Localized All-or-None Calcium Liberation by Inositol Trisphosphate

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Laser confocal microscopy was used to monitor calcium ion ($Ca^{2+}$) liberation from highly localized (micrometer) regions of intact Xenopus oocytes in response to photo-released inositol 1,4,5-trisphosphate (InsP$_3$). Local $Ca^{2+}$ release varied in an all-or-none manner with increasing amount of InsP$_3$, in contrast to signals recorded from larger areas, which grew progressively as the concentration of InsP$_3$ was raised above a threshold. Liberation of $Ca^{2+}$ was restricted to within a few microns of the site of InsP$_3$ release and, in response to agonist activation, localized regions of the oocyte showed asynchronous oscillations in cytoplasmic $Ca^{2+}$ release. Results obtained with this technique provided direct evidence that InsP$_3$-induced $Ca^{2+}$ liberation was quantized and suggest that the InsP$_3$-sensitive $Ca^{2+}$ pool may be a collection of independent, localized compartments that release $Ca^{2+}$ in an all-or-none manner.

Inositol 1,4,5-trisphosphate (InsP$_3$) is a ubiquitous intracellular second messenger that acts in part by liberating $Ca^{2+}$ stored within the cell (1). The properties of the $Ca^{2+}$ release are spatially and temporally complex (1-4) and are important for signal transduction in the cell. However, most quantitative studies of InsP$_3$-evoked $Ca^{2+}$ liberation have been done with suspensions of permeabilized cells (5-8) in which spatial information is lost. We now describe results obtained by the use of an approach that combines flash photolysis of caged InsP$_3$ (9, 10) with confocal fluorescence $Ca^{2+}$ monitoring to allow rapid (millisecond) measurement of $Ca^{2+}$ release from highly localized regions within a single intact cell.

Experiments were performed on oocytes from X. laevis with light flash photolysis to release InsP$_3$ from intracellularly loaded, caged InsP$_3$ (10). The resulting rise in cytoplasmic free $Ca^{2+}$ was monitored simultaneously with voltage-clamp recording of $Ca^{2+}$-activated membrane chloride ($Cl^-$) conductance (11) and with long-wavelength fluorescent $Ca^{2+}$ indicators (12) to monitor $Ca^{2+}$ from either large or minute regions of the cell (13). A threshold amount of InsP$_3$ is required to evoke any $Cl^-$ current, but the current then increases progressively with increasing InsP$_3$ (10). Using the fluorescent indicator, Fluo-3, to monitor $Ca^{2+}$ liberation throughout the area of the cell (10$^4$ µm$^2$) exposed to the photolysis light, we found that intracellular $Ca^{2+}$ release followed a pattern similar to that of the $Cl^-$ current (Fig. 1, A and B). Increasing amounts of InsP$_3$ were released by altering the duration of the light flash (10). No $Ca^{2+}$ signal was detected with flashes shorter than 8 ms, whereas longer flashes evoked progressively larger responses. The abrupt onset of the $Ca^{2+}$ signal with increasing flash duration and the approximately linear relationship with suprathreshold flashes (Fig. 1B) suggest the existence of a threshold in the $Ca^{2+}$ release process and cannot be fitted well by a power function (7). A similar relation was seen for the $Ca^{2+}$-mediated $Cl^-$ current, except that the threshold was slightly higher, suggesting that an elevation in free $Ca^{2+}$ above the resting concentration may be required to evoke a detectable current.

The fluorescence and current signals (Fig. 1, A and B) both reflected an average $Ca^{2+}$ concentration throughout an appreciable volume of cytoplasm. To monitor $Ca^{2+}$ signals from a highly localized region of the cell, we used a confocal optical system with Rhod 2 as the fluorescent indicator of $Ca^{2+}$ concentration (13). A certain threshold flash duration was again needed to evoke a detectable $Ca^{2+}$ signal (Fig. 1, C and D). However, the $Ca^{2+}$ signal varied in an almost all-or-none manner with increasing liberation of InsP$_3$. A small increase above threshold evoked a large signal that grew very little as the flash duration was further lengthened, although the rate of rise and duration of the responses increased. Similar results were obtained in nine oocytes, and the mean increase in size on lengthening the flash from 8 to 40% of threshold to more than three times the threshold was only 18 ± 6% [mean ± standard error of the mean (SEM)]. It was unlikely that saturation of the fluorescent indicator accounted for this behavior, because Rhod 2 has a relatively low (1 µM) affinity for $Ca^{2+}$ (12), and the maximal InsP$_3$-evoked signals were smaller (53 ± 7%; nine oocytes) than the peak fluorescence evoked by lysing the oocytes in a high (12 mM) $Ca^{2+}$ solution. Some oocytes gave $Ca^{2+}$ signals of intermediate size to stimuli just above threshold, but these may have arisen from attenuated diffusion of $Ca^{2+}$ released at a site a few microns from the measuring spot, rather than from a partial release of $Ca^{2+}$. The $Ca^{2+}$ signals evoked by successive suprathreshold stimuli at a given measuring position showed little variability in size; the standard deviation for stimuli of 2 to 10 times the threshold was only 8% of the mean (41 observations, eight oocytes). Membrane currents evoked by the localized

![Fig. 1. Fluorescent $Ca^{2+}$ signals and $Ca^{2+}$-dependent membrane currents evoked by photorelease of varying amounts of InsP$_3$. (A) Records obtained with coincident large-diameter (150 µm) light spots for photolysis and monitoring of $Ca^{2+}$-dependent fluorescence of Fluo-3. The upper trace in each frame shows fluorescence (upward deflection = increasing $Ca^{2+}$) and the lower trace shows membrane current. Flashes of ultraviolet light of various durations (indicated in milliseconds) were given at the arrowheads. (B) Peak sizes of fluorescence signals (filled symbols, thick line) and membrane currents (open symbols, thin line) evoked by flashes of varying durations. Data are from the same oocyte as in (A) and are scaled as a percentage of that evoked by a 20 ms flash. Similar results were obtained in three additional oocytes. (C and D) Results from an experiment like that in (A and B), except that the confocal optical system was used to record $Ca^{2+}$-dependent fluorescence from a near point source, and the photolysis light was restricted to an area of 60 µm$^2$, centered with the monitoring light. Data in (D) are scaled as a percentage of the maximal responses.](image-url)
(50 to 100 μm²) light flashes used in these experiments showed the same thresholds as the Ca²⁺ signals, but grew progressively as the flash duration was further lengthened (Fig. 1D). This graded increase may have arisen because increasing numbers of Ca²⁺ release sites were recruited throughout that area of the membrane covered by the photolysis light.

Confocal Ca²⁺ signals began with a latency that decreased from more than 500 ms with just suprathreshold stimuli to 53 ± 3 ms (SEM; six oocytes) with stimuli of about ten times the threshold. The rise in Ca²⁺ was abrupt, beginning after a period of apparent quiescence and lasting about 50 ms (Fig. 2A). Because photorelease of InsP₃ is virtually complete within 10 ms (14), some intermediate process between InsP₃ formation and Ca²⁺ liberation (possibly regenerative) may be involved. Between the onsets of the Ca²⁺ and membrane current signals, an additional latency of about 50 ms was seen that might be due to buffered diffusion of Ca²⁺ toward the membrane.

To determine the extent to which Ca²⁺ release is localized, Ca²⁺ signals were monitored confocally from a fixed point, and the light spot used to photolyse caged InsP₃ was displaced (Fig. 2B). The Ca²⁺ signal decreased progressively with increasing separation and showed a slower rising phase and longer peak time, as would be expected for diffusional spread. In the example shown, the signal size decreased to about one-half of the maximum value at a distance of 5 μm and was barely detectable at 8 μm.

Calcium mobilizing agonists evoke oscillatory Cl⁻ currents in the oocyte (15), which probably arise through oscillatory liberation of Ca²⁺ (2, 4). However, previous attempts to record oscillations with Ca²⁺ indicators were unsuccessful (16). We simultaneously measured membrane Cl⁻ current (which monitors intracellular Ca²⁺ through the whole oocyte) and localized intracellular Ca²⁺ (confocal monitor) during bath application of agonist. The current response began earlier (30 s) than the confocal Ca²⁺ signal, indicating that regions of the oocyte distant from the measuring point were activated with a shorter latency. Furthermore, the confocal record initially showed a series of fairly regular oscillations in Ca²⁺ concentration that had no obvious relation with the irregular fluctuations in Cl⁻ current. In addition, the Ca²⁺ oscillations died away during agonist application, leaving a more sustained elevation in Ca²⁺ concentration. Some oocytes displayed spontaneous Ca²⁺ signals even in the absence of stimulation. These were of a similar time course to the signals evoked by just suprathreshold light flashes, but were often of smaller amplitude. The small size may have resulted if Ca²⁺ release originated at points that were distant from the measuring spot.

In permeabilized cells, submaximal doses of InsP₃ liberate only a fraction of the available Ca²⁺ that can be released by a maximal dose (5, 6). This has been interpreted as reflecting a quantal process, such that a given submaximal concentration of InsP₃ releases all the Ca²⁺ from a fraction of the Ca²⁺ stores, whereas none is released from the remaining stores (5). However, it was not clear from those experiments whether the effect arose from heterogeneity between cells or within the stores of each cell (6). In this report, we show directly that localized Ca²⁺ liberation within a single cell is quantized (all or none) and indicate further that oscillations in Ca²⁺ are generated independently and asynchronously at different locations within the cell. In the large (1 mm in diameter) oocyte, graded whole-cell responses may arise if different local Ca²⁺-release units show varying thresholds, so that greater numbers are recruited by increasing concentration of InsP₃. On the other hand, the all-or-none current responses to InsP₃ described in hepatocytes (14) could arise if those small cells contain a single release unit or a homogeneous population of units. The identity of the quantal Ca²⁺-release unit, and the mechanism underlying the local quantal release are not yet clear. Quantal release may arise because of depletion of stored Ca²⁺, feedback inhibition of Ca²⁺ release by a rise in cytoplasmic Ca²⁺ (4, 17), or a decrease in intramitochondrial Ca²⁺ (18).

REFERENCES AND NOTES

13. Procedures for preparation of *X. laevis* oocytes, voltage-clamp recording, and flash photolysis were as described [10; K. Sumikawa, I. Parker, R. Miledi, *Methods Neurosci.* 1, 30 (1989)]. Oocytes were loaded intracellularly with $\sim 1$ pmol of caged InsP$_3$ (myo-inositol 1,4,5-trisphosphate, $P^{4(5)}$-1-(2-nitrophenyl) ethyl ester; CalBiochem) and $\sim 0.5$ pmol of Fluo-3 or Rhod-2 (Molecular Probes). Optical measurements were made from restricted regions of the vegetal hemisphere to avoid light absorption by pigment in the animal hemisphere. Two systems were used to monitor intracellular Ca$^{2+}$ with long wavelength fluorescent indicators (12) to minimize photolysis of caged InsP$_3$. Both were constructed from an upright Zeiss microscope fitted with two stacked epifluorescence units. The lower epifluorescence unit provided flashes of near UV light for photolysis, and the upper provided fluorescence excitation for the Ca$^{2+}$ indicator. In the first system (4), Fluo-3 fluorescence was recorded from a relatively large area ($\sim 10^4 \mu m^2$) and coincident with the photolysis light. The second system used confocal optics to monitor Ca$^{2+}$-dependent fluorescence from a virtual point source in the cytoplasm. Light from a 0.2 mW green (543.5 nm) He-Ne laser was focused by a secondary lens in the epifluorescence unit and reimaged as a diffraction-limited spot by a 40 X water immersion objective (numerical aperture, 0.75), about 5 $\mu m$ below the surface of the oocyte. Emitted light at wavelengths $> 590$ nm was collected through the same lens and monitored by a photomultiplier through a 50 $\mu m$ pinhole positioned confocally in the microscope photo-tube. The photolysis light was focused as a square (area, 50 to 100 $\mu m^2$) centered around the monitoring spot. Rhod-2 was used as the indicator in these experiments, as its excitation spectrum matches well the emission of the inexpensive He-Ne laser. From the size of the detector pinhole and the magnification of the objective lens, we estimated that signals were recorded from a spot with a diameter of about 2 $\mu m$, in the plane of the membrane. Measurements obtained by focusing the microscope through a thin ($\sim 1$ $\mu m$) film of rhodamine solution further indicated that the signal was largely restricted to a depth of about 10 $\mu m$ in the cytoplasm. The monitoring spot remained fixed and was not scanned, as in confocal imaging microscopy. Increases in fluorescence of both Fluo-3 and Rhod-2 corresponded to increasing free Ca$^{2+}$, but because neither shows spectral shifts with Ca$^{2+}$-binding, we did not calibrate signals in terms of free Ca$^{2+}$ concentration.


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