# The Influence of Sarcoplasmic Reticulum $Ca^{2+}$ Concentration on $Ca^{2+}$ Sparks and Spontaneous Transient Outward Currents in Single Smooth Muscle Cells

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ABSTRACT Localized, transient elevations in cytosolic $Ca^{2+}$, known as $Ca^{2+}$ sparks, caused by $Ca^{2+}$ release from sarcoplasmic reticulum, are thought to trigger the opening of large conductance $Ca^{2+}$-activated potassium channels in the plasma membrane resulting in spontaneous transient outward currents (STOCs) in smooth muscle cells. But the precise relationships between $Ca^{2+}$ concentration within the sarcoplasmic reticulum and a $Ca^{2+}$ spark and that between a $Ca^{2+}$ spark and a STOC are not well defined or fully understood. To address these problems, we have employed two approaches using single patch-clamped smooth muscle cells freshly dissociated from toad stomach: a high speed, wide-field imaging system to simultaneously record $Ca^{2+}$ sparks and STOCs, and a method to simultaneously measure free global $Ca^{2+}$ concentration in the sarcoplasmic reticulum ($[Ca^{2+}]_{SR}$) and in the cytosol ($[Ca^{2+}]_{CYTO}$) along with STOCs. At a holding potential of 0 mV, cells displayed $Ca^{2+}$ sparks and STOCs. $Ca^{2+}$ sparks were associated with STOCs; the onset of the sparks coincided with the upstroke of STOCs, and both had approximately the same decay time. The mean increase in $[Ca^{2+}]_{CYTO}$ at the time and location of the spark peak was ~100 nM above a resting concentration of ~100 nM. The frequency and amplitude of spontaneous $Ca^{2+}$ sparks recorded at −80 mV were unchanged for a period of 10 min after removal of extracellular $Ca^{2+}$ (nominally $Ca^{2+}$-free solution with 50 μM EGTA), indicating that $Ca^{2+}$ influx is not necessary for $Ca^{2+}$sparks. A brief pulse of caffeine (20 mM) elicited a rapid decrease in $[Ca^{2+}]_{SR}$ in association with a surge in $[Ca^{2+}]_{CYTO}$ and a fusion of STOCs, followed by a fast restoration of $[Ca^{2+}]_{CYTO}$ and a gradual recovery of $[Ca^{2+}]_{SR}$ and STOCs. The return of global $[Ca^{2+}]_{CYTO}$ to rest was an order of magnitude faster than the refilling of the sarcoplasmic reticulum with $Ca^{2+}$. After the global $[Ca^{2+}]_{CYTO}$ was fully restored, recovery of STOC frequency and amplitude were correlated with the level of $[Ca^{2+}]_{SR}$, even though the time for refilling varied greatly. STOC frequency did not recover substantially until the $[Ca^{2+}]_{SR}$ was restored to 60% or more of resting levels. At $[Ca^{2+}]_{SR}$ levels above 80% of rest, there was a steep relationship between $[Ca^{2+}]_{SR}$ and STOC frequency. In contrast, the relationship between $[Ca^{2+}]_{SR}$ and STOC amplitude was linear. The relationship between $[Ca^{2+}]_{SR}$ and the frequency and amplitude was the same for $Ca^{2+}$ sparks as it was for STOCs. The results of this study suggest that the regulation of $[Ca^{2+}]_{SR}$ might provide one mechanism whereby agents could govern $Ca^{2+}$ sparks and STOCs. The relationship between $Ca^{2+}$ sparks and STOCs also implies a close association between a sarcoplasmic reticulum $Ca^{2+}$ release site and the $Ca^{2+}$-activated potassium channels responsible for a STOC.

KEY WORDS: $Ca^{2+}$ spark • spontaneous transient outward current • Mag-fura-2 • $[Ca^{2+}]_{SR}$ • ryanodine receptor

## INTRODUCTION

$Ca^{2+}$ signaling has long been treated in terms of global changes in cytosolic $Ca^{2+}$ even though $Ca^{2+}$ must serve as a signal for many different processes, suggesting that $Ca^{2+}$ elevations might be targeted to different regions of the cell or "microdomains." In recent years, attention has increasingly been drawn to highly localized $Ca^{2+}$ changes within the cell. Such highly localized $Ca^{2+}$ signals are of importance for two reasons (Berridge, 1997). First, in many important processes, the global elevation in $Ca^{2+}$ is actually the sum of highly localized $Ca^{2+}$ elevations due to release from discrete foci in the sarcoplasmic reticulum (SR).[1] Hence, localized elevations can be the "elementary events" underlying a global rise in $Ca^{2+}$. Second, and perhaps even more interestingly, such focal $Ca^{2+}$ signals might perform localized and very specific signaling functions in the absence of a global elevation in cytosolic $Ca^{2+}$. For example, localized elevations in $Ca^{2+}$ are thought to regulate large conductance $Ca^{2+}$-activated potassium channels

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[1]*Abbreviations used in this paper:* BK channel, $Ca^{2+}$-activated potassium channel; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; STOC, spontaneous transient outward current.

(BK channels) in the surface membrane of both neurons and smooth muscle cells. Such elevations in $Ca^{2+}$ were first postulated by Brown et al. (1983), who called them $Ca^{2+}$ "packets" that might serve as "internal transmitters" to explain the spontaneous miniature outward currents caused by activation of groups of BK channels in bullfrog sympathetic ganglion cells.

In cardiac, skeletal, and smooth muscle cells, such transient, localized cytosolic $Ca^{2+}$ elevations are called $Ca^{2+}$ sparks. In smooth muscle, the existence of $Ca^{2+}$ sparks was first inferred from the appearance of spontaneous transient outward currents (STOCs) that are caused by the concerted opening of a number of BK channels and that have been observed in a wide variety of smooth muscle types (Bolton and Imaizumi, 1996). The $Ca^{2+}$ sparks that cause STOCs have now been directly observed by others and ourselves in a number of smooth muscle types (Nelson et al., 1995; Kirber et al., 1996; Mironneau et al., 1996; ZhuGe et al., 1998a).

The regulation of $Ca^{2+}$ sparks in smooth muscle and other cell types has only recently been addressed. Several regulatory factors for $Ca^{2+}$ sparks have been suggested or demonstrated, among them cyclic nucleotides (Porter et al., 1998), cytosolic $Ca^{2+}$ (Cheng et al., 1996), and luminal $Ca^{2+}$ concentration; that is, $Ca^{2+}$ concentration within the sarcoplasmic reticulum ($[Ca^{2+}]_{SR}$) (Lukyanenko et al., 1996). In the case of luminal $Ca^{2+}$, possible evidence for its role in regulating sparks comes in a study of ventricular myocytes from phospholamban-deficient knock-out mice (Santana et al., 1997). However, in that study, the level of luminal $Ca^{2+}$ was inferred rather than measured directly since there was no way to quantify the actual level of luminal $Ca^{2+}$. Moreover, there is no study of the effects of luminal $Ca^{2+}$ on $Ca^{2+}$ sparks in smooth muscle and no direct measure of the effect of luminal $Ca^{2+}$ on STOCs in any preparation.

It has been postulated that the frequency of STOCs in smooth muscle reflects the level of $[Ca^{2+}]_{SR}$ (Bolton and Imaizumi, 1996), but there has been no direct evidence for this since simultaneous measurements of both STOCs and $[Ca^{2+}]_{SR}$ have not been made. In the present study, we use the low affinity $Ca^{2+}$ indicator, mag-fura-2, to make measurements of $[Ca^{2+}]_{SR}$ while monitoring STOCs in a single smooth muscle cell with tight-seal, whole-cell recording. We show that both $Ca^{2+}$ sparks and STOCs are abolished upon depletion of SR $Ca^{2+}$ and that they recover as the SR reloads. Further, the SR recovers much more slowly than the cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_{CYTO}$). We also demonstrate for the first time by direct measurement a steep relationship between the level of luminal $Ca^{2+}$ and the frequency of $Ca^{2+}$ sparks and STOCs over a restricted range of $[Ca^{2+}]_{SR}$. These findings suggest that agents that act to regulate $Ca^{2+}$ sparks and STOCs, and hence the contractile state of smooth muscle, might exert their effects in part by altering $[Ca^{2+}]_{SR}$.

## METHODS AND MATERIALS

### *Preparation of Cells and Electrophysiology*

Single smooth muscle cells were enzymatically dispersed from the stomach of *Bufo marinus* as described previously (Fay et al., 1982). Membrane currents were recorded with either the Axopatch 1D or Axoclamp 2A (Axon Instruments) in the tight-seal, whole-cell recording configuration. Over 50% of the cells displayed STOCs under the conditions employed. Extracellular solution contained (mM): 130 NaCl, 3 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, pH adjusted to 7.4 with NaOH. Pipette solution contained (mM): 137 KCl, 3 $MgCl_2$, 10 Hepes, 3 $Na_2ATP$, pH adjusted to 7.2 with KOH; free $[Mg^{2+}]$ in this solution was calculated to be 0.63 mM. All experiments were carried out at room temperature. Recordings of whole-cell currents were low-pass filtered with the single-pole filter of the Axoclamp 2A (100-Hz cutoff), digitally sampled at 300 Hz, and stored for analysis. In experiments where $Ca^{2+}$ sparks were imaged simultaneously with the current, the low frequency cutoff, using the internal four-pole Bessel filter of the Axopatch 1D, was 200 Hz and the sampling rate was 1 kHz. STOCs were detected using a custom algorithm to detect peaks in the current. Peaks were determined using a running average of 18 ms. Once a peak was found, valleys were then checked on either side also using a running average with a window of 18 ms. The valley after the peak was then used as the next starting point to detect the next peak. Net peak current of any found STOC was calculated from the peak current minus the average of the two valley currents. Outward current transients that exceeded 10 pA were counted as STOCs.

### *Measurements of Global $[Ca^{2+}]$ in Cytosol and SR*

Global $[Ca^{2+}]$ was measured using a high temporal resolution microfluorimeter as described previously (Becker et al., 1989). Mag-fura-2 acetoxymethylester (1 μM) was loaded into the cells as described in RESULTS. For measurements with this dye, fluorescence was converted to $[Ca^{2+}]$ (Grynkiewicz et al., 1985) using a $K_d$ for $Ca^{2+}$-mag-fura-2 of 54 μM; determining $R_{max}$, $R_{min}$, and β as previously described (Becker and Fay, 1987). In the absence of $Mg^{2+}$, this calculation gave a resting mean $[Ca^{2+}]_{SR}$ of 154 μM determined in 172 cells. This calibration depends on a variety of factors, which cannot be determined with certainty in vivo (Golovina and Blaustein, 1997). However, in A7r5 cells, a cell line derived from smooth muscle, Sugiyama and Goldman (1995) found that alterations in $[Mg^{2+}]_{SR}$, ranging from 0 to 20 mM, did not alter the mag-fura-2 fluorescence ratio when $[Ca^{2+}]_{SR}$ was held constant at 100 μM. From these and other observations, Sugiyama and Goldman (1995) concluded that, with the diminished $Mg^{2+}$ sensitivity of mag-fura-2 in the presence of the relatively high $[Ca^{2+}]$ of the SR, measurements of changes in $[Ca^{2+}]_{SR}$ were unlikely to be changed significantly by concomitant changes in $[Mg^{2+}]_{SR}$ (see also Hofer and Schulz, 1996; Quamme et al., 1993). Finally, our conclusions here depend on changes in $[Ca^{2+}]_{SR}$, not on absolute values. In those experiments where global $[Ca^{2+}]_{SR}$ and $[Ca^{2+}]_{CYTO}$ were measured simultaneously in the same cell, we used a custom-built, high-speed multiple-wavelength microfluorimeter equipped with a 150-W xenon lamp. In such experiments, $Ca^{2+}$ Green-1 dextran, potassium salt (mol wt 3000, 10 μM) was introduced via the patch pipette into cells preloaded with mag-fura-2 acetoxymethylester (see RESULTS) and excited at 490 nm. Mag-fura-2 was excited at 340 and 380 nm. Every 20 ms, the fluorescence was mea-

sured at 535 nm for a period of 5 ms for each excitation wavelength. For $Ca^{2+}$ Green measurements, $[Ca^{2+}]_{CYTO}$ was calculated as described previously (Hernandez-Cruz et al., 1990), taking the resting $[Ca^{2+}]_{CYTO}$ as 100 nM based on previous measurements in these cells using fura-2 (Drummond and Fay, 1996).

### *Digital Imaging of $Ca^{2+}$ Sparks*

Fluorescence images of cytosolic free $Ca^{2+}$ using fluo-3 as a calcium indicator were achieved using a custom-built wide-field digital imaging system or ultrafast microscope (see Fig. 1). The system can acquire images at a maximum speed of 543 Hz, thus providing a temporal resolution comparable to the confocal line-scan technique, but with a much larger observed area. Such rapid imaging 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; see Fig. 1, legend). The camera was interfaced to a custom made inverted microscope. The 488 nm line of an Argon Ion laser (Coherent) provided fluorescence excitation, and a laser shutter controlled the exposure duration. 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 a Silicon Graphics workstation. $Ca^{2+}$ images were derived on a pixel to pixel basis from the equation $\Delta F/F_0\ (\%) = 100 \times [F(x,y,t) - F_0(x,y)]/F_0(x,y)$, where $F(x,y,t)$ is the fluorescence at each pixel in the time series and $F_0$ is an image of the "resting" level given by the average of several images over time in the absence of sparks. The change in fluorescence provides only a relative, not an absolute, measurement of free $Ca^{2+}$ concentration. An increase in fluorescence was considered to be a $Ca^{2+}$ spark when it was ≥5.0% and lasted for at least two 10-ms frames. The root mean square (rms) noise, following smoothing of the images with a three-by-three pixel approximation to a Gaussian, was both measured from the data and calculated from the noise properties of the CCD camera; it was 1.5% in each case. Thus, the threshold for a spark, 5%, was more than three times greater than the rms noise.

### *Data Analysis and Reagents*

Data are reported as mean ± SEM, and $n$ refers to the number of cells. Statistical analysis of difference was made with paired or unpaired Student's $t$ test, as appropriate, with $P < 0.05$ considered significant. Mag-fura-2, fluo-3, and $Ca^{2+}$ Green-1 were purchased from Molecular Probes, Inc., and all other chemicals from Sigma Chemical Co.

## RESULTS

### *$Ca^{2+}$ Sparks and STOCs Recorded in the Same Cell*

To establish the relationship between $Ca^{2+}$ sparks and STOCs, we first recorded both events simultaneously in the same cell using the ultrafast microscope diagrammed in Fig. 1 and standard patch clamp method-

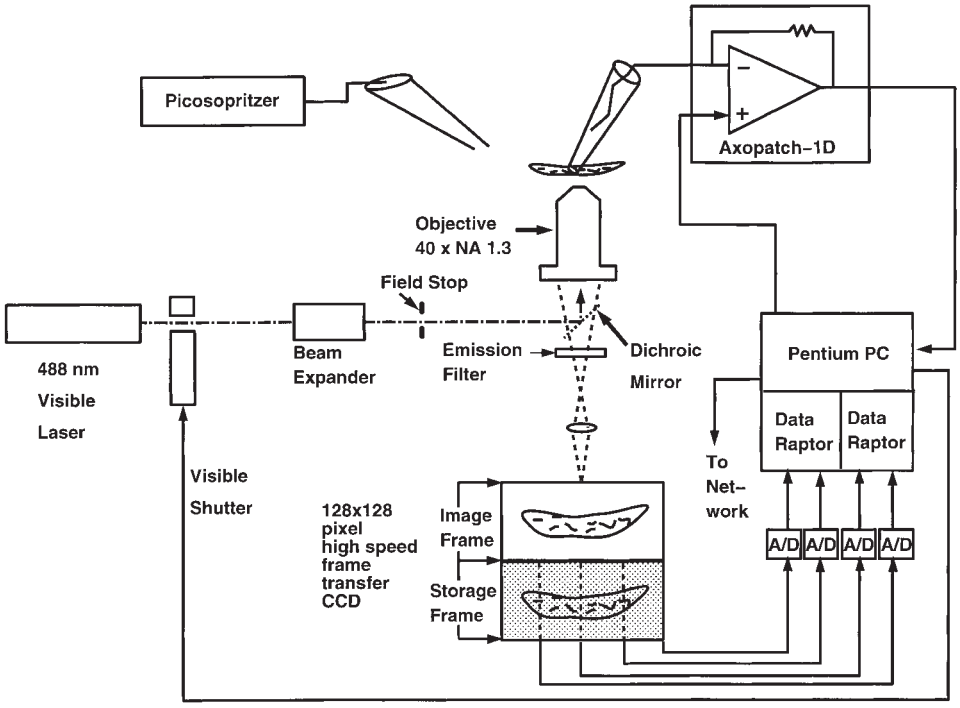


FIGURE 1. Schematic illustration of the ultrafast digital imaging system used to collect two-dimensional images of fluo-3 fluorescence. The beam of an Argon laser, tuned to λ = 488 nm, is passed through an electronically controlled shutter and telescopic beam expander. A field stop iris diaphragm typically limits the illumination to a region of the specimen ~100 μm in diameter. The expanded beam is reflected by a dichroic mirror in the body of a custom-built epifluorescence microscope, passes through the objective (40× 1.3 NA Nikon oil immersion) and illuminates the cell. The fluo-3 fluorescence is collected by the objective, passes through the dichroic mirror and a 500-nm long-pass emission filter, and is imaged onto a high-speed, low-noise (70% quantum efficiency, 6.5 electrons root mean square readout noise), thermoelectrically cooled frame transfer CCD camera. The camera image frame is an array of 128 × 128 24-μm square pixels; the storage frame is of identical size. The detected image is shifted from the image to the storage frame in 50 μs, and the storage frame read out in 1.8 ms. Since acquisition of a new image can begin as soon as the image frame is cleared, the maximum camera rate is 543 frames/s. In this experiment, images were integrated for 10 ms and read out immediately, resulting in an image rate of 100 images/s. Each pixel samples a 333-nm square of the specimen. The camera has four digital outputs connected to two frame grabber boards (Data Raptor; Bitflow Inc.) connected to the PCI bus of a Pentium 90 MHz PC. The images are collected and stored in frame grabber memory, which can hold 8 MB of information, and later transferred to disk archival storage. The PC has an analogue to digital converter that digitizes the whole cell patch clamp current as well as the position of the objective, the image readout times, and membrane potential. Thus, the exact timing of the electrophysiological events in relation to the acquired images is known. Patch clamp recordings are done with an Axopatch 1D patch clamp amplifier. Agents are applied by pressure ejection with a Picosopritzer II (General Valve Corp.).

ology (Hamill et al., 1981). The $Ca^{2+}$ indicator fluo-3 (50 μM) was loaded into the cells through the patch pipette. At a holding membrane potential of 0 mV, the smooth muscle cells displayed $Ca^{2+}$ sparks and coincident STOCs as illustrated in Fig. 2. Each spark was associated with a STOC in this sequence, with both spark and STOC rising simultaneously. (However, the spark for each STOC is not evident since whole-cell patch recording registers all STOCs in the cell, whereas the image captures only a portion of the cell.) In this smooth muscle cell type, the mean half time of spark decay (~20 ms) was close to that of the STOCs (17.0 ± 1.7 ms; $n$ = 15 cells). The mean amplitudes of $Ca^{2+}$ sparks and STOCs were 10.8 ± 0.2% and 27.2 ± 5.2 pA ($n$ = 15 cells), respectively.

The $Ca^{2+}$ sparks were visible as distinct, isolated events restricted to a small area of the cell, usually covering <3 $\mu m^2$ at the time of their peak amplitude. Hence, they are quite different from the $Ca^{2+}$ waves seen in many cell types. For a given cell, there were multiple spark-generating foci, with each focus discharging in an apparently random way. Moreover, it appeared that some foci discharged at a much higher rate than others and thus may constitute "hot spots," similar to those first identified in esophageal smooth muscle cells (Kirber et al., 1998; see also Gordienko et al.,

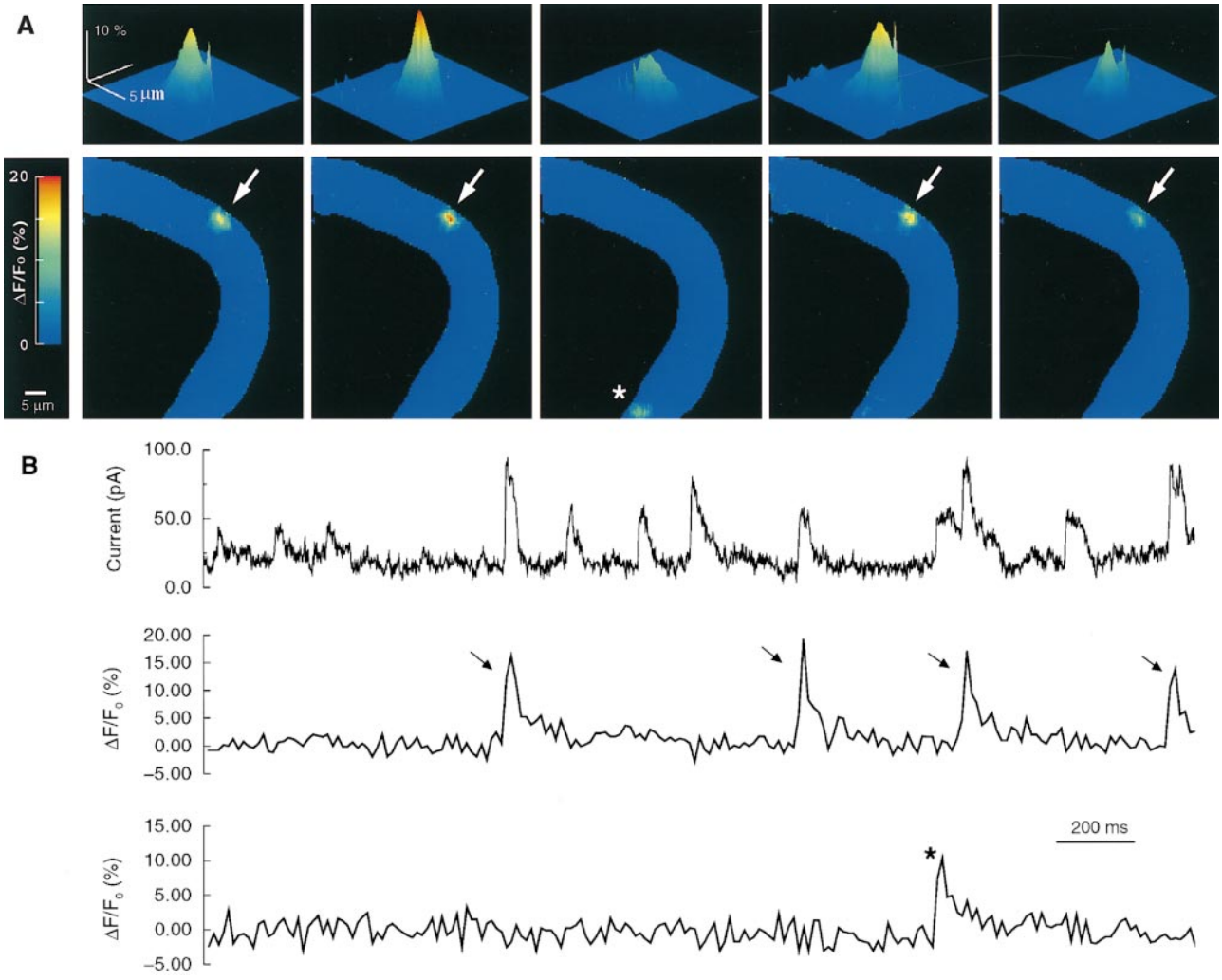


FIGURE 2. $Ca^{2+}$ sparks and STOCs recorded simultaneously in a single smooth muscle cell. $Ca^{2+}$ sparks were monitored with the ultrafast microscope while membrane currents were recorded in the tight-seal, whole-cell mode as described in METHODS. The patch pipette contained 50 μM $K_5$ fluo-3. Approximately 10 min after rupturing the patch membrane, two-dimensional fluorescence images were acquired continuously at a rate of 100 Hz. After low-pass filtering at 200 Hz, the membrane current was digitally sampled at 1 kHz at a holding potential of 0 mV. (A) Images (10-ms exposures) of fluo-3 fluorescence showing five $Ca^{2+}$ sparks from two sites, four at one site (arrows) and one at the other (*), during a 2-s recording period. These images were obtained at the time of the peak spark intensity (B, middle and bottom). The images in A, top, show the $Ca^{2+}$ sparks below with a surface plot at higher spatial magnification. (B, top) Continuous records of whole-cell currents. (Middle and bottom) Continuous records of fluorescence intensity for two different pixels, each 333 × 333 nm. (Middle) Fluorescence for the pixel at the center of the sparks indicated by the arrows; (bottom) fluorescence for the pixel at the center of the spark indicated by the asterisk.

1998). For example, in Fig. 2, there were two spark-generating foci in the approximately one third of the cell surveyed in this case during the 2-s time period shown. Whereas one focus discharged only once, the other discharged four times.

The coincidence and similar time course of $Ca^{2+}$ sparks and STOCs provide strong evidence that sparks are responsible for generating STOCs. To further examine this point, we tested the effect of altering spark frequency on STOC generation. To do so, we employed caffeine, which is known to cause $Ca^{2+}$ release from internal stores through activation of ryanodine receptors (RyRs), which presumably underlie $Ca^{2+}$ sparks in smooth muscle, as is the case in other preparations (Cheng et al., 1993; Xu et al., 1994; Nelson et al., 1995; Tsugorka et al., 1995). In the presence of 0.5 mM caffeine at a holding potential of 0 mV, a lower concentration than was used to deplete the SR and abolish sparks and STOCs in these cells (see below), there was an increase in frequency of both sparks (a 2.2-fold increase from 1.3 ± 0.4/s to 2.9 ± 0.4/s; 184 sparks in five cells; $P < 0.05$) and STOCs (a 2.4-fold increase from 2.5 ± 0.7/s to 5.9 ± 1.1/s; 443 STOCs in the same five cells; $P < 0.05$). Hence, consistent with earlier studies on other smooth muscle cells (Nelson et al., 1995; Mironneau et al., 1996), STOCs in these cells are due to $Ca^{2+}$ sparks. That STOCs are caused by sparks does not imply, however, that every spark causes a STOC; in some instances we observed sparks that failed to cause STOCs (see also Kirber et al., 1998).

### *STOCs are Due to BK Channels*

In other types of smooth muscle, STOCs are thought to result from coincident openings of a cluster of BK channels (Bolton and Imaizumi, 1996). To establish the identity of channels underlying the STOCs in these cells, we examined the effects of extracellular $K^+$ and iberiotoxin, a specific inhibitor of BK channels (Galvez et al., 1990). In normal (3 mM) extracellular $K^+$, STOC activity was apparent at holding potentials of −60 mV or more positive, with greater amplitude at more positive potentials (Fig. 3 A, top), consistent with the voltage dependence of STOCs in other smooth muscle preparations (Bolton and Imaizumi, 1996). In 45 mM extracellular $K^+$ (Fig. 3 A, middle), the STOCs reversed in sign in the region of −20 mV, close to the calculated $K^+$ reversal potential of −25 mV for these cells. Moreover, the STOCs induced by depolarization were eliminated by 100 nM iberiotoxin (Fig. 3 B), as expected for events caused by BK channels. Finally, it is of considerable interest that STOCs occur at a potential of −80 mV (Fig. 3 A, bottom), given the $Ca^{2+}$ sensitivity of BK channels in these cells (see DISCUSSION).

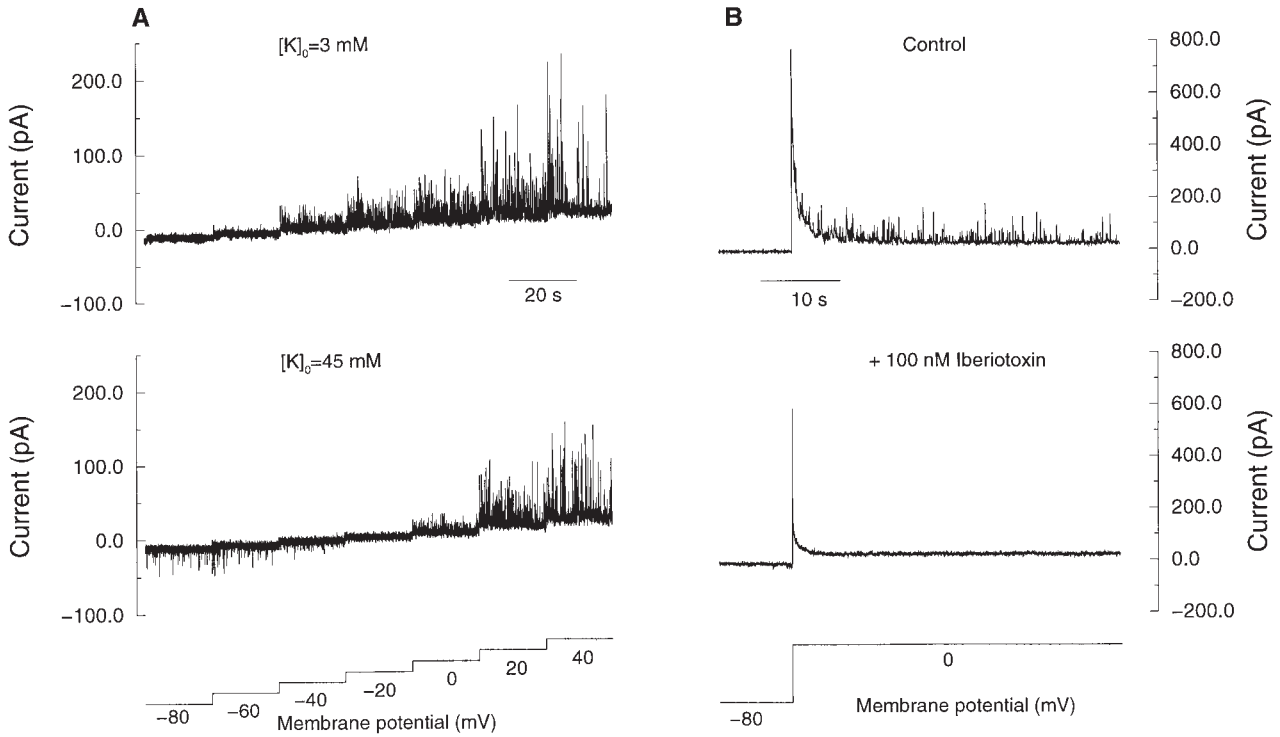


FIGURE 3. STOCs arise from openings of BK channels. (A) Dependence of STOCs on extracellular $K^+$ and membrane potential. The voltage was increased in stepwise fashion, as shown, at two different concentrations of external $K^+$. (B) Blockade of STOCs by iberiotoxin (100 nM).

### *$Ca^{2+}$ Sparks Occur in the Absence of Extracellular $Ca^{2+}$*

Considerable evidence has accumulated in other smooth muscle cells and in neurons that the $Ca^{2+}$ causing STOCs is from an intracellular source as opposed to entry through the surface membrane (Brown at al., 1983; Bolton and Imaizumi, 1996). This is consistent with the idea that STOCs are caused by $Ca^{2+}$ sparks and that $Ca^{2+}$ sparks are due to release of $Ca^{2+}$ from intracellular stores. To establish the source of $Ca^{2+}$ sparks in these cells, we tested whether spontaneous $Ca^{2+}$ sparks that occur at −80 mV are independent of extracellular $Ca^{2+}$. As shown in Fig. 4, the frequency and amplitude of the sparks were unchanged in the presence and absence of extracellular $Ca^{2+}$ at this holding potential. (To eliminate extracellular $Ca^{2+}$, 50 μM EGTA was added to a nominally $Ca^{2+}$-free solution. Sparks were then monitored for a period that ranged from 3 to 10 min after superfusion with this solution. Longer periods in $Ca^{2+}$-free solution were not employed to avoid effects due to possible depletion of intracellular stores.) Spark amplitude and rate were 10.9 ± 0.93% and 0.5 ± 0.24/s, respectively, in the presence of extracellular $Ca^{2+}$, values that were not significantly different from 9.4 ± 0.9% and 0.45 ± 0.30/s in its absence ($n$ = 4 cells). Hence, $Ca^{2+}$ sparks do not require $Ca^{2+}$ entry through the surface membrane. We then went on to establish in direct fashion that SR $Ca^{2+}$ is the source of $Ca^{2+}$ sparks and therefore the cause of STOCs.

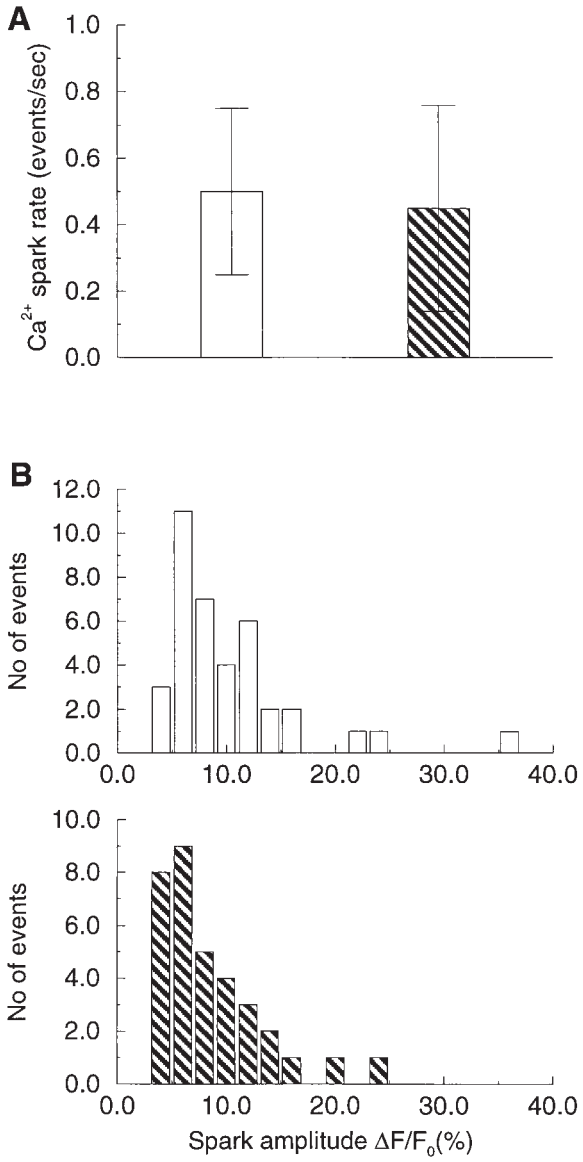


FIGURE 4. $Ca^{2+}$ sparks occur without $Ca^{2+}$ entry through the surface membrane. At a holding potential of −80 mV, $Ca^{2+}$ sparks were recorded from each cell in the presence of extracellular $Ca^{2+}$ (1.8 mM), and then for a period of 10 min or less after removal of extracellular $Ca^{2+}$. The $Ca^{2+}$-free environment was established by superfusing cells with nominally $Ca^{2+}$-free solution containing 50 μM EGTA. The spark rate (A) and amplitude (B) were the same in the presence (open bars) and absence (striped bars) of extracellular $Ca^{2+}$.

### *Mag-Fura-2 Measures $[Ca^{2+}]_{SR}$*

To directly evaluate the role of $[Ca^{2+}]_{SR}$ in the generation of $Ca^{2+}$ sparks and STOCs, we employed the following method to measure $Ca^{2+}$ in internal stores based on techniques used in other cell types (Hofer and Machen, 1993; Chatton et al., 1995; Mlinar and Fay, 1995). Cells were incubated with the esterified form of the low affinity calcium indicator, mag-fura-2. Upon entering the cells, mag-fura-2 acetoxymethylester was hydrolyzed and trapped both in the cytosol and other intracellular compartments (Williams et al., 1985). A tight-seal, whole-cell patch recording configuration was then used to dialyze the cytosol against the contents of the patch pipette. Upon rupture of the patch membrane, the fluorescence ratio gradually increased (Fig. 5 A), indicating that the nondialyzable dye was trapped in a compartment containing higher free $[Ca^{2+}]$ than that in the bulk cytosol. The following experiments were carried out to determine if this compartment was indeed the SR.

Since caffeine activates RyRs causing $Ca^{2+}$ release from the SR (Xu et al., 1994), the effect of caffeine on the mag-fura-2 signal was examined. As shown in Fig. 5 B, the fluorescence ratio decreased rapidly in response to caffeine (20 mM). After cessation of caffeine application, the fluorescence ratio recovered back towards its prestimulus level, but this recovery was completely blocked in the presence of 1 μM thapsigargin (Fig. 5 B), an inhibitor of SR calcium pumps (Thastrup et al., 1990). In contrast, a mitochondrial uncoupler, the protonophore carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone (1 μM), which inhibits $Ca^{2+}$ uptake by mitochondria in these cells (Drummond and Fay, 1996), had no effect on the mag-fura-2 fluorescence ratio (Fig.

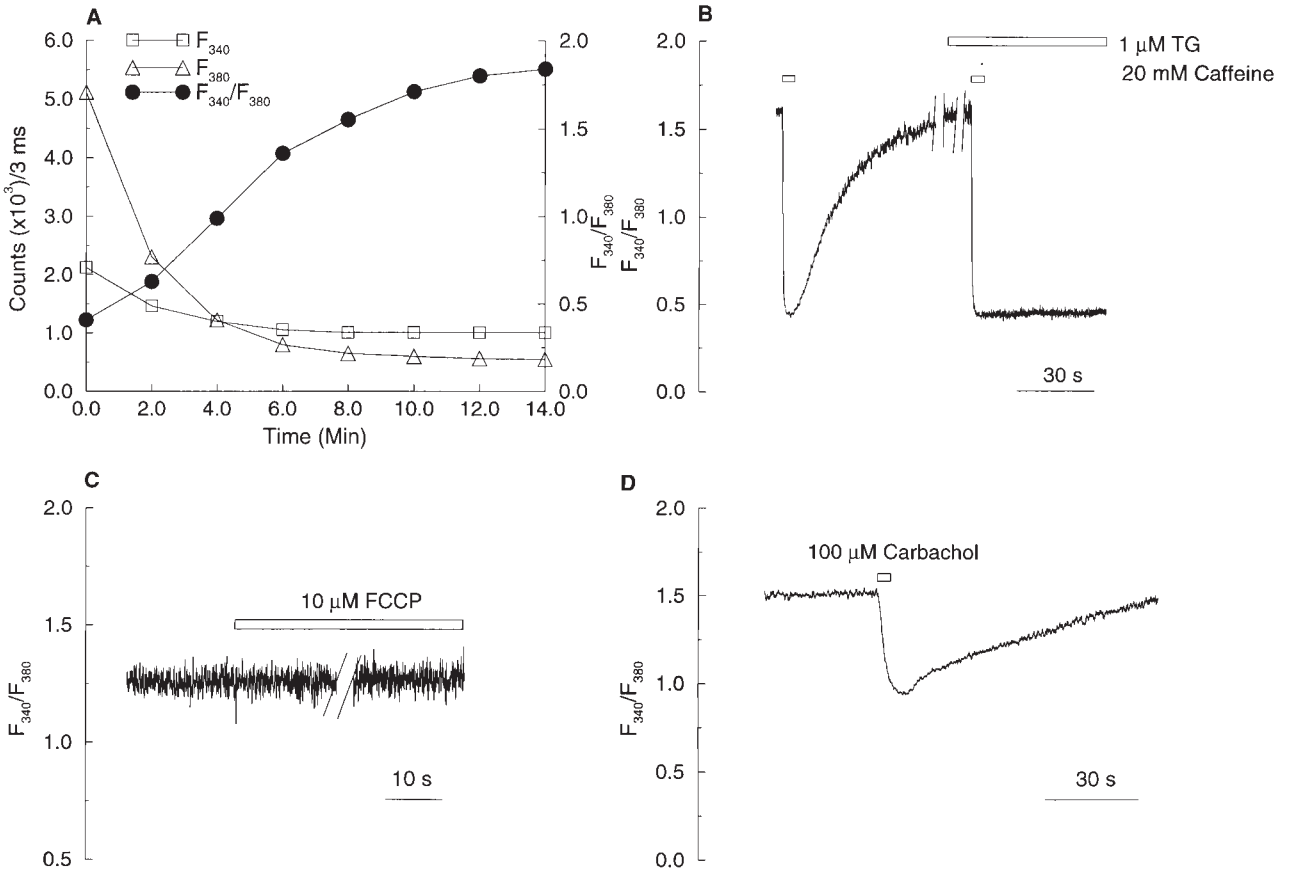


FIGURE 5. Mag-fura-2 detects free $Ca^{2+}$ concentration in sarcoplasmic reticulum. (A) After loading of mag-fura-2 into the cytoplasm and the intracellular stores by incubation with the esterified form of the dye, tight-seal whole-cell recording was begun at time 0, which denotes the occurrence of membrane breakthrough. Fluorescence from a no-nuclear region of the cell was sampled for 10 s at 2-min intervals as shown. The fluorescence ratio increased as dye was dialyzed away, indicating that the dye remaining in the cell was trapped in a compartment with high $[Ca^{2+}]$. (B) Mag-fura-2 signal in single smooth muscle cells responds to caffeine and thapsigargin (TG), consistent with localization of the dye in the SR. Trace shows effect of caffeine before and after treatment with TG on the fluorescence ratio in a single cell dialyzed as in A. The recording was stopped for 5 min (first gap) after the first brief application of caffeine; it was stopped again for 5 min after application of 1 μM TG (second gap). Note that by itself TG had no effect on fluorescence ratio over this 5-min period, as is evident by the fact that the trace did not shift after the second 5-min gap. Caffeine (20 mM) was applied locally from a puffer pipette for 3 s at the times indicated by the bars. (C) Mag-fura-2 signal did not change in response to the application of the mitochondria uncoupler carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone (FCCP; 1 μM). The recording was stopped for 3 min as indicated by the gap in the trace. (D) Carbachol (100 μM) applied locally for 3 s induced a decrease in the mag-fura-2 ratio, consistent with an SR localization of the indicator.

5 C). Carbachol (100 μM), an inositol 1,4,5-trisphosphate–producing agent, caused a decrease followed by a recovery in fluorescence ratio, although this decrease was less than that caused by 20 mM caffeine (Fig. 5 D). In another set of experiments, inclusion of 100 μM ryanodine (Xu et al., 1994) in the patch pipette also led to a decline in the mag-fura-2 fluorescence ratio, albeit more slowly, to about the same level observed with caffeine (data not shown). In summary, the sensitivity of the mag-fura-2 signal to agents known to act on the SR indicates that the mag-fura-2 signal arises principally from the SR in these cells. This conclusion is also supported by an earlier demonstration that mag-fura-2 displays the same submembranous distribution pattern in these cells as does calsequestrin, which is localized to SR (Steenbergen and Fay, 1996). Finally, mag-fura-2 has also been demonstrated to be a reliable indicator of $[Ca^{2+}]_{SR}$ in A7r5 cells, a cell line derived from rat aorta smooth muscle cells (Sugiyama and Goldman, 1995).

### *The Relationship between $[Ca^{2+}]_{SR}$ and STOCs: Simultaneous Measurements of $[Ca^{2+}]_{CYTO}$, $[Ca^{2+}]_{SR}$, and STOCs*

Having established the methods to directly measure $[Ca^{2+}]_{SR}$, we then sought to examine the relationship between $[Ca^{2+}]_{SR}$, $[Ca^{2+}]_{CYTO}$, and the generation of STOCs. To do so, we simultaneously monitored, at high temporal resolution, mag-fura-2 fluorescence originating from the SR and $Ca^{2+}$ Green fluorescence

originating from the cytosol. We used the same experimental approach as above to load mag-fura-2, but for these experiments $Ca^{2+}$ green was included in the patch pipette. Brief exposure to caffeine (Fig. 6) caused a prompt release of $Ca^{2+}$ from the SR, as indicated by a rise in $[Ca^{2+}]_{CYTO}$ and a fall in $[Ca^{2+}]_{SR}$. $[Ca^{2+}]_{CYTO}$ returned rapidly ($t_{1/2}$ = 4.6 ± 0.7 s) to normal resting levels, whereas it took well over 10× as long for the $[Ca^{2+}]_{SR}$ to return to its resting level ($t_{1/2}$ = 62.8 ± 5.8 s; $n$ = 12). As can be seen from Fig. 6, the recovery of the STOCs began well after global $[Ca^{2+}]_{CYTO}$ had returned to an unchanging resting level and hence STOC frequency is not a function of global $[Ca^{2+}]_{CYTO}$ during this period.

We next analyzed STOC activity during the period after $[Ca^{2+}]_{CYTO}$ had returned to rest and while the SR was still refilling. Although the refilling of the SR was always at least an order of magnitude slower than the restoration of $[Ca^{2+}]_{CYTO}$ to resting levels after caffeine application (see DISCUSSION), there was considerable variation from cell to cell in the rate of SR refilling. Based on the rate of restoration of $[Ca^{2+}]_{SR}$, we grouped the cells into two classes: "fast" ($[Ca^{2+}]_{SR}$ restored to the precaffeine level in 200 s or less) and "slow" (full SR recovery not achieved within 200 s). The mean time for recovery to 80% of precaffeine levels for the fast and slow groups was 47.8 ± 18.0 s ($n$ = 4) and 174.9 ± 17.3 s ($n$ = 5), respectively. Representative examples of recovery from a cell in the fast and slow groups are shown in Fig. 7 A. Once STOCs ceased following discharge of $Ca^{2+}$ from the SR, they reappeared at appreciable frequency only after the SR refilled to 60% or more of the resting level. The recovery of STOC frequency and amplitude after caffeine application is plotted as a function of normalized $[Ca^{2+}]_{SR}$ in Fig. 7 B. Despite the substantial variation in recovery time, both the fast and slow groups show the same relationship between $[Ca^{2+}]_{SR}$ and STOCs, indicating that the lapse of time after caffeine application did not account for the change in STOC function. STOC frequency generally showed the most marked recovery at levels in excess of 80% of resting $[Ca^{2+}]_{SR}$ (Fig. 7 B). Thereupon, STOC frequency increased steeply with progressive refilling of the SR so that the relationship between $[Ca^{2+}]_{SR}$ and STOC frequency was most marked as the original precaffeine level was approached. Not only did the frequency of STOCs increase with refilling, but their amplitude also increased as expected on the basis of an increase in the electrochemical gradient for $Ca^{2+}$ across the SR membrane. However, the increase in STOC amplitude was more linear than the increase in frequency.

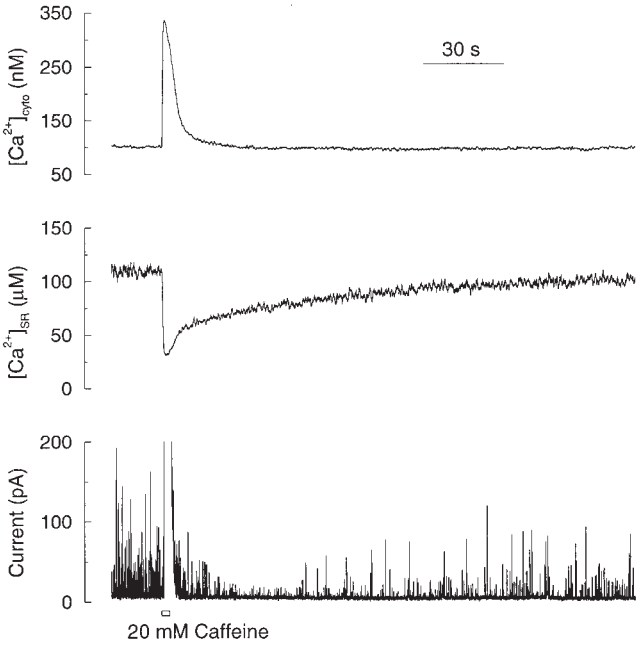


FIGURE 6. The reappearance of STOCs after a brief caffeine stimulation is correlated with refilling of SR with $Ca^{2+}$, but not with global $[Ca^{2+}]_{CYTO}$. $[Ca^{2+}]_{CYTO}$ (top), $[Ca^{2+}]_{SR}$ (middle), and STOCs (bottom) at rest and in response to a brief (3-s) caffeine application measured simultaneously in a single smooth muscle cell voltage clamped at 0 mV. $[Ca^{2+}]_{SR}$ was determined ratiometrically using mag-fura-2 as outlined above. $[Ca^{2+}]_{CYTO}$ was determined from changes in the fluorescence of $Ca^{2+}$ Green using a value for resting $[Ca^{2+}]_{CYTO}$ of 100 nM. (The current record is briefly interrupted shortly after caffeine application as the recording saturated at the gain required to observe STOCs for the majority of the recording.)

### *Relationship between $[Ca^{2+}]_{SR}$ and $Ca^{2+}$ Sparks*

The dependence of STOCs on the degree of SR $Ca^{2+}$ refilling suggests that $Ca^{2+}$ sparks should also show the same dependence. However, since BK channel activity can be affected by factors other than $Ca^{2+}$ sparks, it is possible that the $Ca^{2+}$ sparks might bear a different relationship to $[Ca^{2+}]_{SR}$. Hence, we also examined the time course of $Ca^{2+}$ spark recovery after SR depletion with caffeine. After a 3-s caffeine application, $Ca^{2+}$ sparks were imaged for a 2-s period at intervals of 30 s. The results of these experiments are given in Fig. 8 A, where spark frequency (●) and amplitude (□) are plotted as a function of the time after caffeine application. Software limitations of the digital imaging system presently preclude simultaneous measurements of $Ca^{2+}$ sparks at high time resolution and $[Ca^{2+}]_{SR}$. However, the relationship between the time course of refilling and the level of $[Ca^{2+}]_{SR}$ at each point in time allowed us to determine the approximate relationship between $[Ca^{2+}]_{SR}$ and $Ca^{2+}$ spark recovery. (The recovery of the STOC frequency and amplitude within 200 s in these cells indicated that SR refilling was essentially complete within this time and hence followed a fast time course; see Fig. 7 A.) The pattern of recovery of the $Ca^{2+}$ sparks was qualitatively similar to that of the

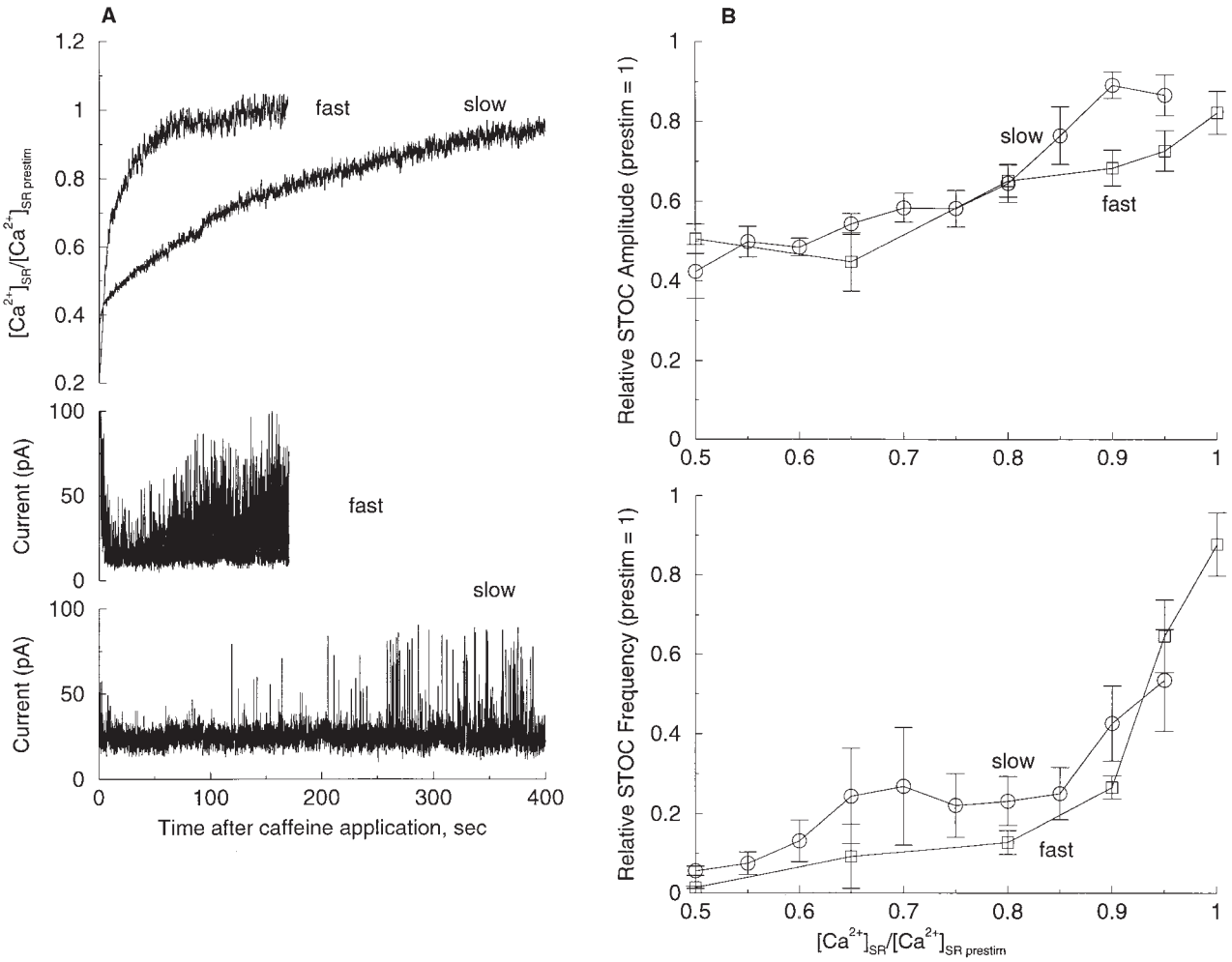


FIGURE 7. STOC frequency and amplitude are functions of luminal $[Ca^{2+}]$ regardless of refilling rate. (A) Recordings from representative cells showing fast and slow recovery of $[Ca^{2+}]_{SR}$ and corresponding STOCs. Time course of normalized $[Ca^{2+}]_{SR}$ for the two cells (top) and STOCs (middle and bottom) after 3-s caffeine application that ended at time 0. (B) STOC amplitude (top) and frequency (bottom) as a function of $[Ca^{2+}]_{SR}$ as the SR reloads after a 3-s caffeine application. STOC frequency and amplitude were determined for intervals in each record corresponding to 5% increases (for slowly refilling cells) and 10 or 15% increases (for fast refilling cells) in $[Ca^{2+}]_{SR}$ during refilling. The data for each of the cells (four in the fast group and five in the slow group) are normalized to the values in each cell before caffeine application.

STOCs (Fig. 8 B). That is, there was a steep relation between $Ca^{2+}$ spark frequency and $[Ca^{2+}]_{SR}$ at levels when the $[Ca^{2+}]_{SR}$ approached control levels; i.e., at $[Ca^{2+}]_{SR}$ in excess of 80% of control levels. As with the STOCs, the amplitude of the $Ca^{2+}$ sparks recovered as the stores reloaded, as expected for an increase in the electrochemical driving force on $Ca^{2+}$ across the SR membrane.

## DISCUSSION

### *$Ca^{2+}$ Sparks Trigger STOCs*

When spontaneous miniature outward currents due to BK channel openings were first observed in neurons almost two decades ago, Brown et al. (1983) demonstrated that the source of the $Ca^{2+}$ was intracellular and speculated that "packets of $Ca^{2+}$" were released from intracellular stores and acted as intracellular messengers. When STOCs were first reported in smooth muscle cells over a decade ago (Benham and Bolton, 1986), a similar explanation was advanced. The observation of $Ca^{2+}$ "puffs" in *Xenopus* oocytes (Parker and Yao, 1991) and $Ca^{2+}$ sparks in cardiac (Cheng et al., 1993) and skeletal (Tsugorka et al., 1995) myocytes was soon followed by the observation of similar $Ca^{2+}$ sparks in single smooth muscle cells by Nelson et al. (1995), and then by Mirroneau et al. (1996). In the present study, we used a high-speed imaging system to record $Ca^{2+}$ sparks and STOCs simultaneously in the same cell, which allowed us to compare them in detail. We found that there is a close association between the oc-

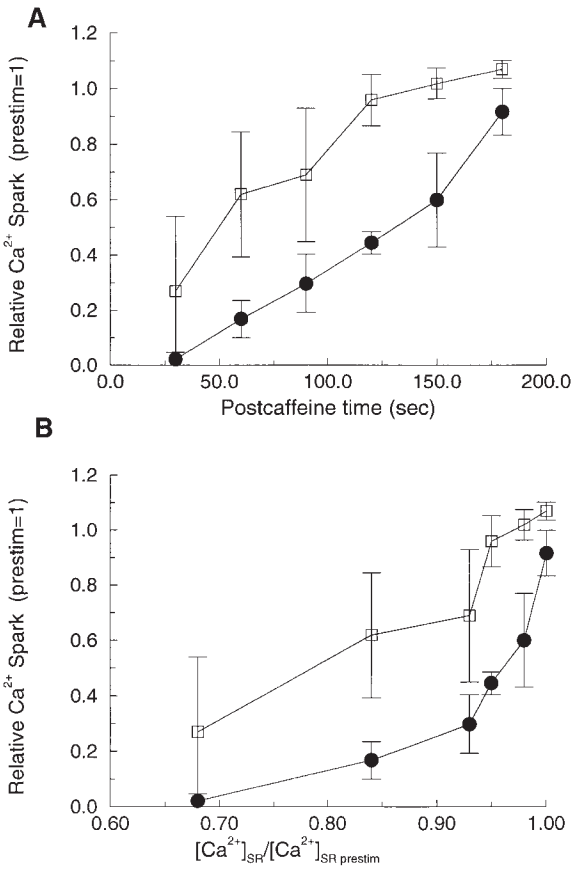


FIGURE 8. Relationship between $[Ca^{2+}]_{SR}$ and $Ca^{2+}$ sparks. (A) Relationship between time after a 3-s caffeine application and amplitude (□) and frequency (●) for $Ca^{2+}$ sparks. After the caffeine application, $Ca^{2+}$ sparks ceased, and then recovered as the SR refilled. (B) Relationship between fractional $[Ca^{2+}]_{SR}$ and spark frequency (●) and amplitude (□) determined as described in the text. Images were acquired as in Fig. 2, and the amplitude and frequency of $Ca^{2+}$ sparks were normalized to values before the application of caffeine ($n = 4$ cells).

currence of $Ca^{2+}$ sparks and STOCs and that $Ca^{2+}$ sparks and their corresponding STOCs have similar time courses. (In other smooth muscle types, however, the $Ca^{2+}$ sparks have a longer time course than the STOCs they cause; Kirber et al., 1998). Furthermore, changes in $Ca^{2+}$ spark frequency and amplitude were always paralleled by changes in STOC frequency and amplitude when the SR was depleted by caffeine and subsequently refilled. Thus, our findings provide additional evidence for the causal link between $Ca^{2+}$ sparks and STOCs.

### *How Can a 100-nM Increase Over the Resting Cytosolic $[Ca^{2+}]$ Cause a STOC?*

The peak spark amplitude measured with fluo-3 averaged ~10% ($\Delta F/F_0 \times 100$), and the resting $[Ca^{2+}]_{CYTO}$ measured ratiometrically with fura-2 in these cells is consistently ~100 nM (Drummond and Fay, 1996). Using this value for resting $[Ca^{2+}]$, the mean $\Delta F/F_0$ converts to a mean change in $[Ca^{2+}]$, at the peak, of ~100 nM,[2] leading to a mean total $[Ca^{2+}]$ at the peak of ~200 nM in the brightest pixel in a given spark, assuming equilibrium between $Ca^{2+}$ and fluo-3. Is this sufficient to cause openings of BK channels in this cell type at 0 mV, the potential at which we measured STOCs and $Ca^{2+}$ sparks simultaneously? The best answer to this question comes from consideration of earlier studies of BK channels in excised, inside-out patches in the same cells used here (Singer and Walsh, 1987). There, the probability of a channel's being in the open state ($P_o$) at 0 mV in the presence of 100 nM $Ca^{2+}$ was essentially 0 (see Fig. 6 in Singer and Walsh, 1987), and an order of magnitude increase in $[Ca^{2+}]$ to 1 μM caused a $P_o$ of only ~0.1. Thus, it appears that a $[Ca^{2+}]_{CYTO}$ of ~200 nM is not sufficient to cause substantial BK channel opening at 0 mV.

How are we to explain this apparent contradiction? Three considerations lead strongly to the explanation that the BK channels lie close enough to the SR $Ca^{2+}$ release site so that the channels sense a much higher concentration than the average we measured. First, there is considerable work on modeling the diffusion-reaction events that occur near a point source of $Ca^{2+}$ release in the presence of calcium buffering (Stern, 1992; Naraghi and Neher, 1997). The results from such studies indicate that the fluo-3 is not in equilibrium with $Ca^{2+}$ emerging from the point of SR release at distances smaller than our pixel sizes (333 × 333 nm). Thus, $[Ca^{2+}]_{CYTO}$ very close to the point of SR release could be many micromolar, given that $Ca^{2+}$ currents through the release channels are in the range of 1 pA (Mejia-Alvarez et al., 1998). If the BK channels are very close to an SR release site, then they will sense a $[Ca^{2+}]$ in the micromolar range that is sufficient to activate them at 0 mV.

[2]The ability of the ultrafast microscope to resolve and measure highly localized calcium signals was examined using a computer simulation of $Ca^{2+}$ sparks of known peak $[Ca^{2+}]$ as imaged inside a model cell. Fluorescence ratios ($\Delta F/F_0$) were calculated from simulated images of a range of spark $[Ca^{2+}]$ amplitudes, both in and out of focus. From these simulations, we estimated that an observed average spark amplitude of 10% ($\Delta F/F_0$) is consistent with a peak spark $[Ca^{2+}]$ of 200 nM, or 100 nM above resting $[Ca^{2+}]$. This estimate was made in the following way.

First, the fluorescence intensity distribution of a typical $Ca^{2+}$ spark inside a smooth muscle cell was simulated. Custom software was used to calculate the three-dimensional image of a model smooth muscle cell filled with 50 μM fluo-3 ($K_d$ = 390 nM) in equilibrium with a resting $[Ca^{2+}]$ of 100 nM. The cell was modeled as a cylinder with cross-sectional diameters of 10 μm in the transverse direction and 6 μm in the axial direction, the direction of focus in the microscope, and of infinite length with respect to the imaging. These dimensions were previously derived from three-dimensional reconstructions of toad gastric smooth muscle cells (our unpublished data). The three-dimensional fluorescence intensity distribution was calculated assuming bound fluo-3 was 100× as fluorescent as the free species. At resting $[Ca^{2+}]$,

Second, in earlier studies on excised inside-out patches in these cells, we calculated the minimum density of the BK channels to be on the order of 1 channel/$\mu m^2$, based on an assumption of uniform channel density (Singer and Walsh, 1987). But such a uniform distribution would place only three BK channels in the 3-$\mu m^2$ region, which is the area over which the $Ca^{2+}$ elevation occurs during the spark. Since even at a $[Ca^{2+}]_{CYTO}$ of 1 $\mu$M, the $P_o$ is 0.1, less than one BK channel would be open at any one time in this region. And in most of this 3-$\mu m^2$ region, the increase in $[Ca^{2+}]$ is less than that at the center of the spark, where it averages 200 nM. But the mean STOC amplitude is ~30 pA, requiring six BK channels to be open simultaneously.

Third, and perhaps most convincingly, is the simple observation that inverted STOCs of substantial amplitude (20–30 pA) can be recorded even at −80 mV (Fig. 3) when the external $K^+$ is elevated. At this potential, the $Ca^{2+}$ sparks average 10% (Fig. 4). However, from earlier studies on excised patches, we know that 10 $\mu$M $[Ca^{2+}]_{CYTO}$ is required for a $P_o$ of 0.1 at this potential (see Fig. 6 in Singer and Walsh, 1987). Hence, the BK channels must lie close to the SR release site. In summary, these considerations lead to two conclusions: an SR $Ca^{2+}$ release site causing a $Ca^{2+}$ spark must lie close to BK channels, and the BK channels responsible for a STOC must be clustered. Thus, the spark-STOC site may be a distinct morphological specialization much like a synaptic vesicle release site.

### *Cytosolic $[Ca^{2+}]$ Recovers an Order of Magnitude Faster than SR $[Ca^{2+}]$ after Caffeine-induced SR Depletion*

This study provides the first direct measure of the temporal relationship between $[Ca^{2+}]_{CYTO}$ and $[Ca^{2+}]_{SR}$ in response to depletion of SR $Ca^{2+}$ stores in smooth muscle cells and in myocytes of any type. After stimulation with caffeine, recovery of SR $Ca^{2+}$ was ~10× slower than cytosolic $Ca^{2+}$. Thus, the correspondence of $Ca^{2+}$ spark and STOC frequency with $[Ca^{2+}]_{SR}$ rather than with $[Ca^{2+}]_{CYTO}$ was readily apparent. Since the rise in $[Ca^{2+}]_{CYTO}$ due to release from the SR fell back to rest well before $[Ca^{2+}]_{SR}$ recovered, much of the cytosolic $Ca^{2+}$ must be either bound to myoplasmic buffering sites with slow off rates or cleared into another, optically silent compartment before gradually reappearing as SR $Ca^{2+}$. Some fraction of the $Ca^{2+}$ that refills the SR may come directly from the cell exterior, although we detected no macroscopic inward current at a holding potential of −80 mV after caffeine-induced depletion. There is evidence in these cells to indicate that mitochondria constitute the third compartment. It has been demonstrated that mitochondria sequester a portion of the elevated $Ca^{2+}$ caused by activation of voltage-gated $Ca^{2+}$ channels in these cells (Drummond and Fay, 1996). More recently, it has been shown that mitochondria sequester $Ca^{2+}$ released from the SR so that the time course or recovery of SR and mitochondrial $Ca^{2+}$ parallel one another as the sequestered $Ca^{2+}$ exits the mitochondria and recharges the SR (Drummond et al., 1997). This mechanism is consistent with observations that SR and mitochondria are located in close apposition in smooth muscle cells (Nixon et al., 1994; see also Rizzuto et al., 1998). Given these facts, it is also possible that mitochondria make a contribution to the regulation of $Ca^{2+}$ sparks, although there is no evidence for this as yet.

### *What Is the Link between $[Ca^{2+}]_{SR}$ and the Frequency of $Ca^{2+}$ Sparks and STOCs?*

Studies on RyRs in artificial lipid bilayers support our conclusion that at least a portion of the increase in fre-

~25% of the fluo-3 was bound to $Ca^{2+}$ and the fluorescence signal at rest was ~20% of the maximum attainable with saturating $Ca^{2+}$.

Second, the spatial $[Ca^{2+}]$ profile of a $Ca^{2+}$ spark was added to the simulated resting cell. A $Ca^{2+}$ spark was modeled as a stationary, Gaussian spot of calcium with a known peak $[Ca^{2+}]$ and a spatial full width at half-maximum amplitude of 1.7 $\mu$m. This simulates the spark at a single point in time, corresponding to the observed images of sparks at the time of peak fluorescence intensity. The $Ca^{2+}$ spark was added to the resting cell model with the spark peak at the center of the cell, and the corresponding three-dimensional image of fluo-3 distribution was calculated as for the resting cell alone. The center of the cell was used to avoid having to simulate the effect of the cell membrane on diffusion. Since the fluorescence background due to resting $[Ca^{2+}]$ is likely highest at the center, where the cell is thickest, this is probably the worst case, for our purposes, for measuring $\Delta F/F_0$.

Next, the image formation and acquisition was simulated. The three-dimensional fluorescence image of the cell and spark was blurred with the three-dimensional image of a theoretical, wide-field, point spread function, for a 1.3 NA objective lens (Nikon Inc.) calculated at 530 nm wavelength (Tella, 1985). The image resolution was decreased to 300-nm pixels, by adding three-by-three groups of pixels, in order to simulate the image formation (point spread function) and acquisition (camera pixelization) process. The resulting three-dimensional image contained images of the spark in and out of focus, as seen against the fluorescence background arising from the global resting $[Ca^{2+}]$.

Lastly, using the blurred images of the cell with and without the spark, the fluorescence ratios ($\Delta F/F_0$) were calculated at the pixel corresponding to the spark center, at 200-nm focus steps through the 6-$\mu$m depth of the cell. The effect of uncertainty in focus was examined by weighting the $\Delta F/F_0$ calculated at each depth through the cell by the probability of a spark occurring at that depth. Although the modeled spark was located in the cell center, the model used for spark spatial distribution assumed that sparks were constrained to occur at the outer edge of the cell, adjacent to the plasma membrane, and were equally likely to occur anywhere along the plasma membrane. A spark with a known peak $[Ca^{2+}]$ of 200 nM (100 nM above resting $[Ca^{2+}]$) yielded a $\Delta F/F_0$ of 18% when in focus (centered in depth) and 5.5% when 3 $\mu$m out of focus (top or bottom of cell). After accounting for the effects of spark location on focus, the average $\Delta F/F_0$ was 10%, a value equivalent to the average observed spark peak amplitude described in this report.

quency of $Ca^{2+}$ sparks (and consequently STOCs) that we observe at higher $[Ca^{2+}]_{SR}$ is due to regulation of RyR gating by $[Ca^{2+}]_{SR}$. RyRs from the cells used in the present study have been partially purified from microsomal membranes and reconstituted into lipid bilayers, where they gave rise to single channel currents whose frequency of opening increased as the $[Ca^{2+}]$ was elevated on the side of the bilayer corresponding to the luminal surface (Xu et al., 1994). Moreover, the RyRs from this amphibian preparation appear to be quite like those in mammalian cardiac cells, although not identical to them (Xu et al., 1994). In both cardiac and skeletal muscle, there is a great deal of evidence from studies in artificial bilayers that luminal $[Ca^{2+}]$ increases the probability of RyR channels being in the open state, although the precise site of this action remains in doubt (Ikemoto et al., 1991; Gilchrist et al., 1992; Sitsapesan and Williams, 1994; 1995; Donoso et al., 1995; Lukyanenko et al., 1996; Tripathy and Meissner, 1996). Nevertheless, we cannot exclude the possibility that the lower apparent frequency of $Ca^{2+}$ sparks at lower SR $Ca^{2+}$ levels is due to small amplitude events (resulting from decreased driving force on SR $Ca^{2+}$) that escape detection (Song et al., 1997). However, if this explanation is true, then the same measurement bias affected two separate and independent measures; that is, electrophysiological recording of STOCs and optical detection of $Ca^{2+}$ sparks, in the same way. Finally, the precise mechanism of SR $Ca^{2+}$ action might matter little as far as the physiological outcome is concerned. That is, an increase in $[Ca^{2+}]_{SR}$ leads to an increase in total outward current whether due to an increase in STOC frequency or amplitude or both, and an increase in outward current will lead to hyperpolarization of the membrane with all the attendant consequences (see Nelson et al., 1995).

### *Physiological Role of $[Ca^{2+}]_{SR}$ as a Regulator of $Ca^{2+}$ Sparks in Smooth Muscle Cells*

The present study makes it clear that as the SR stores attain higher levels of free $Ca^{2+}$ there will be an increase in both the frequency and amplitude of $Ca^{2+}$ sparks and the STOCs that they cause. Moreover, the relationship between $[Ca^{2+}]_{SR}$ and spark (and STOC) frequency becomes quite steep when the SR refills to 80% or more of its resting level. Thus, $[Ca^{2+}]_{SR}$ is potentially an important regulator of spark (and STOC) frequency. However, this study should not be taken to mean that $[Ca^{2+}]_{SR}$ is the only regulator of sparks and STOCs. This caveat is quite important when considering the role of voltage-activated $Ca^{2+}$ channels in regulating sparks. For there is now evidence that $Ca^{2+}$ entry through voltage-gated $Ca^{2+}$ channels in smooth muscle can elicit $Ca^{2+}$ sparks even when the depolarizations used to activate these $Ca^{2+}$ channels are quite brief (Arnaudeau et al., 1997; ZhuGe et al., 1998b). This sort of spark induction by depolarization would appear to be due to a local control mechanism, perhaps together with increased SR $Ca^{2+}$ load, as is the case in cardiac cells (see Cannell et al., 1995). In other instances, however, it may be that global $[Ca^{2+}]_{SR}$ is the dominant intermediary in regulation of spark frequency. For example, it is possible, although as yet unproven, that some neurotransmitters or cyclic nucleotides, which alter spark frequency (Porter et al., 1998), act in part by altering $[Ca^{2+}]_{SR}$.

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