SCIENCE

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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 $18 \pm 6\%$ [mean $\pm$ 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 ($53 \pm 7\%$; 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
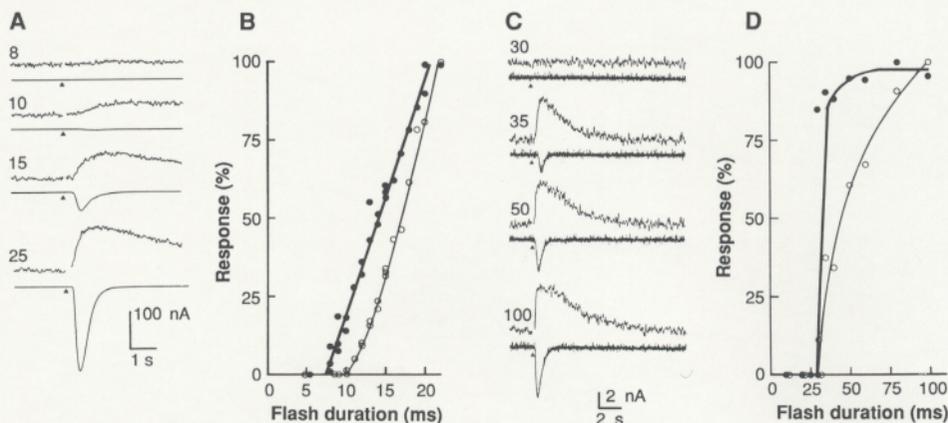

Fig. 1. Fluorescent Ca^{2+} signals and Ca^{2+}-dependent membrane currents evoked by photorelease of varying amounts of $InsP_3$. (**A**) Records obtained with coincident large-diameter (150 μm) light spots for photolysis and monitoring of Ca^{2+}-dependent fluorescence of Fluo-3. The upper trace in 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, concentric with the monitoring light. Data in (D) are scaled as a percentage of the maximal responses.
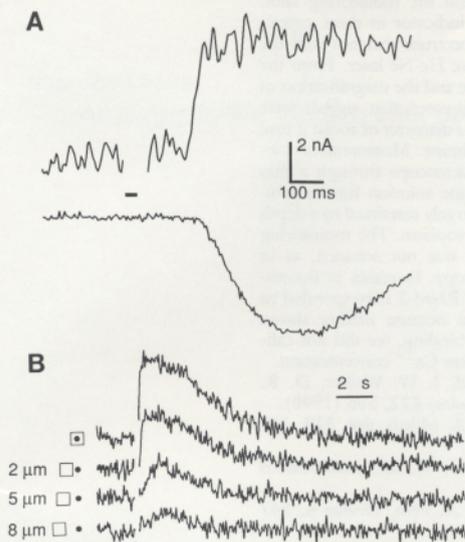
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A

B

2 μm	☐ •
5 μm	☐ •
8 μm	☐ •

Fig. 2. Latency and spatial spread of confocal Ca^{2+} signals. (**A**) Latency of confocal fluorescence (upper trace) and membrane current (lower trace) signals evoked by photorelease of $InsP_3$. Bar indicates duration of the light flash. The fluorescence trace is blanked out during and shortly after the flash because stray light saturated the photomultiplier. (**B**) Lateral spread of the Ca^{2+} signal evoked by local photorelease of $InsP_3$. Traces show confocal records obtained from a fixed point (indicated by a dot in the diagrams), while the photolysis light (square) was displaced by different distances. Numbers indicate the distance in microns from the monitoring spot to the edge of the photolysis light. Flashes of constant intensity and duration were given at each position of the photolysis light, at the time marked by the arrowhead. Data presented are from a single oocyte. Similar results were obtained in two additional oocytes. Control records (with the photolysis light centered on the monitoring spot) obtained before and after the experiments indicated that the diminution in response size was not due to photobleaching of Rhod-2.

(50 to 100 μm²) light flashes used in these experiments showed the same thresholds as the Ca^{2+} signals, but grew progressively as the flash duration was further lengthened (Fig. 1D). This graded increase may have arisen because increasing numbers of Ca^{2+} release sites were recruited throughout that area of the membrane covered by the photolysis light.

Confocal Ca^{2+} 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 Ca^{2+} was abrupt, beginning after a period of apparent quiescence and lasting about 50 ms (Fig. 2A). Because photorelease of $InsP_3$ is virtually complete within 10 ms (14), some intermediate process between $InsP_3$ formation and Ca^{2+} liberation (possibly regenerative) may be involved. Between the onsets of the Ca^{2+} and membrane current signals, an additional latency of about 50 ms was seen that might be due to buffered diffusion
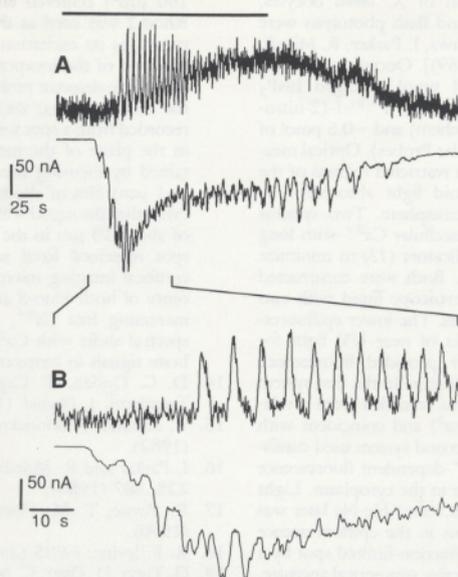
A

B

Fig. 3. Oscillatory fluorescence and current signals evoked with serum as an agonist to activate phosphoinositide signaling (19). (**A**) The upper trace is the confocal fluorescence monitor and the lower trace is the membrane current. Serum (10^3 dilution) was bath-applied for the time indicated by the bar. (**B**) Section of the record in (A) shown with an expanded time scale.

of Ca^{2+} toward the membrane.

To determine the extent to which Ca^{2+} release is localized, Ca^{2+} signals were monitored confocally from a fixed point, and the light spot used to photolyse caged $InsP_3$ was displaced (Fig. 2B). The Ca^{2+} 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 Ca^{2+} (2, 4). However, previous attempts to record oscillations with Ca^{2+} indicators were unsuccessful (16). We simultaneously measured membrane Cl^- current (which monitors intracellular Ca^{2+} throughout the whole oocyte) and localized intracellular Ca^{2+} (confocal monitor) during bath application of agonist. The current response began earlier (30 s) than the confocal Ca^{2+} 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 Ca^{2+} concentration that had no obvious relation with the irregular fluctuations in Cl^- current. In addition, the Ca^{2+} oscillations died away during agonist application, leaving a more sustained elevation in Ca^{2+} concentration. Some oocytes displayed spontaneous Ca^{2+}

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 Ca^{2+} release originated at points that were distant from the measuring spot.

In permeabilized cells, submaximal doses of $InsP_3$ liberate only a fraction of the available Ca^{2+} 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 $InsP_3$ releases all the Ca^{2+} from a fraction of the Ca^{2+} 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 Ca^{2+} liberation within a single cell is quantized (all or none) and indicate further that oscillations in Ca^{2+} 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 Ca^{2+}-release units show varying thresholds, so that greater numbers are recruited by increasing concentration of $InsP_3$. On the other hand, the all-or-none current responses to $InsP_3$ 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 Ca^{2+} release unit, and the mechanism underlying the local quantal release are not yet clear. Quantal release may arise because of depletion of stored Ca^{2+}, feedback inhibition of Ca^{2+} release by a rise in cytoplasmic Ca^{2+} (4, 17), or a decrease in intraluminal Ca^{2+} (18).

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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 ~1 pmol of caged InsP$_3$ (*myo*-inositol 1,4,5-trisphosphate, P$^{4(5)}$-1-(2-nitrophenyl) ethyl ester; CalBiochem) and ~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 (~10^4 μm^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× water immersion objective (numerical aperture, 0.75), about 5 μm 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 μm pinhole positioned confocally in the microscope photo-tube. The photolysis light was focused as a square (area, 50 to 100 μm^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 μm, in the plane of the membrane. Measurements obtained by focusing the microscope through a thin (~1 μm) film of rhodamine solution further indicated that the signal was largely restricted to a depth of about 10 μm 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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