Regenerative release of calcium from functionally discrete subcellular stores by inositol trisphosphate

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[Plate 1]

SUMMARY

Fluorescence imaging was used to determine the spatial and temporal patterns of subcellular calcium (Ca\(^{2+}\)) liberation induced in Xenopus oocytes by photorelease of inositol 1,4,5-trisphosphate (Ins\(_P_3\)) from a caged precursor. Increasing levels of Ins\(_P_3\) evoked Ca\(^{2+}\) release that began in a graded manner but, at varying threshold levels of Ins\(_P_3\), localized sites then showed transient and asynchronous ‘puffs’ of Ca\(^{2+}\) release. With higher levels of Ins\(_P_3\), Ca\(^{2+}\) from adjacent sites formed a focus for initiation of a propagating Ca\(^{2+}\) wave. The results show that Ins\(_P_3\)-sensitive Ca\(^{2+}\) stores are arranged as distinct and functionally independent units, and that Ca\(^{2+}\) is released in both graded and regenerative fashions.

1. INTRODUCTION

Inositol 1,4,5-trisphosphate (Ins\(_P_3\)) is an intracellular messenger that mediates responses to many neurotransmitters and hormones by liberating Ca\(^{2+}\) ions stored within the cell (Berridge & Irvine 1989). The spatial organization of Ca\(^{2+}\) liberation is complex, and may encode or determine the way in which information is passed along the signal transduction pathway (Berridge et al. 1988; Cheek 1989; O’Sullivan et al. 1989; Lechleiter et al. 1991; Delisle 1991; Meyer 1991). The result that Ca\(^{2+}\) release from permeabilized cell preparations varies in a stepwise manner with successive increments of Ins\(_P_3\) concentration (Mullem et al. 1989; Taylor & Potter 1990; Meyer et al. 1990; Meyer & Stryer 1990) has led to the proposal that Ins\(_P_3\)-sensitive Ca\(^{2+}\) stores are organized as multiple subcellular quanta, displaying different sensitivities to Ins\(_P_3\) (Parker & Ivrrea 1990; Mualllem et al. 1989). However, alternative interpretations are possible, including heterogeneity between cells (Taylor & Potter 1990), or modulation of Ins\(_P_3\) receptors by intraluminal Ca\(^{2+}\) within a homogeneous store (Irvine 1990; Tregear et al. 1991). We have tested the ‘quantal’ hypothesis by using video imaging of oocytes loaded with a fluorescent indicator to visualize subcellular Ca\(^{2+}\) release with micrometre resolution, and to determine the dependence of Ca\(^{2+}\) release upon Ins\(_P_3\) concentration.

2. MATERIALS AND METHODS

Experiments were done on oocytes of Xenopus laevis. These were obtained from albino frogs to avoid interference during optical recordings by the pigment that is normally present in the animal hemisphere of the cells. Oocytes were each injected with about 50 pmol of the Ca\(^{2+}\) indicator fluo-3 (Minta et al. 1989) and about 5 pmol caged Ins\(_P_3\) (meioinositol 1,4,5-trisphosphate, \(\text{P}^{\text{N}}\text{SO}_{4}\text{-1-(2-nitrophenyl)}\) ethyl ester) (McCray & Trentham 1989), resulting in respective final intracellular concentrations of approximately 30 \(\mu\)M and 5 \(\mu\)M. Injections were made by pneumatic pressure pulses applied to a glass micropipette filled with 5 mM fluo-3 and 0.5 mM caged Ins\(_P_3\) (Parker & Miledi 1989), and an interval of 30–90 min was allowed before recording to allow even distribution of the compounds throughout the cell.

The optical system for simultaneous flash photolysis of caged Ins\(_P_3\) and recording of Ca\(^{2+}\)-dependent changes in fluo-3 fluorescence is shown in figure 1. This was based on an upright Zeiss microscope fitted with two stacked epifluorescence units and a 40 \(\times\) water immersion objective (numerical aperture 0.75). The lower epifluorescence unit provided flashes of near UV light (wavelengths about 340–420 nm) for photolysis, by means of a continuous Xenon arc lamp and an electronically controlled shutter. A variable rectangular slit diaphragm allowed the photolysis light to be focused on the oocyte as a square or rectangle of desired size. The upper epifluorescence unit was fitted with a 100 W quartz halogen lamp, and provided blue excitation light for the fluo-3. The excitation light was restricted to circle of about 100 \(\mu\)m diameter on the oocyte surface, as the curvature of the cell did not allow larger regions to be viewed in sharp focus. Because of the long excitation and emission wavelengths of fluo-3, the fluorescence excitation light caused no detectable photorelease of Ins\(_P_3\), and photolysis light flashes were almost completely blocked by the barrier filter in the recording path.

Fluorescence images were detected by an intensified CCD camera (Quantex Corp., Sunnyvale, California, U.S.A.) and stored on a domestic super VHS videocassette recorder at video frame rate (30 s\(^{-1}\)). The intensifier and camera were operated at their minimum gain settings, and the intensity of
the fluorescence excitation light was adjusted so that the camera operated within its linear range. Video frames were captured with 8 bit (256 level) resolution and processed using the JAVA image processing system (Jandel Scientific, Corte Madre, California, U.S.A.). The images in figure 2 show regions of about 200 x 200 pixels (100 µm x 100 µm) in the centre of the recording field. Control frames of resting fluorescence were subtracted and, to reduce noise, the lowest four intensity levels were set to zero and the images were low-pass filtered through a 3 x 3 convolution mask. Timecourses of Ca²⁺ signals were monitored by using a photodiode and collector lens to measure intensities from small spots on a monochrome display screen. Because fluo-3 does not permit the use of ratio measurements to determine absolute free Ca²⁺ levels, data are presented in arbitrary units. However, comparison of fluorescence signals within each experiment provides a relative indication of free Ca²⁺ levels.

Figures illustrate results from individual oocytes but, with the exception of figure 4, are representative of findings in at least five oocytes.

3. RESULTS

(a) Punctate release of Ca²⁺

The spatial distribution of subcellular Ca²⁺ liberation induced by InsP₃ was monitored by recording images of fluo-3 fluorescence from small (ca. 100 µm diameter) regions of the oocyte after photorelease of InsP₃.

Despite the considerable thickness of the oocyte (1 mm or more in diameter), the fluorescence signals reflect changes in free Ca²⁺ occurring only in a thin layer close to the plasma membrane. This is because the turbidity of the cytoplasm severely restricts light transmission, and measurements with a confocal microscope showed that the recorded fluorescence declined to one-half at a distance of about 15 µm into the cell. Photolysis flashes of varying duration were used to release different amounts of InsP₃. A roughly linear relation is expected because the photolysis reaction involves single-photon absorption, and even the longest flashes used would have photolyzed only a small fraction of the available caged InsP₃ (Parker 1991). The timecourses of the evoked Ca²⁺ transients were slow (several hundred ms) as compared both to the flash durations used and the kinetics of photorelease of InsP₃ (< 10 ms; McGraw & Trentham 1989).

Figure 2a illustrates typical patterns of Ca²⁺ liberation evoked by stimulus flashes of various durations. In this oocyte, a flash of 6 ms duration evoked a single, localized "puff" of Ca²⁺, beginning after a latency of 3.2 s and lasting less than 1 s. The same site responded
Figure 2. Spatial patterns of subcellular Ca\(^{2+}\) liberation induced by photoreleased InsP\(_3\). Increasing free Ca\(^{2+}\) levels are depicted on a pseudocolour scale from blue to red, after subtraction of resting images captured before stimulation. Fluorescence images were recorded from a circular field, just encompassed within the frame borders. The photolysis light was arranged as a square slightly larger than the frames, to ensure uniform liberation of InsP\(_3\) throughout the recording area. A. Ca\(^{2+}\) release occurs at discrete sites, showing different thresholds for InsP\(_3\). All frames show Ca\(^{2+}\) release within the same recording area, evoked by photolysis flashes of various durations. The images were captured at intervals of 3-2 s following a 6 ms flash; 1.6 s and 2.4 s following a 7 ms flash; and 1.7 s, 2.3 s, 3.3 s and 6.3 s following an 8 ms flash. B. Active wavefront of propagating Ca\(^{2+}\) release. Images were formed by sequential subtraction of video frames captured at ten-frame intervals, and thus show increases in Ca\(^{2+}\) occurring during the preceding 333 ms. A 20 ms photolytic flash given 1.6 s before the first frame evoked a focus of Ca\(^{2+}\) release near the center of the recording area. C. Punctate release of Ca\(^{2+}\) following a strong (duration about 3-times threshold) light flash. Images show successive video frames (33 ms intervals) beginning two frames after the end of a 20 ms flash.
out of 13 oocytes from another donor frog, Ca²⁺ waves were triggered as soon as one or a group of adjacent release sites were activated, so that it was not possible in these cells to observe transient puffs of Ca²⁺ release.

Following stimulation by longer light flashes, Ca²⁺ release occurred nearly simultaneously at multiple discrete loci, spaced about 10–20 μm apart (figure 2c). ‘Spots’ of Ca²⁺ first became apparent within one or two video frames following the flash, and subsequently became brighter and more diffuse over the next several frames. Even at early times, when diffusion of Ca²⁺ would be minimal, the Ca²⁺ spots did not appear as point sources. Instead, they were often shaped as elongated blobs or as rings, with dimensions of approximately 5 μm. No Ca²⁺ waves were apparent, probably because Ca²⁺ was released simultaneously from all of the InsP₃-sensitive stores within the recording area.

(b) Timecourse of Ca²⁺ puffs

Figure 3a shows the timecourses of cytoplasmic free Ca²⁺ monitored at three points within the same recording field as figure 2a. Site i was the most sensitive and showed a Ca²⁺ transient in response to a 6 ms flash, whereas site ii began to respond only when the flash was lengthened to 7 ms. Site iii did not show Ca²⁺ puffs, but was invaded by the wave of Ca²⁺ initiated following an 8 ms flash. Free Ca²⁺ levels during the wave rose higher than during the puffs, and decayed more slowly.

An important result was that different subcellular sites operated independently, and generated asynchronous puffs of Ca²⁺. Another example of this is shown in figure 3k, obtained using prolonged exposure to UV light of low intensity to produce a gradually rising level of InsP₃ within the cell. Two sites about 30 μm apart gave repetitive puffs of Ca²⁺, superimposed on a gradual rise in baseline Ca²⁺.

(c) Spontaneous Ca²⁺ puffs

Oocytes did not usually show any changes of Ca²⁺ in the absence of stimulation. However, by chance, we observed one oocyte which displayed spontaneous puffs of Ca²⁺. These occurred apparently at random, at multiple sites within the recording field. Figure 4 shows traces of Ca²⁺ transients recorded at two sites. One site (upper trace) gave frequent puffs, whereas other sites (e.g., lower trace) were less active.

(d) Pacemaker and regenerative Ca²⁺ release

In addition to the release of Ca²⁺ as puffs or waves, brief flashes evoked also a smaller, gradual elevation of Ca²⁺. This ‘pacemaker’ Ca²⁺ was not resolved in images such as figure 2a, but is evident in several traces in figure 3a, where it can be seen to precede the appearance of puffs, as well as occurring at sites that failed to show puffs. Because the fluorescence signals from the pacemaker Ca²⁺ were faint, we integrated measurements over wider (several hundred μm²) areas.

of the cell to obtain better resolution. For example, the oocyte in figure 5a first showed a detectable response to a 10 ms flash, and the Ca\textsuperscript{2+} signal then increased progressively as the flash was lengthened to 12 ms. However, the response to a 14 ms flash showed an inflexion after about 2 s, after which the Ca\textsuperscript{2+} level rose abruptly as a Ca\textsuperscript{2+} wave propagated through the recording area. Increasing the flash duration to 15 ms increased the size of the response and shortened the latency to the inflexion but, although a further increase to 17 ms gave a still shorter latency, the response size increased little. Measurements from a different oocyte of peak sizes of Ca\textsuperscript{2+} signals evoked by flashes of various durations are plotted in figure 5b, and fit a curve with three distinct components: (i) an initial ‘foot’ increasing in a graded manner with flash duration; (ii) an abrupt rise; and (iii) a more gradual rise over which the signal grew only 30%, for a fivefold increase in flash duration.

At present we do not know whether the pacemaker Ca\textsuperscript{2+} is liberated diffusely, or in a punctate manner like the regenerative Ca\textsuperscript{2+} release. The faint Ca\textsuperscript{2+} fluorescence signals did not allow good spatial resolution and, in any case, Ca\textsuperscript{2+} ions would become diffusely distributed during the slow rising phase of the pacemaker Ca\textsuperscript{2+}.

4. DISCUSSION

Our results show that InsP\textsubscript{3} caused punctate release of sequestered Ca\textsuperscript{2+} in Xenopus oocytes. Individual Ca\textsuperscript{2+} release units functioned independently and showed varying sensitivities to InsP\textsubscript{3}, as predicted by a "quantal" model of Ca\textsuperscript{2+} release (Parker & Ivorra 1990; Muirlem et al. 1989). The morphological correlate of the release units is presently unclear; they may, for example, be discrete organelles, or represent ‘hot spots’ within an extensive reticulum (Rossier & Putney 1991). In the latter case, possible explanations for heterogeneity in sensitivity to InsP\textsubscript{3} include clustering of InsP\textsubscript{3} receptors, or variations in affinity of InsP\textsubscript{3} receptors resulting from differences in either cytoplasmic (Bzeprozynsky et al. 1991) or intraluminal (Irvine 1990) free Ca\textsuperscript{2+} level. Fine structure was evident in patterns of Ca\textsuperscript{2+} from single units, suggesting they have dimensions of a few μm, rather than being point sources. High speed confocal imaging should provide better resolution, and it will be interesting to correlate the foci of Ca\textsuperscript{2+} release with the distributions of InsP\textsubscript{3} receptors and other endoplasmic reticulum marker proteins.

From experiments like figure 2c we estimate that the oocyte may contain a few thousand Ca\textsuperscript{2+} release units if these are arranged as a single layer under the cell membrane, and this number will obviously be greater if additional units are present deeper in the cytoplasm where they are not visible in our recordings. The incremental contribution made by each unit is, therefore, only a small fraction of the total, so that the whole cell may show an apparently smoothly graded response as increasing numbers of units are recruited by increasing concentrations of InsP\textsubscript{3}. However, the Xenopus oocyte is an extraordinarily large cell (> 1 mm diameter). If we assume that the architecture of the Ca\textsuperscript{2+} pools in the oocyte is similar to that in other cells, it seems that there may be room for no more than one release unit in a cell of "normal" size. In such cases, individual cells may show all-or-none responses, and observations of quantal or incremental Ca\textsuperscript{2+} release in cell populations (Muirlem et al. 1989; Taylor & Potter 1990; Mete et al. 1990; Meycr & Stryer 1990) could result from inter-rather than intra-cellular heterogeneity.

In one oocyte we were fortunate to observe spontaneous puffs of Ca\textsuperscript{2+} release, which resembled those evoked by InsP\textsubscript{3}. This activity presumably underlies the spontaneous oscillations sometimes seen in recordings of the Ca\textsuperscript{2+}-activated chloride membrane current (Kusano et al. 1982) and may also be related to the spontaneous outward currents in smooth muscle cells that appear to result from quantal release of Ca\textsuperscript{2+} from intracellular stores (Benham & Bolton 1986). The spontaneous current oscillations are abolished by caffeine, an inhibitor of InsP\textsubscript{3} action (Parker & Ivorra 1991), but it is not yet clear whether these oocytes showing spontaneous Ca\textsuperscript{2+} release have elevated resting levels of InsP\textsubscript{3} or whether the InsP\textsubscript{3}-induced Ca\textsuperscript{2+} release process is sensitized.

Photorelease of increasing amounts of InsP\textsubscript{3} by light flashes of increasing duration evoked three different modes of Ca\textsuperscript{2+} liberation. We refer to these, respectively, as pacemaker Ca\textsuperscript{2+}, Ca\textsuperscript{2+} puffs and Ca\textsuperscript{2+} waves. After brief flashes, the level of Ca\textsuperscript{2+} rose and fell gradually over a few seconds, with an amplitude that was graded with flash duration. However, when the flash was lengthened beyond a certain threshold an abrupt regenerative release of Ca\textsuperscript{2+} was triggered, which appeared either as transient puffs restricted to particular release sites, or as a wave of Ca\textsuperscript{2+} that propagated throughout the stimulated area. The all-or-none dose-response relation we had previously observed (Parker & Ivorra 1990) using confocal optics to monitor Ca\textsuperscript{2+} at a point source probably reflected the initiation of a regenerative response, whereas the pacemaker Ca\textsuperscript{2+} may have been below the limit of resolution in those experiments. Regenerative Ca\textsuperscript{2+} release has also been observed in mammalian oocytes (Miyazaki 1988; Peres 1990).

The pacemaker Ca\textsuperscript{2+} signal appeared to increase as a steeper than linear function of the amount of photorelease InsP\textsubscript{3}. This might reflect cooperative binding of InsP\textsubscript{3} to its tetrameric receptor (Meycr et al. 1988; Meycr & Stryer 1990), or could arise through a subthreshold regenerative effect. However, it is clear that cooperativity cannot explain the abrupt appearances of Ca\textsuperscript{2+} puffs and waves. Instead, the striking resemblance of these regenerative responses to a nerve action potential points to the existence of positive feedback in the release process. Ca\textsuperscript{2+} ions released into the cytosol have been proposed to exert positive feedback at various stages in the messenger pathway, including: (i) stimulation of phospholipase C resulting in increased formation of InsP\textsubscript{3} (Swann & Whitaker 1986; Haroutunian et al. 1991); (ii) Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release from InsP\textsubscript{3}-insensitive stores (Beridge 1991;
Goldbeter et al. 1990); and (iii) Ca\textsuperscript{2+} acting as a coagonist to potentiate InsP\textsubscript{3}-mediated Ca\textsuperscript{2+} liberation (Lino 1990; Finch et al. 1991; Missiaen et al. 1991; Bezprozvanny et al. 1991). Of these models we favour the latter as a possible explanation for our results, as intracellular injections of Ca\textsuperscript{2+} ions into Xenopus oocytes evoke smoothly graded membrane current responses (Miledi & Parker 1984) and do not evoke propagating Ca\textsuperscript{2+} waves (unpublished data). Thus puffs of Ca\textsuperscript{2+} may result from regenerative positive feedback localized to individual release units, and a wave will be initiated if regenerative release is triggered from surrounding units. The organization of InsP\textsubscript{3}-sensitive Ca\textsuperscript{2+} release units is, therefore, probably important not only in determining spatial and temporal aspects of InsP\textsubscript{3} signalling, but also its dose-dependence.

We thank Ricardo Miledi for helpful discussion. This work was supported by grant GM39831 from the U.S. Public Health Service.

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Submitted by R. Miledi; received 16 September 1991; accepted 30 September 1991

Plate 1 was printed by George Over Ltd, Rugby and London, U.K.