‘Trigger’ Events Precede Calcium Puffs in *Xenopus* Oocytes

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ABSTRACT The liberation of calcium ions sequestered in the endoplasmic reticulum through inositol 1,4,5-trisphosphate receptors/channels (IP$_3$Rs) results in a spatiotemporal hierarchy of calcium signaling events that range from single-channel openings to local Ca$^{2+}$ puffs believed to arise from several to tens of clustered IP$_3$Rs to global calcium waves. Using high-resolution confocal linescan imaging and a sensitive Ca$^{2+}$ indicator dye (fluo-4-dextran), we show that puffs are often preceded by small, transient Ca$^{2+}$ elevations that we christen ‘trigger events’. The magnitude of triggers is consistent with their arising from the opening of a single IP$_3$ receptor/channel, and we propose that they initiate puffs by recruiting neighboring IP$_3$Rs within the cluster by a regenerative process of Ca$^{2+}$-induced Ca$^{2+}$ release. Puff amplitudes (fluorescence ratio change) are on average 6 times greater than that of the triggers, suggesting that at least six IP$_3$Rs may simultaneously be open during a puff. Trigger events have average durations of 12 ms, as compared to 19 ms for the mean rise time of puffs, and their spatial extent is 3 times smaller than puffs (respective widths at half peak amplitude 0.6 and 1.6 μm). All these parameters were relatively independent of IP$_3$ concentration, although the proportion of puffs showing resolved triggers was greatest (~80%) at low [IP$_3$]. Because Ca$^{2+}$ puffs constitute the building blocks from which cellular IP$_3$-mediated Ca$^{2+}$ signals are constructed, the events that initiate them are likely to be of fundamental importance for cell signaling. Moreover, the trigger events provide a useful yardstick by which to derive information regarding the number and spatial arrangement of IP$_3$Rs within clusters.

INTRODUCTION

Inositol 1,4,5-trisphosphate (IP$_3$) is a major intracellular messenger that functions by binding to IP$_3$ receptors/channels (IP$_3$Rs) to liberate Ca$^{2+}$ ions sequestered in the endoplasmic reticulum (ER) (1). Opening of these channels is regulated not only by IP$_3$ but also biphasically by cytosolic Ca$^{2+}$ itself, leading to a regenerative process of Ca$^{2+}$-induced Ca$^{2+}$ release (CICR) (2–5). As a result of this positive feedback, in conjunction with the clustered organization of IP$_3$Rs in the ER membrane (6–8), cytosolic Ca$^{2+}$ signals in many cell types display a hierarchical spatiotemporal organization, ranging from Ca$^{2+}$ ‘blips’ that represent putative openings of single IP$_3$Rs (9–11), through ‘puffs’ that involve concerted openings of several clustered IP$_3$Rs (10–13), to saltatory Ca$^{2+}$ waves that propagate globally across cells by successive cycles of CICR and Ca$^{2+}$ diffusion between clusters (12,14).

Ca$^{2+}$ puffs serve as the basic ‘building blocks’ from which global Ca$^{2+}$ signals (waves) are constructed (5,10,12,14) and may also regulate local signaling functions in their own right. It is, therefore, important to understand the fundamental mechanisms by which they arise. However, several key questions remain unresolved: notably, the number of IP$_3$Rs/channels that open during a puff, the density and spatial distribution of these channels within a cluster, and the triggering mechanism by which the initial opening of one channel may rapidly induce the concerted opening of closely neighboring IP$_3$Rs within a cluster.

The *Xenopus* oocyte has proved a highly advantageous model cell system in which to study the elementary events of Ca$^{2+}$ signaling by virtue of its large size and lack of ER Ca$^{2+}$ release channels (e.g., ryanodine receptors (RyRs) and cADP-ribose receptors) other than IP$_3$Rs (15–19). Here, we have explored the microarchitecture of puffs in the oocyte at improved resolution, employing confocal linescan microscopy together with a high- dynamic range fluorescent Ca$^{2+}$ indicator (fluo-4-dextran). A major finding is the discovery of novel IP$_3$-Ca$^{2+}$ signaling events, which we christen Ca$^{2+}$ ‘triggers’. These are small, transient (~12 ms) Ca$^{2+}$ elevations that arise at puff sites immediately preceding the onset of puffs. We interpret them as arising from the opening of a single IP$_3$R and propose that the resulting Ca$^{2+}$ liberation acts as a trigger to induce an explosive concerted opening of multiple adjacent IP$_3$Rs to generate a puff. The Ca$^{2+}$ triggers offer a useful yardstick by which to estimate the number of channels involved in a puff, and comparison of the spatial spread of trigger and puff Ca$^{2+}$ signals provides information regarding the distribution of channels at a puff site. This work presents experimental findings on the spatiotemporal dynamics of triggers and puffs, and in an accompanying work (20) we use these experimental data in a mathematical simulation to predict the architecture of an IP$_3$R cluster.

MATERIALS AND METHODS

Preparation and microinjection of oocytes

*Xenopus laevis* were anesthetized by immersion in 0.17% MS-222 for 15 min and sacrificed by decapitation in accordance with protocols approved by the UC Irvine Animal Care and Use Committee. Stage V and VI oocytes

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were manually plucked and then collagenase treated (0.5 mg/ml) for 30 min. Oocytes were stored in Barth’s solution (in mM: NaCl, 88; KCl, 1; NaHCO3, 2.4; MgSO4, 0.82; Ca(NO3)2, 0.33; CaCl2, 0.41; Hepes, 5; gentamicin, 0.1 mg/ml; at pH 7.4) for 1–4 days before use. A Drummond (Broomall, PA) microinjector was used to inject oocytes to final concentrations (assuming 1-μL cytosolic volume) of 25–40 μM fluo-4-dextran (low affinity, Ka = −3 μM or high affinity, Ka = 0.8 μM: manufacturer’s data); 8 μM caged IP3 (D-myo-inositol 1,4,5-triphosphate, P(4)-1(02-nitrophenyl)ethyl ester), and 90 μM or 300 μM EGTA. EGTA was used to functionally isolate puffs by inhibiting propagation of Ca2+ waves (21); puff characteristics were similar for the two buffer concentrations. After allowing >45 min for intracellular diffusion of compounds, oocytes were imaged using confocal linescan microscopy. The temporal and spatial characteristics of puffs collected with low- versus high-affinity fluo-4-dextrans were indistinguishable, although puff amplitudes were somewhat greater using the low-affinity version fluo-4–dextran (8.8 ± 0.33 ΔF/F0, n = 75 vs. 7.13 ± 0.30 ΔF/F0, n = 37; p < 0.005). Data from the low-affinity and high-affinity versions of the fluo-4-dextran are combined in the results; the majority of the results were obtained with the high-affinity version (Ka = 0.8 μM).

Calcium indicators and caged IP3 were obtained from Invitrogen (Carlsbad, CA); all other reagents were from Sigma Chemical (St. Louis, MO).

Confocal linescan microscopy

Confocal calcium images were obtained using a custom-built linescan confocal scanner interfaced to an Olympus IX70 inverted microscope (Melville, NY) (22). Experiments were performed at room temperature in Ringer’s solution (in mM: NaCl, 120; KCl, 2; CaCl2, 1.8; Hepes, 5; pH 7.4). The microscope was focused in the granule layer of the animal hemisphere where IP3R density and Ca2+ signals are maximal (6), and linescan images were acquired using custom routines written in Labview (National Instruments; Austin, TX). Temporal resolution was optimized by using a relatively short optical density of the ND filters. Caged IP3 was photolyzed by ultraviolet light focused uniformly over a spot 200 μm in diameter surrounding the scan line (22). The stimulus strength was varied by regulating the flash duration with electronic shutter and/or changing light intensity by neutral density (ND) filters. We express stimulus strength on a linear relative scale calculated as Fp/102ND, where Fp = flash duration in milliseconds and ND = optical density of the ND filters. Image data were processed in IDL (Research Systems; Boulder, CO) using custom-written software routines and exported to Microcal Origin, v. 6 (Northampton, MA) for analysis and graphing. Pearson’s correlation coefficient (r) was used to determine the relationship between relative stimulus strength and specific response characteristics, and the Student’s t-test was used to determine if two independent populations were significantly different (p < 0.05 required to achieve significance). Data are presented as mean ± SE.

Calculation of fluorescence ratio signals

A major goal of this study was to quantify the magnitudes of fluorescence signals during triggers and puffs as a basis for subsequent mathematical modeling. For this purpose we needed to correct the “raw” fluorescence measurements for artifactual distortions. The procedure is illustrated in Fig. 1, which diagrams the various factors contributing to the total fluorescence recorded from an oocyte. Fig. 1A shows a raw fluorescence linescan image of two calcium puffs evoked by photorelease IP3, processed only by a 3 × 3 smoothing, and Fig. 1B shows corresponding temporal profiles of fluorescence at the center of a puff (a), and at a location with no Ca2+ response (b). Fluorescence signals from nonratiometric indicators such as fluo-4 are conventionally expressed as ratios (F/F0 or ΔF/ΔF0) of the fluorescence (F) at each pixel relative to the mean resting fluorescence before stimulation (F0).

FIGURE 1 Confocal linescan imaging of Ca2+ puffs in Xenopus oocytes and factors contributing to the fluorescence signal. (A) Representative “raw” fluorescence image showing two puffs. Distance along the scan line is depicted vertically, and time runs from left to right. Increasing fluorescence (F) in arbitrary units on an 8-bit scale is depicted in pseudocolor as indicated by color bar. The shutter controlling the laser excitation light was opened when marked by the first arrow, and a photolysis flash was delivered to photorelease IP3 at the second arrow. Data are unprocessed excepting a 3 × 3 pixel smoothing. (B) Temporal profile of raw fluorescence levels monitored at the two sites marked by white arrows in A. Measurements were averaged over three pixels (∼0.2 μm). (C) Components contributing toward the total fluorescence signal at rest and during a puff: (i) photon and dark noise offset in the absence of laser excitation; (ii) oocyte autofluorescence; (iii) Ca2+-independent fluorescence of indicator dye; (iv) Ca2+-dependent fluorescence of indicator at basal cytosolic free [Ca2+]; and Ca2+-evoked fluorescence increase during a puff. Calculations of fluorescence ratio changes (F/F0) were made after subtracting factors (i) and (ii) from the “raw” basal fluorescence to obtain a corrected value of F0. Fluorescence steps and peak puff amplitude (v) are not to scale. Representative values (in raw fluorescence units) are i = 2.5, ii = 2.0, iii = 2.9, iv = 19, v = 132. (D) Pseudocolored fluorescence ratio image (F/F0) derived from the record in A after correcting as described in C. (E) Temporal profile of the puff in D (arrow). The profile appears nearly identical to B but actually represents small critical differences for measuring small amplitude events, which will be the focus of this work. Timescale is the same as D.
A remaining source of \( \text{Ca}^{2+} \)-independent fluorescence originates from the fluorescence of the indicator dye remaining at zero \([\text{Ca}^{2+}]\) (Fig. 1 C). We estimated this by comparing the fluorescence of droplets of low-affinity fluo-4-dextran (25 \( \mu \text{M} \), prepared from the same lot as that used for imaging; together with 100 mM KCl, 5 mM HEPES at pH 7.4) in \( \text{Ca}^{2+} \)-free medium (no added \( \text{Ca}^{2+} \) and 5 mM EGTA) and in saturating \([\text{Ca}^{2+}]\) (100 \( \mu \text{M} \) CaCl\(_2\)). The residual fluorescence in \( \text{Ca}^{2+} \)-free medium was 2.2\% of that in saturating \( \text{Ca}^{2+} \). We did not apply any correction for \( \text{Ca}^{2+} \)-independent fluo-4 fluorescence here, but the value is important for the companion work where we model puff and trigger generation (20).

**RESULTS**

'Trigger' events often precede puffs

Puffs are believed to arise because CICR coordinates the concerted opening of several IP\(_3\)Rs within a cluster. It is likely, therefore, that a puff is initiated when \( \text{Ca}^{2+} \) liberated by the opening of a single IP\(_3\)R causes neighboring channels to open. In previous experiments we had failed to detect such putative single-channel trigger events (5,9) (but see Sun et al. (11) for first mention of possible trigger events). However, using higher-resolution imaging and a \( \text{Ca}^{2+} \) indicator dye (fluo-4-dextran) with high-dynamic range together with intracellular loading of EGTA to functionally uncouple puff sites (22), we now show that puffs do not always display a smoothly monotonic rising phase but in many instances are preceded by discrete brief low-amplitude events.

Representative examples of temporal profiles of individual puffs are shown in Fig. 2 A. Those on the left were selected as displaying trigger events (red shading), whereas those on the right show puffs that appear to rise monotonically from baseline without any detectable inflection in the rising phase. Fig. 2 B presents linescan images illustrating puffs without (i) and with (ii) trigger events, formed in both cases by averaging selected individual images after aligning puffs to their location of maximum intensity and time of maximal rate of rise, and Fig. 2 C shows temporal profiles of fluorescence ratio during these averaged events.

Given the brief duration and small amplitude of trigger events, we were concerned to exclude the possibility that they might be subjectively misidentified from random noise spikes that happened to precede a puff. Several observations argue against that possibility. First, the amplitudes of triggers were generally above the baseline noise level, even in individual traces (Fig. 2 A) and were much larger than baseline noise after averaging several events (Fig. 2 C). Second, if the triggers simply represented upward noise spikes, then puffs selected as failing to show triggers would be expected to display a corresponding initial negative deflection—which was not the case (Fig. 2, A and C). Third, distinct inflections in the rising phase were still apparent in time-aligned averages of randomly selected puffs (e.g., *inset* to Fig. 3), where only a portion of the averaged puffs had trigger events.
The question then arises as to whether these small Ca\textsuperscript{2+} signals are specifically associated with puffs or whether we identify triggers as random events that coincidentally arise at the start of a puff. We addressed this by counting the number of times upward baseline fluctuations exceeded a threshold of 1 \(\Delta F/F_0\) (the amplitude of an average-sized trigger) for \(\geq 4\) ms in 12-ms bins immediately preceding puff onset and at sequentially earlier times. Trigger events exceeding this threshold were seen immediately preceding 75% (8/12) of unselected puffs evoked by weak stimuli (see below), whereas we observed no corresponding random events at unselected puffs evoked by strong stimuli (see below). To further examine whether the likelihood of observing triggers was blind to the stimulus strength when determining which events in each group are indicated. The inset shows averages of \(\Delta F/F_0\) where only \(\sim 20\%\) of puffs showed triggers (inset, Fig. 3).

### Relative amplitudes of puffs and triggers

Ninety-two calcium puffs (48 with trigger events and 44 without) were selected for analysis of the spatial and temporal characteristics of the triggers and puffs. Table 1 summarizes average values of their fluorescence ratio amplitudes, spatial widths at half peak amplitude, and durations.

Fig. 4A shows a scatter plot of corrected trigger amplitude \(\Delta F/F_0\) measured at the point of inflection before the rapid rate of rise of a puff as a function of relative flash strength. No correlation was apparent \((r = 0.15;\) slope of linear regression \(= 0.01 \pm 0.01; n = 29)\), and we pooled data from all flash strengths to derive the amplitude distribution of triggers (Fig. 4B), yielding a mean amplitude \(\Delta F/F_0 = 1.26 \pm 0.10\) \((n = 48)\). The peak amplitudes of puffs (including events both with and without triggers) also showed only a weakly positive correlation with flash strength (Fig. 4C, \(r = 0.37;\) slope = 0.09 \pm 0.03; \(n = 73)\). We thus pooled data across different flash strengths to obtain the distribution of puff amplitudes (Fig. 4D; mean \(\Delta F/F_0 = 7.3 \pm 0.35; n = 92)\). Measurements were made using fluo-4-dextran to simultaneously resolve trigger events and puffs, but the relatively high affinity of these indicators introduced concern that the fluorescence signal may have approached saturation during the much larger puffs. To exclude this possibility, puff amplitudes were separately measured using a low-affinity calcium indicator (Oregon Green 5N; \(K_d = 20\) \(\mu\)M). This again revealed only a weakly positive correlation between puff amplitude and flash strength \((r = 0.46;\) slope = 0.01 \pm 0.003; \(n = 57)\); data not shown), indicating that the maximal \(\text{Ca}^{2+}\) concentration attained during a puff indeed shows relatively little dependence on [IP\textsubscript{3}]. Trigger events were not resolved with Oregon Green 5N.

To use the trigger events as a yardstick by which to estimate the minimum number of channels opening during a puff, we calculated the puff/trigger amplitude ratios from

### Table 1: Average amplitude, spatial, and kinetic characteristics of puffs and triggers

<table>
<thead>
<tr>
<th>(\Delta F/F_0) (corrected)</th>
<th>(n)</th>
<th>(\pm)</th>
<th>(\Delta F/F_0) (corrected)</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplitude</td>
<td>7.27</td>
<td>(\pm)</td>
<td>1.26</td>
<td>(\pm)</td>
</tr>
<tr>
<td>Puff rise time or trigger duration</td>
<td>18.97</td>
<td>(\pm)</td>
<td>11.74</td>
<td>(\pm)</td>
</tr>
<tr>
<td>FWHMA</td>
<td>1.51</td>
<td>(\pm)</td>
<td>0.57</td>
<td>(\pm)</td>
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<tr>
<td>FDHA</td>
<td>70.14</td>
<td>(\pm)</td>
<td>3.11</td>
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\(n\) indicates the number of events in each group.
paired measurements on puffs that displayed triggers. The distribution of ratios is plotted in Fig. 4 F, and the ratio showed no obvious correlation with flash strength (Fig. 4 E; \( r = 0.37; \) slope = 0.16 ± 0.08; \( n = 29 \)). The mean puff/trigger ratio derived from paired individual measurements was 8.44 ± 0.78 (\( n = 48 \)); but this value is likely to be exaggerated by instances where very small trigger amplitudes gave rise to large ratios. Thus, we calculated a more reliable estimate of the puff/trigger amplitude ratio from the average puff and trigger amplitudes, yielding a value of 5.8.

**Trigger and puff kinetics**

Trigger durations were measured from the apparent onset of the trigger event to the start of the fast rise of the puff. The distribution of trigger durations ranged between ~5 ms and 25 ms (Fig. 5 B), and there was no apparent dependence of trigger duration on flash strength (Fig. 5 A; \( r = -0.01; \) slope = -0.003 ± 0.05; \( n = 29 \)). The average trigger duration was 11.7 ± 0.6 ms (\( n = 48 \)). Puff rise times were measured from the beginning of the puff onset (or the end of the trigger event in those with triggers) until the time when the puff reached ~95% peak amplitude. Again, there was no significant dependence of puff rise time on relative flash strength (Fig. 5 C; \( r = -0.15; \) slope = -0.10 ± 0.07; \( n = 73 \)), and the distribution of rise times (Fig. 5 D) gave a mean of 19.0 ± 0.80 ms, \( n = 92 \). Puff durations were measured as full duration at half peak amplitude (FDHA). There was a weak negative correlation between FDHA and relative flash strength (Fig. 5 E; \( r = -0.30; \) slope = -0.62 ± 0.24; \( n = 73 \)), and the distribution of all values is shown in Fig. 5 F. The mean FDHA was 70.1 ± 3.1 ms, \( n = 92 \).

**Spatial distribution of fluorescence signal during triggers and puffs**

Fig. 6 A shows averaged spatial profiles derived from representative puffs and trigger events. Because the baseline
noise level was appreciable in relation to the amplitude of triggers, we were able to obtain reliable measurements of trigger width (FWHA) from only a subset of events (Fig. 6 B; \( n = 22 \)), yielding a mean width of 0.57 \( \pm 0.09 \) \( \mu \)m. Puff widths measured in the same way showed no dependence (\( r = -0.07 \); slope = -0.003 \( \pm 0.03 \); \( n = 73 \)) on photolysis flash strength (Fig. 6 C), and pooled measurements displayed a roughly Gaussian distribution of puff widths (Fig. 6 D), with a mean of 1.51 \( \pm 0.05 \) \( \mu \)m (\( n = 92 \)); almost three times wider than trigger events.

**Are puffs with triggers the same as puffs without triggers?**

The mean corrected amplitude of puffs with trigger events (7.46 \( \pm 0.71 \) \( \Delta F_0^0; n = 30 \)) tended to be greater than events without detectable triggers (6.06 \( \pm 0.34 \) \( \Delta F_0^0; n = 38 \)); but the difference just failed to reach statistical significance (\( p = 0.06 \)). No significant differences were apparent in puff widths (puffs with trigger = 1.42 \( \pm 0.09 \) \( \mu \)m, \( n = 30 \); without trigger = 1.56 \( \pm 0.09 \) \( \mu \)m, \( n = 38 \); \( p = 0.25 \)), durations (FDHA of puffs with trigger = 73.94 \( \pm 4.81 \) ms, \( n = 30 \); without trigger = 84.15 \( \pm 4.47 \); \( p = 0.13 \)), or rise times (19.86 \( \pm 1.32 \) ms, \( n = 30 \) vs. 19.79 \( \pm 1.46 \) ms, \( n = 38 \); \( p = 0.97 \)).

**DISCUSSION**

We show that IP\(_3\)-evoked \( \text{Ca}^{2+} \) puffs in *Xenopus* oocytes are often preceded by brief, small amplitude \( \text{Ca}^{2+} \) signals, which we name “trigger” events. Although both puffs and trigger events exhibit variability in their spatial and temporal characteristics, these characteristics are largely independent of IP\(_3\) concentration. As discussed below, the trigger events most probably represent the opening of a single IP\(_3\)R/ channel and thus provide a useful yardstick by which to estimate the number and spatial distribution of IP\(_3\)Rs involved in an average puff (20) and provide clues as to the mechanism of puff initiation.
Trigger events likely represent single IP3R channel openings

Our experiments were done using fluo-4 dextran as the Ca2+ indicator because its fluorescence in the absence of calcium is extremely low but increases ~30-fold upon binding calcium. This large dynamic range, together with fast (2 ms per line) confocal imaging allowed us to resolve trigger events preceding puffs, whereas previous attempts to identify such “pacemaker” activity during the rising phase of puffs had failed (5).

Several observations suggest that the trigger events likely represent the initial opening of a single IP3R channel. Most directly, fluorescence signals generated by Ca2+ flux through individual N-type voltage-gated channels, expressed in oocytes and imaged with the same indicator and microscope system as used here, showed amplitudes (∆F/FO 1–2) and spatial widths (FWHM 0.55–0.7 μm) similar to the trigger events (23). The Ca2+ currents through the N-type channels were ~0.3 pA, which is comparable to or smaller than the current (~0.5 pA) estimated to pass through the IP3R channel under physiological conditions (24). Moreover, modeling simulations that take into account factors including cytosolic Ca2+ buffering and the microscope point-spread function also indicate that the trigger events are consistent with an underlying Ca2+ flux equivalent to a single-channel current of 0.3–0.5 pA (20,25). The mean channel open dwell time derived from patch-clamp measurements of IP3R in Xenopus oocyte nuclei is ~6 ms (26), so it might well be that triggers arise from a single opening event, given that our measure of the mean trigger duration (12 ms) is probably overestimated owing to a failure to resolve brief signals. Alternatively, the trigger may represent a burst of repeated openings with an overall high open probability, whereby repetitive opening of a single-channel results from positive feedback by Ca2+ passing through that channel (27).

How many IP3R channels open during a puff?

Strong evidence indicates that Ca2+ puffs arise through the concerted opening of IP3Rs arranged in clusters (5,7,10,12). However, experimental data have still not fully resolved questions regarding how many channels are in a cluster, how many of those channels open during a puff, and the spacing between IP3Rs; although these issues have been addressed in modeling studies (8,28). By comparing the characteristics of a presumptive single IP3R/channel opening (trigger) to the opening of many channels (a puff) at the same site, we are in a unique position to estimate such parameters. Given that the mean puff fluorescence amplitude is 5.8 times greater than that of triggers (Table 1), we can conclude that at least six channels open during a puff.

However, calculating the potential channel number based upon a puff/trigger amplitude ratio alone is clearly simplistic and assumes a linear superposition of channel signals, which is likely not the case. An alternative method, which is less sensitive to local indicator saturation, is to estimate the respective Ca2+ fluxes (currents) underlying both the trigger and puff by determining the fluorescence “signal mass” (fluorescence ratio change × volume) of each event (11).

Here, we followed the procedure of Hollingworth et al. (29)
and calculated signal mass as peak fluorescence ratio × 1.2 × the cube of the width (FWHA) of a Gaussian function fitted to the distribution of fluorescence along the scan line at the peak of the event. The mean signal mass of the puff and trigger was then divided, respectively, by the average puff rise time and trigger duration to obtain a relative measure of calcium flux during each of these events. This yielded an average puff/trigger flux ratio of ~60. However, this estimate is subject to considerable uncertainties, in particular because of the large errors introduced by cubing what is already an uncertain measurement of the width of the small trigger events.

The problems associated with both of these methods led us to consider a more complex deterministic mathematical model (20) incorporating the major variables involved in calcium release from single and multiple channels. By using the experimental values described in this work in conjunction with consideration of other factors such as intracellular buffers and the relationship between actual calcium ions released and the confocal signal observed, we show in a companion work that a few tens of channels likely open during a puff (20).

**Blips, trigger events, and the initiation of puffs**

IP$_3$R-mediated Ca$^{2+}$ “blips” have been described previously (9–11) and are generally observed temporally separate from puffs, yet often at the same locations (11). This study marks the first time, to our knowledge, that these putative precursors to puffs. We propose that these trigger events are the foundation on which puffs are initiated and built, and as such they should prove helpful for determining the mechanisms underlying intracellular calcium signaling.

Opening of IP$_3$Rs/channels requires the presence of both IP$_3$ and calcium (2,4). At basal [Ca$^{2+}$] and at the relatively small IP$_3$ concentrations used here to evoke puffs, the probability of any individual IP$_3$R channel becoming activated will be very low. When such an event does occur, we hypothesize that the channel may either i), open once, or as a burst of several openings sustained by Ca$^{2+}$ flux through the channel, but then terminate when IP$_3$ dissociates, to generate a discrete blip; or ii), trigger the regenerative opening by CICR of neighboring channels within the cluster to generate a puff. The observations of trigger events with durations of 10 ms or longer suggest that the functional coupling between IP$_3$Rs in a cluster is relatively weak, so that the enormous Ca$^{2+}$ microdomain in the immediate vicinity of an open channel does not invariably evoke an immediate regenerative opening of multiple adjacent channels. On the other hand, a majority of puffs evoked by higher [IP$_3$] showed an abrupt rising phase, without discernable triggers. In these cases, it may be that the latency between opening of an initial channel and regenerative triggering of CICR became too short to resolve because a greater number of channels had bound IP$_3$ and were thereby in an “activatable” state. It remains to be determined to what extent the coupling efficiency is limited by the spacing between IP$_3$Rs or by their inherent kinetics of channel opening after binding of activating Ca$^{2+}$.

Trigger events were followed by a rapid inflection into the rising phase of the puff proper, with a mean duration of ~19 ms from the time of inflection to the peak of the puff. Most puffs showed a smooth, monotonic rising phase after the trigger. However, a small subset did have slower, “step-like” onsets that might correspond to the successive recruitment of IP$_3$R channels. Because the number of such events was small and there are potential confounding interpretations (e.g., out of focus fluorescence also will show a slower rate of rise), we did not attempt to analyze this limited subset of data. In general, the regenerative recruitment of channels appears to proceed rapidly once more than one channel is open, and the release process already begins to terminate after ~20 ms.

**Puff characteristics are relatively independent of [IP$_3$]**

The amplitude, spatial spread, and duration of puffs all showed only a slight dependence on the amount of photo-released IP$_3$, in agreement with earlier findings (30) that local IP$_3$-evoked Ca$^{2+}$ liberation approximates an “all-or-none” process. On the other hand, the probability of puff occurrence grew steeply as a function of increasing photorelease (14), as would be expected if a greater number of IP$_3$Rs within a cluster bound IP$_3$, thereby increasing the likelihood that one would open after the random binding of a Ca$^{2+}$ ion and trigger a puff. The cumulative Ca$^{2+}$ liberation during a puff seems, therefore, not to be delimited simply by the extent of IP$_3$ binding to receptors in a cluster but, instead, may involve some negative feedback mechanism—possibly including inhibition by locally elevated cytosolic Ca$^{2+}$ or by depletion of luminal [Ca$^{2+}$]. Although puff parameters showed little average dependence on the amount of photo-released IP$_3$, there was still considerable variability in puff amplitudes and durations between individual events, probably reflecting both stochastic variations in the numbers and kinetics open IP$_3$R channels and differences in IP$_3$R number and distribution among different puff sites (11).

**Comparison with RyR-mediated Ca$^{2+}$ signals**

The hierarchy of IP$_3$-evoked Ca$^{2+}$ signals encompassing single-channel events (blips, triggers), multi-channel events (puffs), and global cell waves (10,11) is closely replicated in muscle and other cells where RyRs mediate CICR from the sarco/endoplasmic reticulum. Thus, analogous to puffs, Ca$^{2+}$ “sparks” are now generally believed to arise from the concerted opening of several clustered RyRs (31), whereas smaller events (“quarks”) (10,32) are thought to be generated.
by the opening of one, or a small number of, RyRs. Of particular interest here are the Ca\textsuperscript{2+} ‘‘embers’’, which were originally described as faint tails after sparks in frog skeletal muscle (32) but are more prevalent in rat muscle where they are seen to precede as well as trail a fraction of spontaneous sparks (32). Thus, the trigger and ember events are likely to continue to provide important insights into the fundamental mechanisms involved in initiation of the elementary puffs and sparks from which Ca\textsuperscript{2+} signals are constructed in numerous cell types. This work was supported by grants GM 48071 and GM 65830 from the National Institutes of Health.

**REFERENCES**


