sun et al. (1998) and developed for analysis of Ca$^{2+}$ sparks using wide-field microscopy by ZhuGe et al. (2000). A brief summary of the signal mass method, which is described in detail in ZhuGe et al. (2000) follows. During a syntilla, free Ca$^{2+}$ (diffusion coefficient, $D = 250$ $\mu$m$^2$/sec) and Ca$^{2+}$ bound to fluo-3 ($D = 22$ $\mu$m$^2$/sec) (Smith et al., 1998) quickly diffuse in three dimensions away from the release site as Ca$^{2+}$ continues to be discharged. Therefore, to quantify the increase in total fluorescence, i.e., the Ca$^{2+}$ signal mass ($\Delta$CaFl) resulting from the binding of fluo-3 to the discharged Ca$^{2+}$, fluorescence must be collected from a sufficiently large volume surrounding the release site. The optimal value for this volume was found to be that subtended by an area of 116 $\mu$m$^2$ ($81 \times 81$ pixels). Because photons must be collected from a three-dimensional (3-D) volume and not simply from a single plane, wide-field microscopy is well suited to this method.

To relate the measured increase in fluorescence to the total amount of Ca$^{2+}$ released in a single syntilla (Ca$_T$), a calibration curve was constructed that related measured fluorescence to the amount of Ca$^{2+}$ bound to fluo-3 ($\Delta$CaFl) (O’Reilly et al., 2003). The same illumination conditions, optical pathway, and CCD camera configuration were used as in measurements on the terminals. Glass capillaries (Vitro Dynamics, Rockaway, NJ), with an internal rectangular cross section 20-40 $\mu$m-wide, were loaded with solutions containing known concentrations of fluo-3 (1, 5, 25, 50, 75, 100, and 150 $\mu$M) used to calibrate the photomultiplier tube and saturating [Ca$^{2+}$] (1.45 mM). Data were fitted with a straight line ($r = 0.85$) having a slope of 0.072 intensity units per fluo-3 molecule per msec exposure (14 molecules of bound fluo-3 per count per millisecond).

Figure 1. Relationship between measured fluorescence and amount of Ca$^{2+}$ bound to fluo-3 (CaFl). To determine the total amount of Ca$^{2+}$ released in a syntilla (Ca$_T$), measured fluorescence was determined as a function of the amount of Ca$^{2+}$ bound to fluo-3 (CaFl). Images were acquired of glass capillaries (internal rectangular cross section 20-$\mu$m deep and 200-400 $\mu$m wide) loaded with known concentrations of fluo-3 (1, 5, 25, 50, 100, and 150 $\mu$M) used to calibrate the photomultiplier tube and saturating [Ca$^{2+}$] (1.45 mM). Data were fitted with a straight line ($r = 0.85$) having a slope of 0.072 intensity units per fluo-3 molecule per msec exposure (14 molecules of bound fluo-3 per count per millisecond).
example, each intensity unit measured in a 5 msec imaging period corresponds to ~2.79 molecules of Ca\(^{2+}\) bound to fluo-3.

Because “whole-terminal” patch recording was used at the same time that images were acquired, endogenous mobile buffers were presumably dialyzed away, and so can be neglected. In the absence of all other buffers, 50 \(\mu M\) fluo-3 binds >97% of Ca\(^{2+}\) entering the cytosol for Ca\(^{2+}\) currents ranging from 0.1–10 pA (ZhuGe et al., 2000). Such a Ca\(^{2+}\) current entering the cytosol from intracellular stores in the case of a syntilla is designated by \(I_{Ca(syntilla)}\). Thus, the peak signal mass provides a faithful indicator of Ca\(^{2+}\) in the absence of buffers other than fluo-3. However, endogenous non-mobile buffers alter the magnitude and time course of the total amount of Ca\(^{2+}\) released in a single syntilla, as \(\Delta [Ca] = \Delta ([Ca(Fl)] + \Delta ([CaBu]) \times \text{Volume}, \text{with} [CaFl] \text{the concentration of Ca}^{2+} \text{bound to fluo-3}, [CaBu] \text{the concentration of free Ca}^{2+},\) and [CaBuf] the concentration of Ca\(^{2+}\) bound to endogenous non-mobile buffers.

Noting that \(\Delta [CaFl] = \kappa_{fluor,0} \Delta [Ca^{2+}]\) and \(\Delta [CaBu] = \kappa_{surf} \Delta [Ca^{2+}]\), then \(Ca_T = \Delta CaFl + \Delta CaBu\). Thus, \(\kappa_{fluor,0}\) is determined by simulating syntillas and examining the spatial and temporal profile of Ca\(^{2+}\). Simulations of the spatiotemporal profile of Ca\(^{2+}\) arising from a syntilla

To gain insight into the possible role of syntillas, we modeled a terminal and simulated syntillas, and examined the spatial and temporal profile of free Ca\(^{2+}\) that resulted at the plasma membrane where vesicle fusion together with either 200 \(\mu M\) or 1 \(mM\) extracellular EGTA, we observed Ca\(^{2+}\) syntillas, spontaneous, brief, miniature Ca\(^{2+}\) transients of the type illustrated in Figure 2, A and B (see supplementary material for movie, available at www.jneurosci.org). The Ca\(^{2+}\)-free condition establishes the intracellular origin of the Ca\(^{2+}\) responsible for the syntillas. In these and subsequent experiments, the exposure of the terminals to a Ca\(^{2+}\)-free environment was kept to a minimum, as described in Materials and Methods, to avoid depletion of the intracellular stores. Ca\(^{2+}\) syntillas were also observed at ~80 mV in the presence of normal extracellular Ca\(^{2+}\) (Fig. 2D) and in the presence of both extracellular Ca\(^{2+}\) (100 \(\mu M\)) and Cd\(^{2+}\), the latter applied by puffer pipette (200 \(\mu M\)).
Ca\(^{2+}\) signal mass is directly proportional to the total amount of Ca\(^{2+}\) released into the cytosol in a single syntilla (Ca\(_{syntilla}\)), as given by: Ca\(_{T}\) = \(\Delta C a F l (1 + 1/\kappa_{flu-3} + \kappa_{buf}/\kappa_{flu-3})\), where \(\Delta C a F l\) is the increase in amount of Ca\(^{2+}\) bound to fluo-3, and \(\kappa_{buf}\) and \(\kappa_{flu-3}\) are the "binding ratios" (Naraghi and Neher, 1997) of fluo-3 and non-mobile endogenous buffers (Stuenkel, 1994), respectively, as defined in Materials and Methods. Hence, the signal mass is given in moles of Ca\(^{2+}\) (Fig. 2C), as calculated in Materials and Methods. Because the signal mass is not a measure of Ca\(^{2+}\) concentration ([Ca\(_{syntilla}\)]) but an integrated signal giving Ca\(_{T}\) (Fig. 2C, black lines), its derivative (Fig. 2C, red lines) is a function of the underlying Ca\(^{2+}\) current (I\(_{Ca(syntilla)}\)) entering the cytosol from internal stores. (The ability to determine Ca\(_{T}\) depends on the assumption of negligible removal of Ca\(^{2+}\) from the cytosol before the peak of the signal mass; this appears justified because the rise of the signal mass is considerably faster than its decay and because [Ca\(^{2+}\)], is high only in a small region for a short time).

If syntillas arise solely from intracellular stores, they should not differ in the presence or absence of extracellular Ca\(^{2+}\). This was in fact the case. Spontaneous Ca\(^{2+}\) syntillas at a holding potential (V\(_{h}\)) of \(-80\) mV had the same frequency and the same Ca\(^{2+}\) signal mass in the presence of extracellular Ca\(^{2+}\) as in its absence (Fig. 2D). Because the signal mass of each syntilla at its plateau (Fig. 2C) is directly proportional to the total Ca\(^{2+}\) released and hence to the product of the mean I\(_{Ca(syntilla)}\) and its duration, it is unlikely that either changed substantially in the presence or absence of external Ca\(^{2+}\); otherwise the change in the magnitude of I\(_{Ca(syntilla)}\) must compensate exactly for the change in its duration.

**Ca\(^{2+}\) syntillas are mediated by RyRs**

Four lines of evidence indicated that syntillas are mediated by RyRs. First, ryanodine, a blocker of RyRs at the concentration used (100 \(\mu\)M in the bathing solution) (ZhuGe et al., 1999; Llano et al., 2000), decreased spontaneous syntilla frequency, but did not alter the total amount of Ca\(^{2+}\) released by an individual syntilla (Fig. 3A) (see Discussion). Second, brief (4 sec) applications of caffeine, which increases the probability of RyR opening and inhibits IP\(_3\) receptors (Ehrlich et al., 1994), increased both syntilla frequency and the total Ca\(^{2+}\) released by an individual syntilla (see Discussion). Furthermore, ryanodine blocked this increase in frequency, again without affecting the total Ca\(^{2+}\) released by an individual syntilla (Fig. 3A). Third, ryanodine at a lower concentration (10 \(\mu\)M in the puffer pipette) increased syntilla frequency, similar to the increase in Ca\(^{2+}\) spark frequency.

---

**Figure 2.** Spontaneous Ca\(^{2+}\) syntillas in isolated nerve terminals in Ca\(^{2+}\)-free solution at a membrane potential of \(-80\) mV. A, B, Images of one Ca\(^{2+}\) syntilla in a single isolated terminal in Ca\(^{2+}\)-free solution (200 \(\mu\)M EGTA) at a holding membrane potential, V\(_{h}\) = \(-80\) mV. Contour plots (A) show same syntilla as B. C, Time course, corresponding to the images in A and B, of signal mass (black) and its first derivative (red); expanded time scale on right. D, Neither syntilla frequency (N = 13 and 18, with and without Ca\(^{2+}\), respectively) nor Ca\(^{2+}\) signal mass (\(n = 22\) and 27, with and without Ca\(^{2+}\), respectively) were altered by extracellular calcium at \(V_{h}\).
seen in striated muscle (Gonzalez et al., 2000; Hui et al., 2001), whereas it decreased the total amount of Ca\(^{2+}\) released by an individual syntilla (Fig. 3B) (see Discussion). Fourth, RyRs were identified immunocytochemically in the terminals, and they were not confined to one region of the terminal (Fig. 3C). However, the RyRs were not uniformly distributed throughout the terminal but had a somewhat higher density near the periphery, as is evident from the stereo pair in Figure 3C. Similarly, syntilla sites were not confined to one region of the terminal (Fig. 3D,E). Figure 3D shows images of six different syntillas arising from different sites in the same terminal, and Figure 3E is a map indicating the site of all the syntillas recorded from that terminal. The right panel of Figure 3E indicates the magnitude of each of these syntillas, with the diameter of each circle proportional to the total amount of Ca\(^{2+}\) released in that syntilla. Moreover, thapsigargin (TG; 2 \(\mu\)M in the bathing solution) greatly decreased syntilla frequency from 0.31 \(\pm\) 0.06 \((N = 25)\) to 0.05 \(\pm\) 0.03 \((N = 14)\) \((p = 0.004)\) in the presence of extracellular Ca\(^{2+}\). (The average amount of Ca\(^{2+}\) released per syntilla was \(35.23 \pm 8.10 \times 10^{-20}\) moles \((n = 29)\) in controls versus \(40 \pm 21\) \((n = 2)\), but these values could not be adequately compared because there were so few syntillas in the presence of TG.) The effects of TG indicate that syntilla generation involves a cellular compartment containing SERCA pumps that are generally considered to be confined to the ER. Finally, mitochondria do not appear to be necessary for syntilla generation, because syntillas were still present after 10 min exposure to 2 mM cyanide and 5 mM azide. This treatment was observed to collapse the mitochondrial membrane potential, as measured with a fluorescent probe in parallel experiments (Drummond et al., 2000).

**Quantitative characterization of the Ca\(^{2+}\) syntilla using the signal mass approach**

The signal mass method permits the characterization of the Ca\(^{2+}\) syntilla in a quantitative manner. The distribution (Fig. 4A) of the total amount of Ca\(_T\) per syntilla is exponential with a mean of \(40 \times 10^{-20}\) moles of Ca\(^{2+}\) or \(-250,000\ Ca\ ions released per syntilla. The averaged derivative of signal mass traces (Fig. 4B) gives the minimum value for the peak \(I_{Ca(syntilla)}\) and the maximal duration of \(I_{Ca(syntilla)}\) because buffers act as a filter to decrease the peak and increase the duration of the derivative (see Materials and Methods). Hence, the peak \(I_{Ca(syntilla)}\) is at least 1.88 pA on average, and its duration is at most tens of milliseconds. Despite the exponential distribution of Ca\(_T\), \(I_{Ca(syntilla)}\) does not appear to be caused by a single RyR channel opening for various durations, because the magnitude of Ca\(^{2+}\) current flowing through a single RyR in an artificial bilayer is 0.35 pA (Mejia-Alvarez et al., 1999), considerably smaller than the peak current calculated here (Fig. 4B). Thus, it appears that a syntilla...
and absence of Na⁺ potential the rates were not significantly different in the presence of a low caffeine concentration (1 mM), which increased syntilla frequency but does not alter the amount of Ca²⁺ stores. The importance of Na⁺ influx, which might affect Ca²⁺ in the physiology of these terminals, whereas it was found in 44% of the terminals when Na⁺ was present. The resting level of total fluorescence at −80 mV was not different in the presence versus absence of Na⁺ (2.7 ± 0.3 (arbitrary units; N = 55) versus 2.8 ± 0.2 (N = 57), respectively. In sum, at least part of the increase in syntilla frequency, and perhaps all of it, is found in the absence of external Na⁺ and is therefore independent of Na⁺ influx. In contrast, the increase in global [Ca²⁺] at −40 mV is dependent on the presence of extracellular Na⁺ (see Discussion).

Depolarizations to levels more than or equal to −40 mV or prolonged application of caffeine cause a global increase in cytosolic [Ca²⁺] in the absence of Ca²⁺ influx

As mentioned in the previous section, in 44% of the terminals, depolarization from −80 to −40 mV caused an increase in global cytosolic [Ca²⁺] (Fig. 6A), and this increase was attenuated by ryanodine (Fig. 6B). When the depolarization caused an increase
in global Ca\(^{2+}\), one or several sites responded first with a local release of Ca\(^{2+}\) that was joined by other regions resulting in a rise in global Ca\(^{2+}\) (Fig. 6A). In a given terminal the same site or sites were the first to respond after each of a series of successive depolarizations separated by a waiting interval. After depolarization from −80 to 0 mV, the increase in global cytosolic Ca\(^{2+}\) was greater in magnitude than that at −40 mV, and it was attenuated by ryanodine (Fig. 6A,B). As with the global increase in Ca\(^{2+}\) at −40 mV, the same focus or foci responded first during a series of successive depolarizations.

As described above, brief application of caffeine (4 sec, at −80 mV) from a puffer pipette located 50 μm from a terminal caused an increase in both syntilla frequency and the signal mass of individual syntillas, but not an increase in global Ca\(^{2+}\). Longer application (1–5 min) by bath perfusion (20 mM caffeine) caused a global increase in Ca\(^{2+}\), as illustrated in Figure 6C. As is
apparent in these images, individual localized events could be seen leading up to the global increase and within it. It is also clear from the images of Figure 6C that the global increase is not due to a wave-like spread from an initiation site or sites. As with the global increase induced by depolarization, the caffeine-induced global increase in [Ca^{2+}] was attenuated by ryanodine (Fig. 6D).

**Discussion**

The regulation of Ca^{2+} syntillas by membrane potential

Ca^{2+} syntillas are unique in a variety of ways other than their origin in nerve terminals. The most striking of these distinctive features is the regulation of syntilla frequency by membrane potential in the absence of Ca^{2+} influx. Although CICR has often been suggested as a mechanism to cause Ca^{2+} release from ryanodine-sensitive Ca^{2+} stores in neurons, regulation of stores by membrane potential in the absence of Ca^{2+} influx has not been demonstrated in any neuronal structure or in any exocytotic cell type, to the best of our knowledge. The existence of voltage regulation in the nerve terminals studied here suggests that this mechanism may be present elsewhere in neurons. Regulation of miniature Ca^{2+} release events by membrane potential has been firmly established only in skeletal muscle fibers (Tsugorka et al., 1995), but the results of the present study suggest that an analogous mechanism may exist in nerve terminals. L-type Ca^{2+} channels have been identified in neurohypophysial terminals from the rat (Lemos and Nowycky, 1989), and these may possibly serve as a voltage sensor. This idea is supported by the finding of Chavis et al. (1996) in cerebellar granule cells that RyRs are tightly coupled to L-type Ca^{2+} channels and can enhance their activity. Alternatively, the effect may be the result of an indirect coupling between L-type Ca^{2+} channels and RyRs of the type seen in smooth muscle, which is also independent of Ca^{2+} influx (del Valle-Rodriguez et al., 2003).

In the absence of both extracellular Na^+ and Ca^{2+}, depolarization from −80 to −40 mV elicited an increase in syntilla frequency so that voltage-activated Na^+ influx cannot account for the frequency increase. However, in the presence of extracellular Na^+, there was an additional effect of depolarization that led to a greater increase in global [Ca^{2+}]. This effect of external Na^+ reinforces the importance of Na^+ in the physiology of these terminals (Stuenkel and Nordmann, 1993; Turner and Stuenkel, 1998).

The spatiotemporal profile of Ca^{2+} syntillas

The ability of membrane potential in the absence of Ca^{2+} influx to increase syntilla firing rate suggests that the site of Ca^{2+} release in a syntilla resides quite close to the plasma membrane, a conclusion that is reinforced by maps of syntilla sites (Fig. 3E). Hence, we sought to determine the spatiotemporal profile of [Ca^{2+}] caused by a syntilla in the region of the plasma membrane. To do so, we performed simulations to determine the spatiotemporal profile, as outlined in Materials and Methods, and the results are shown in Figure 7 with the input waveform shown in Figure 7A. The plots in Figure 7, B and C, present information in the same format as confocal line-scans, with time along the x-axis and distance along the y-axis and color providing the scale for concentration. Iso-concentration lines are used, as opposed to a continuous color depiction (as in line-scan images), because they make it possible to present a greater range of concentrations. Figure 7B shows the simulation of a syntilla directly under the PM. The green line is 10 μM (see legend), a concentration believed to be necessary for neuropeptide release (Lee et al., 1992). The 10 μM isoconcentration line reaches its maximum spatial extent, a radius of ~500 nm, at ~10 msec after the peak of I_{Ca}. At a radius of 300 nm, [Ca^{2+}] remains ≥ 10 μM for >40 msec. At distances >500 nm, the concentration will not reach 10 μM. Hence, it appears possible for a syntilla to activate proteins in its microdomain without triggering exocytosis at a site a few hundred nanometers away. This, in turn, means that the nerve terminal may be divided into more Ca^{2+} microdomains than those arising from Ca^{2+} influx alone. The same is true for syntilla sites not at the PM. For example, Figure 7C shows the simulation of a syntilla located 400 nm away from the plasma membrane. In this case, the isoconcentration lines show the values of free [Ca^{2+}] taken along a line directly.
The simulations demonstrate that syntillas are likely to result in supermicromolar concentrations over hundreds of nanometers for tens of milliseconds. Hence, syntillas may affect Ca\(^{2+}\) targets that are sensitive to supermicromolar concentrations of Ca\(^{2+}\) within a small region. A supermicromolar concentration of Ca\(^{2+}\) is necessary to affect some components of vesicle cycling (see below) (Sudhof and Rizó, 1996). Thus, it is possible for syntillas to affect targets of low Ca\(^{2+}\) sensitivity in the syntilla microdomain without acting on targets in different microdomains, perhaps, for example, microdomains inhabited by plasmalemmal Ca\(^{2+}\) channels where exocytosis is triggered.

The nature of the intraterminal stores providing Ca\(^{2+}\) for the syntillas

It is clear that neurohypophyseal nerve terminals contain functional ryanodine receptors, as is evident from the sensitivity of syntillas to both caffeine and ryanodine. Because ryanodine affects both gating and conductance of RyRs in a concentration-dependent way, with an additional level of complexity added by the interaction of RyRs with one another and with other proteins in generating focal Ca\(^{2+}\) release, for example in striated muscle (Fill and Copello, 2002), it is not surprising that its action on syntillas is complicated. At a lower concentration (10 \(\mu\)M in the puffer pipette positioned 50 \(\mu\)m from the terminal; see Materials and Methods), ryanodine increased the syntilla frequency just as low concentrations increase the frequency of Ca\(^{2+}\) sparks in cardiac and skeletal muscle which are also mediated by RyRs (Satoh et al., 1998; Gonzalez et al., 2000; Hui et al., 2001). At this activating concentration of ryanodine, the amount of Ca\(^{2+}\) released per syntilla was decreased. This effect is reminiscent of the presence of low-amplitude focal Ca\(^{2+}\) transients in striated muscle at a concentration in which ryanodine increased spark frequency and is consistent with the induction by lower ryanodine concentrations of low conductance, high Po states of RyRs (Fill and Copello, 2002). At a higher concentration (100 \(\mu\)M in the bathing solution), ryanodine decreased the frequency of the syntillas, as might be expected since it blocks RyRs at higher concentrations (Fill and Copello, 2002). However, at this concentration ryanodine had no effect on the amount of Ca\(^{2+}\) released per syntilla, suggesting perhaps that syntilla sites were silenced by ryanodine after the generation of a syntilla, consistent with the use dependence of ryanodine action. Finally the ability of caffeine to increase the amount of Ca\(^{2+}\) released per syntilla could result from recruitment of more RyRs at a given release site or from an other effect of caffeine, for example an anti-phosphodiesterase action. The results with ryanodine and caffeine are supported by the immunocytochemical identification of RyRs whose distribution, like that of the syntillas, is not confined to one region of the terminal.

The failure of cyanide and azide to affect the syntillas suggests that mitochondria are not the source of the Ca\(^{2+}\) release reported here, although they may eventually turn out to provide some modulatory influences (Koizumi et al., 1999). One intriguing candidate for the source of syntilla Ca\(^{2+}\) is the neurosecretory granules in which preliminary studies using immunogold labeling have disclosed both RyRs and IP\(_3\)Rs (Salzberg et al., 2000). Moreover, in these neurosecretory granules, Ca\(^{2+}\) is stored at high levels that may be altered in the process of depolarization-induced secretion (Thirion et al., 1995). However, it is not clear how these results relate to our finding that TG, which is specific for SERCA pumps, drastically reduced the frequency of syntilla because SERCA pumps are generally considered to reside only on ER membrane. Hence, the possibility arises that more than one intracellular compartment may be involved in the generation of syntillas. Finally, the present study demonstrates that Ca\(^{2+}\) stores within nerve terminals must be considered as more than buffering mechanisms (Castonguay and Robitaille, 2001), because they can act to release Ca\(^{2+}\) in a quantal manner after physiological levels of depolarization.

The effect of Ca\(^{2+}\) syntillas on neurosecretion

It is well known that in nerve terminals extracellular Ca\(^{2+}\) provides the source that triggers neurotransmitter release. How then might Ca\(^{2+}\) from internal stores act to affect the secretion of neuropeptide in the terminals used here? The neurohypophysial nerve terminals possess at least two functionally distinct acutely releasable secretory granule pools that differ in size, rate, and Ca\(^{2+}\) sensitivity of exocytosis (Giovannucci and Stuenkel, 1997). The “immediately releasable pool” is characterized by an immediate jump in and rapid recovery of the resting membrane capacitance, and the “readily releasable pool” is typified by a slower membrane capacitance rise that persists after the stimulus. Giovannucci and Stuenkel (1997) suggested that the activation of unknown Ca\(^{2+}\)-dependent steps prepare the granules of the ready releasable pool for secretion. Indeed, in the trafficking route of recycling vesicles it is not well understood how vesicles that are not competent for release are freed from their restraints and how they travel to the membrane (Betz and Angleson, 1998). In this context several possibilities exist for the function of syntillas. First, it has been suggested that if motors are involved in vesicle mobilization, they must be activated selectively by nerve stimulation (Betz and Angleson, 1998; Ryan, 1999). Syntillas might provide the link between depolarization and motor driven vesicle mobilization. Second, in vitro studies have shown that phosphorylation by Ca\(^{2+}\)-calmodulin kinase II of synapsin I reduces its affinity for the synaptic vesicle (De Camilli et al., 1990), and syntillas might provide the Ca\(^{2+}\) to elicit this phosphorylation and release the vesicle from the restraint imposed by synapsin I. In summary, Ca\(^{2+}\) is thought to have effects on many stages of the synaptic cycle (Sudhof and Rizó, 1996), including the processes by which vesicles are maintained in clusters, mobilized, and recruited for fusion with the plasma membrane, and the Ca\(^{2+}\) provided by syntillas may act on one or more of these processes to alter the level of neurosecretion.

References


Zhuge R, Fogarty KE, Tuft RA, Walsh JV (2002) Spontaneous transient outward currents arise from microdomains where BK channels are expressed to a mean Ca(2+) concentration on the order of 10 micromolar during a Ca(2+) spark. J Gen Physiol 120:15–28.