

CONFOCAL MICROFLUORIMETRY OF Ca^{2+} SIGNALS EVOKED IN *XENOPUS* OOCYTES BY PHOTORELEASED INOSITOL TRISPHOSPHATE

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(Received 16 March 1992)

SUMMARY

1. The subcellular characteristics of inositol 1,4,5-trisphosphate (InsP_3)-induced Ca^{2+} liberation were studied in *Xenopus* oocytes by the use of confocal microfluorimetry to monitor Ca^{2+} signals from minutely localized region of the cell in response to photorelease of InsP_3 from a caged precursor.

2. Photorelease of increasing amounts of InsP_3 by progressively longer light flashes evoked transient Ca^{2+} responses that appeared abruptly at a certain threshold duration, and then grew steeply over a narrow range of flash durations to reach a maximum. Further lengthening of flash duration gave no increase in size of the Ca^{2+} signals, but their rate of rise continued to increase and their duration became longer. Simultaneous measurements of Ca^{2+} -activated Cl^- currents showed a slightly higher threshold than the Ca^{2+} signal, and a more graded dependence upon flash duration.

3. The threshold flash durations required to evoke Ca^{2+} and membrane current signals grew by more than 100-fold as the area of the oocyte exposed to photolysis light was reduced from a square of $140\ \mu\text{m}$ to $5\ \mu\text{m}$.

4. Ca^{2+} signals evoked by photoreleased InsP_3 began following a dose-dependent latency that was as long as several seconds with low intensity light, but shortened to about 50 ms at maximum intensity. The extrapolated minimum latency with infinite photorelease of InsP_3 was about 30 ms.

5. InsP_3 -evoked membrane currents began 30 ms or longer after the corresponding Ca^{2+} signals, whereas currents evoked by photorelease of Ca^{2+} from a caged precursor began within 5 ms of the onset of the light flash.

6. No differences in duration of InsP_3 -evoked Ca^{2+} signals were apparent when the confocal measuring spot was positioned close to the plasma membrane or about $10\ \mu\text{m}$ more deeply into the oocyte. At both locations the Ca^{2+} signals were more prolonged than the associated membrane current signals.

7. Ca^{2+} signals to a test light flash were suppressed for about 2 s following a conditioning suprathreshold flash, but recovered almost completely after 6 s. The associated membrane current signals were facilitated at short intervals, suppressed

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at intervals between 0.5 and 3 s, and subsequently recovered more slowly than the Ca^{2+} signals.

8. Photorelease of InsP_3 during 30 s exposures of low intensity evoked trains of repetitive Ca^{2+} spikes. The overall amplitudes of these responses changed little with increasing photolysis intensity, but the spikes began following shorter latencies, increased in frequency, and became smaller and superimposed on a more sustained elevation of Ca^{2+} . At high light intensities spikes could not be discerned and, after an initial abrupt increase, the Ca^{2+} level declined monotonically over several seconds. The Ca^{2+} -activated membrane current reflected the Ca^{2+} spikes, but not the maintained elevation of Ca^{2+} .

9. We conclude that InsP_3 -sensitive Ca^{2+} stores in the oocyte are arranged in a 'quantal' manner, as multiple functionally independent units. These release their contents with nearly all-or-none dose dependence at varying threshold levels of InsP_3 , producing single spikes of Ca^{2+} following transient elevation of InsP_3 and repetitive spikes during maintained elevation of InsP_3 .

INTRODUCTION

Inositol, 1,4,5-trisphosphate (InsP_3) is a ubiquitous second messenger, which is formed in many different cell types in response to receptor-mediated activation of phospholipase C, and functions primarily by liberating Ca^{2+} ions from intracellular stores (Berridge & Irvine, 1989). Studies of InsP_3 action in intact cells have been greatly facilitated by the availability of a photolabile caged InsP_3 (McCray & Trentham, 1989), that allows rapid and precisely controlled elevations of intracellular InsP_3 to be generated following flashes of UV light. We had previously employed this technique in *Xenopus* oocytes, and monitored the resulting changes in cytosolic free Ca^{2+} level by recording a Ca^{2+} -activated chloride membrane conductance, and by measuring the fluorescence of intracellularly loaded Ca^{2+} indicator dyes (Parker & Miledi, 1989; Parker & Ivorra, 1990*a*, 1991, 1992; Ivorra & Parker, 1990). However, there is increasing evidence that InsP_3 -mediated Ca^{2+} liberation does not occur homogeneously throughout the oocyte. Specifically, intracellular injections of InsP_3 produce a localized depression of responses to subsequent injections (Berridge, 1989), agonist activation evokes complex patterns of Ca^{2+} waves travelling across the oocyte (Lechleiter, Girard, Peralta & Clapham, 1991*a, b*; Yao & Parker, 1991), and video imaging of Ca^{2+} signals evoked by uniform photorelease of InsP_3 reveals that Ca^{2+} is released at discrete 'hot spots' spaced several micrometres apart, which each function autonomously (Parker & Yao, 1991). Thus, interpretation of Ca^{2+} -evoked chloride currents is difficult, because they arise across the entire membrane area of the cell, and probably reflect the summation of asynchronous Ca^{2+} transients from many different regions. Furthermore, Ca^{2+} indicator dyes suffer the same limitation if fluorescence is monitored by a photomultiplier from the whole cell, or from an appreciable fraction of the cell.

To study better the characteristic of InsP_3 -induced Ca^{2+} release at a subcellular level, we developed a point confocal optical system (Pawley, 1990) that allows fluorescence signals from Ca^{2+} indicators to be monitored from a minute volume within the oocyte. The use of an indicator (rhod-2) excited by green light (Minta, Kao & Tsien, 1989) permits simultaneous flash photolysis of caged InsP_3 by UV light.

Unlike commercial confocal microscopes, which form images by scanning the confocal spot, our system monitors from only a single fixed spot. Thus, although spatial information is lost, Ca^{2+} transients at a fixed location are followed with good time resolution. We describe the use of this system to investigate the functional characteristics of individual InsP_3 -sensitive stores in the oocyte and, in particular, the dependence of the size and time course of cytoplasmic Ca^{2+} signals on the level of InsP_3 . A brief account of some of the results has appeared (Parker & Ivorra, 1990b).

METHODS

Preparation of oocytes and electrophysiology

Experiments were done on ovarian oocytes of *Xenopus laevis* obtained after killing donor frogs by decerebration and pithing. Isolated oocytes were treated with collagenase to remove enveloping cells, and membrane currents were recorded using a two-electrode voltage clamp as described previously (Sumikawa, Parker & Miledi, 1989). During recordings, oocytes were continually superfused with Ringer solution (composition (mM): NaCl, 120; KCl, 2; CaCl_2 , 1.8; Hepes, 5; pH about 7.0) at room temperature (21–25 °C).

Electrodes were impaled while viewing the oocyte through a low power ($6.3\times$) objective lens that provided a conveniently long working distance. After voltage-clamping, a further micropipette was inserted to allow pneumatic pressure injection of caged InsP_3 together with the fluorescent Ca^{2+} indicator dye rhod-2 (Minta *et al.* 1989). This pipette was filled with an aqueous solution including 1 mM caged InsP_3 (myo-inositol, 1,4,5-trisphosphate, $P^{4(5)}$ -1-(2-nitrophenylethyl ester)), 0.5 mM rhod-2 free acid and 5 mM Hepes at pH 7. The volume of fluid ejected by each pressure pulse was estimated by measuring the size of the fluid droplet expelled with the pipette tip in the air, and a total volume of about 500 pl was usually injected into each oocyte. The injection pipette was then withdrawn, and the objective lens was replaced by a $40\times$ water immersion lens (Zeiss; numerical aperture 0.75) fitted with an insulating collar to avoid interference with measurements of clamp current. To allow for the short working distance of the immersion objective, the voltage-clamp electrodes were impaled at shallow angles.

Caged InsP_3 and DM-nitrophen (dimethoxynitrophenamine; caged Ca^{2+}) were obtained from Calbiochem (La Jolla, CA, USA) and rhod-2 from Molecular Probes Inc. (Eugene, OR, USA). All other reagents were from Sigma Chemical Co. (St. Louis, MO, USA).

Optical system

Figure 1 shows a diagram of the optical system used to generate flashes of near UV light for photolysis of caged InsP_3 , and to allow simultaneous confocal recording of rhod-2 fluorescence. This was based on an upright Zeiss microscope, fitted with two epi-fluorescence units stacked one above the other.

The lower unit provided the photolysis light flashes, and has previously been described (Parker, 1992). Briefly, the light source was a 75 W continuous xenon arc lamp fitted with a high speed electronic shutter (Newport Corp., Fountain Valley, CA, USA) to provide flashes of any desired duration greater than about 2 ms. Wavelengths between about 340 and 450 nm were selected through a Schott UG5 filter and reflected toward the objective lens by a dichroic mirror in the epifluorescence unit. A variable rectangular slit diaphragm (Leitz) allowed the photolysis light to be focused onto the oocyte as a square or slit of variable size, positioned anywhere within the field of view. In some experiments the intensity of the photolysis light was varied by means of neutral density filters, which were calibrated at a wavelength of 350 nm. The irradiance of the system at maximal output (4 mW mm^{-2}) was estimated to photolyse $< 1\%$ of the caged InsP_3 present in the illuminated volume of the oocyte during a flash of 25 ms duration (Parker & Ivorra, 1992). Unless otherwise noted, the light was normally arranged as a square of $15 \mu\text{m}$ side, concentric with the confocal monitoring spot.

The upper epifluorescence unit was modified to provide point-source excitation for confocal measurement of rhod-2-fluorescence. A 0.2 mW green (547.5 nm) helium–neon laser (Melles Griot, Irvine, CA, USA) was used as the light source. The parallel output beam was focused to a point at the image plane of the microscope objective by a lens (focal length 10 mm) cemented onto the adjustable holder normally used to mount the field diaphragm in the epifluorescence unit. The

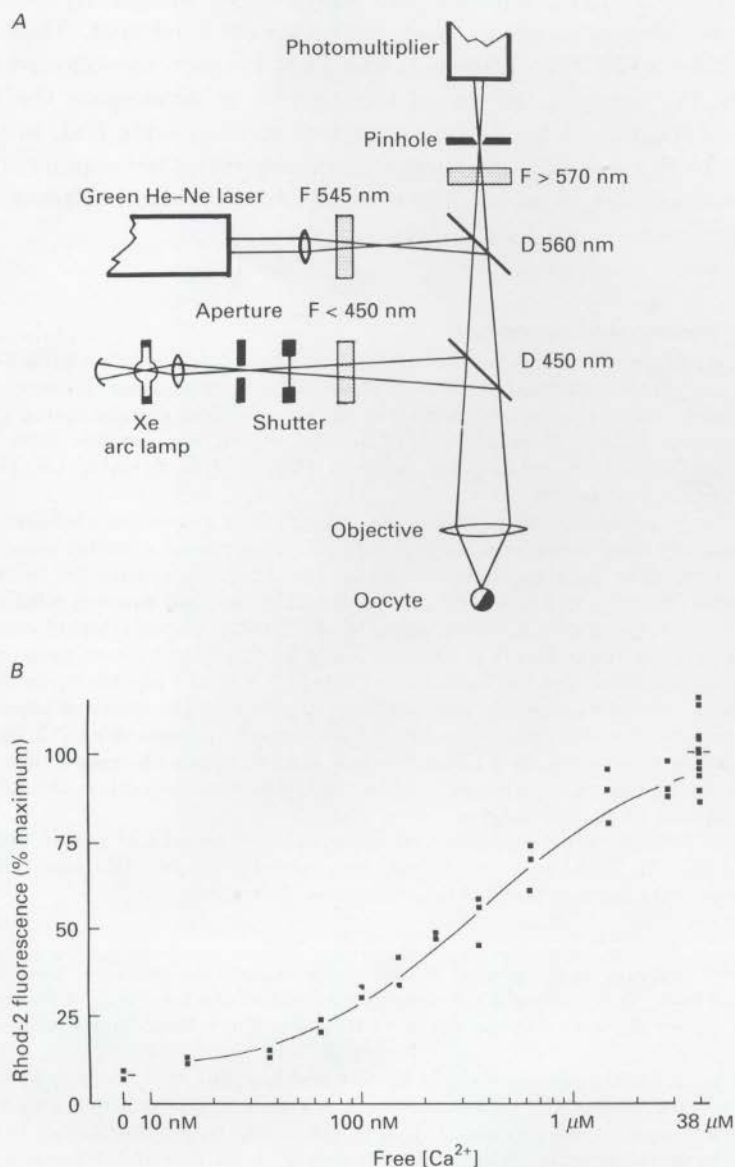


Fig. 1. *A*, schematic diagram of the optical system for simultaneous flash photolysis and confocal monitoring of Ca^{2+} transients. See text for details. F = filter, D = dichroic mirror; with wavelengths indicated in nm. *B*, rhod-2 fluorescence measured by the confocal microfluorimeter as a function of free Ca^{2+} concentration. Fluorescence is expressed as a percentage of the mean maximum fluorescence obtained in repeated readings from droplets containing $38 \mu M$ free Ca^{2+} . The curve was calculated using a K_d of 340 nM for binding of Ca^{2+} to rhod-2, and a minimum fluorescence in Ca^{2+} -free solution 8% of that in saturating Ca^{2+} .

diverging cone of laser light was then reflected by a dichroic mirror and was re-focused by the microscope objective as a near diffraction limited spot on the oocyte. A green filter in the excitation path selected only the main output line of the laser and rejected the red glow from the plasma tube.

Light emitted by fluorescence of the rhod-2 was collected through the same objective and brought to a focus at a pinhole (Melles Griot, Irvine, CA, USA) mounted in an eye-piece in the microscope phototube. A barrier filter blocked wavelengths shorter than 570 nm, and the intensity of light passing through the pinhole was monitored by a photomultiplier. The optimal size of the pinhole was determined empirically. Most experiments were done with a 50 μm hole, in order to have sufficient light through-put to give a good signal-to-noise ratio. However, this was replaced by a 20 μm pinhole when improved depth discrimination was required. The pinholes were mounted approximately in the centre of the optical axis, and fine adjustments to bring the light source and detector pinhole into optimal confocal alignment were made using the positioning screws holding the focusing lens in front of the laser. Alignment was achieved by focusing the microscope on a thin film of rhodamine solution, sandwiched between a slide and coverslip, and adjusting the lateral and axial position of the focusing lens to obtain maximum photomultiplier signal. The anode current of the photomultiplier was measured through a current-to-voltage convertor, and records were low-pass filtered through an eight pole Bessel filter. Most traces were recorded after filtering at 20 Hz, but in cases where improved temporal resolution was required (Fig. 7), the cut-off was raised to 400 Hz. Simultaneous records of photomultiplier current and voltage-clamp current were stored on floppy disks by a digital oscilloscope for subsequent analysis by computer.

Oocytes were usually positioned so that the photolysis and recording light spots lay just to the vegetal side of the equator. This was to avoid light absorption by pigment in the animal hemisphere, whilst maximizing the sensitivity to InsP_3 , which is greater near the animal pole (Berridge, 1988; Parker, 1992). However, a few experiments were done using oocytes from albino frogs, which lack pigment, and these oocytes were oriented with the animal hemisphere facing the objective lens.

Estimates given in the text of rates of diffusion of InsP_3 and Ca^{2+} in the oocyte cytoplasm were made assuming respective diffusion coefficients of 2×10^{-6} and $6 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}$ (Meyer & Stryer, 1991).

RESULTS

The first sections of the Results concern the characteristics of the confocal optical system. Subsequent sections describe observations of InsP_3 -evoked Ca^{2+} release made with this system.

Ca^{2+} dependence of rhod-2 fluorescence

The Ca^{2+} dependence of rhod-2 was measured using calibration solutions containing 20 μM rhod-2, 100 mM KCl, 10 mM MOPS at pH 7.2, and various ratios of 10 mM EGTA/CaEGTA (Calcium Calibration Buffer Kit 1; Molecular Probes, Eugene, OR, USA). The resulting free Ca^{2+} concentrations were calculated assuming a dissociation constant of 150 nM for Ca^{2+} binding to EGTA. Fluorescence measurements were made using the confocal system to record from 5 μl droplets of these solutions, and are plotted in Fig. 1B as a function of free Ca^{2+} concentration. The fluorescence in saturating Ca^{2+} was 12-times greater than that in Ca^{2+} -free solution, and the curve in Fig. 1B was calculated using an effective K_d of about 340 nM for Ca^{2+} binding to rhod-2. These values differ substantially from those (dissociation constant, K_d , = 1 μM , fluorescence ratio excess *vs.* zero Ca^{2+} = 3.40) originally reported by Minta *et al.* (1989).

Spatial resolution of the confocal system

A confocal microscope records fluorescence from within a minute volume at the focus of the objective lens. The confocal spot has the shape of a (American) football, elongated along the optical axis, with dimensions that are determined principally by the numerical aperture of the objective and the diameter of the detector pinhole

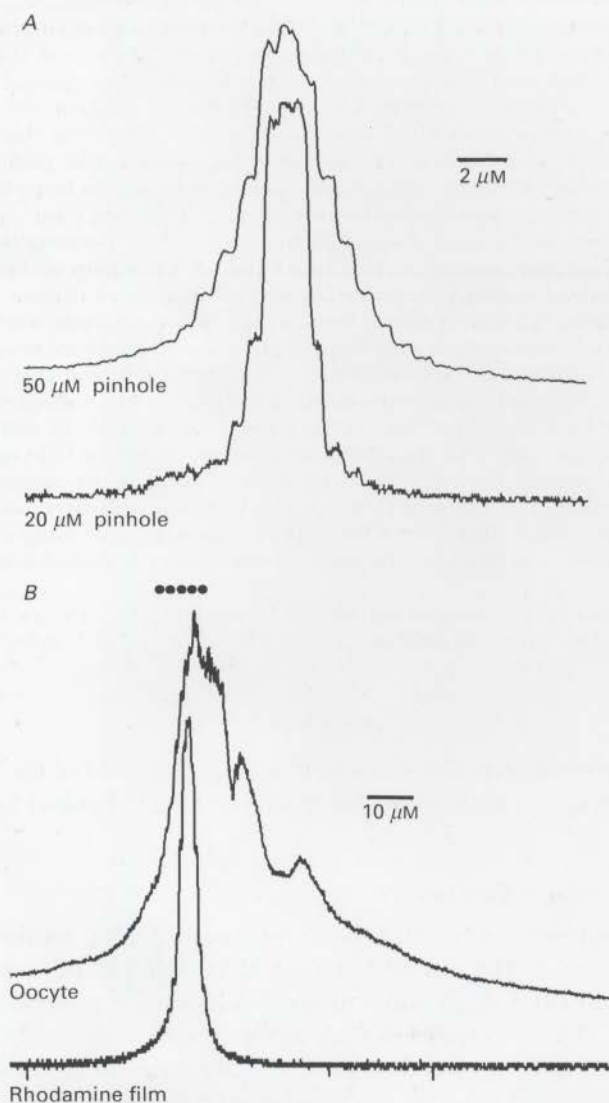


Fig. 2. Depth resolution of the confocal system. *A*, traces show the photomultiplier signal as the microscope was focused in increments through a thin film of rhodamine solution sandwiched between a slide and coverslip. The upper trace was obtained using a 50 μm pinhole and the lower with a 20 μm pinhole. For ease of comparison, both traces were scaled to similar peak heights; the signal with the 20 μm pinhole was 5.4 times smaller than with the 50 μm pinhole. *B*, upper trace shows confocal fluorescence recorded as the microscope was focused down into an oocyte that had been loaded with rhodamine. Dots above the trace indicate the range of positions over which pigment granules were in focus. The lower trace shows for comparison the signal recorded while focusing through a thin film of rhodamine (as in *A*). Both traces were obtained with a 50 μm pinhole.

(Brakenhoff, Bisscher & van der Voort, 1990). In our system the pinhole was larger than the diffraction limit (to increase light throughput), so that the lateral size of the spot could be estimated from the power of the objective and the pinhole size. For

most experiments a $40\times$ objective was used together with a $50\text{ }\mu\text{m}$ diameter pinhole, resulting in a lateral spot diameter of $1.25\text{ }\mu\text{m}$. With a $20\text{ }\mu\text{m}$ pinhole the corresponding value was $0.5\text{ }\mu\text{m}$. To measure the axial resolution, the photomultiplier current was recorded whilst focusing the microscope through a film of rhodamine solution made as thin as possible by squeezing it between a slide and coverslip. Figure 2A shows traces obtained using 20 and $50\text{ }\mu\text{m}$ detector pinholes. As expected, the smaller pinhole provided a sharper resolution; the focus distance over which the signal was greater than half-maximal was about $2\text{ }\mu\text{m}$, as compared to about $4\text{ }\mu\text{m}$ for the $50\text{ }\mu\text{m}$ pinhole. Furthermore, the true resolution of the system was probably somewhat better than implied by these measurements, since they include the unknown, but finite, thickness of the rhodamine film.

Recordings with the $20\text{ }\mu\text{m}$ pinhole would therefore arise mainly within an ellipsoid measuring less than $1\text{ }\mu\text{m}$ laterally and about $2\text{ }\mu\text{m}$ axially. This corresponds to a volume of roughly 1 fl. By comparison the volume of an oocyte is about $1\text{ }\mu\text{l}$; greater by a factor of 10^9 .

Optical profile of the oocyte

The oocyte cytoplasm is highly turbid, so that light will be attenuated as it passes into and out of the cell. For two reasons, we wanted to determine the extent of this attenuation. The first was that loss of light would limit the depth into the cell at which confocal signals could be recorded with acceptable signal-to-noise ratio. The second reason was to estimate how rapidly the UV photolysis light was attenuated by passage into the oocyte, and thus to what depth photorelease from caged InsP_3 would occur.

Measurements of confocal fluorescence were made while slowly focusing the microscope down into oocytes that were loaded with rhodamine. Figure 2B shows a typical profile, together with a profile obtained focusing through a thin film of rhodamine to give an indication of the intrinsic resolution of the optical system. As the confocal spot was advanced into the oocyte the fluorescence rose rapidly to a peak, and then declined more gradually, with several bumps and troughs. Irregularities like those shown were seen consistently in other oocytes, and probably arose from cortical granules and yolk platelets, which have dimensions of a few micrometres and are expected to exclude the hydrophilic dye. By visually interpolating a smooth curve between the various irregularities, the displacement required for the fluorescence to fall to one-half of the peak value was estimated to be about $15\text{ }\mu\text{m}$. Because the detected fluorescence was reduced both by attenuation of the incident excitation light and the emitted fluorescence, the signal would decrease as the square of the single-pass attenuation. Thus, assuming similar attenuation at the excitation and emission wavelengths, light travelling into the oocyte would be reduced to one-half of its original intensity after about $30\text{ }\mu\text{m}$, and to one-tenth after about $100\text{ }\mu\text{m}$. Attenuation of the photolysis light was probably more rapid than this, since the absorbance of the oocyte cytoplasm increases at shorter wavelengths.

Except where otherwise noted, all Ca^{2+} recordings in this paper were made with the microscope focused at the position that gave maximal resting fluorescence.

Dye bleaching

The intensity of the spot of laser light formed by the objective lens was extremely high (about 3×10^{22} photons $\text{cm}^2 \text{s}^{-1}$; assuming 50% transmittance of the 0.2 mW laser beam and a beam waist $0.5 \mu\text{m}$ diameter), and would thus rapidly photobleach the fluorescent probe (Tsien & Waggoner, 1990). However, we expected that diffusional exchange of fresh dye from outside the illuminated area should rapidly replenish dye bleached in the minute focal spot. For example, assuming a diffusion coefficient for rhod-2 in free solution of $2 \times 10^{-6} \text{cm}^2 \text{s}^{-1}$, a dye molecule would move an average distance of $1 \mu\text{m}$ within about 3 ms. In agreement, fluorescence signals recorded from aqueous solutions of rhod-2 showed almost no decline after turning on the laser illumination, either in the presence or absence of Ca^{2+} (Fig. 3B). Recordings from oocytes loaded with rhod-2, on the other hand, gave very different results. As shown in Fig. 3A, a large fluorescence signal was obtained immediately after opening the laser beam, but the fluorescence then declined over a few seconds to reach a steady value only about 13% of the peak. This decline was almost irreversible, since little recovery was seen after the laser beam was interrupted for 1 min.

A probably explanation for the bleaching effect in Fig. 3A was that it arose because a proportion of the rhod-2 loaded into the oocyte was not in free aqueous solution, but was bound or compartmentalized by some fixed structures within the cell. The question then arose as to whether the bound dye showed a change in fluorescence in response to Ca^{2+} . This was tested by evoking Ca^{2+} liberation in response to photorelease of InsP_3 . Figure 3C shows fluorescence responses to identical light flashes delivered shortly after opening the laser beam and following continuous laser illumination for 1 min. The sizes of the Ca^{2+} -evoked fluorescence transients were not appreciably different, even though the background fluorescence just before the stimuli differed by a factor of 2.5. Thus, the Ca^{2+} -dependent fluorescence signal appears to arise largely from that fraction of the rhod-2 which we presume to be in free solution, whereas the immobile, bleachable fraction of the dye appears to be insensitive or only weakly sensitive to Ca^{2+} . Recordings of Ca^{2+} transients were therefore always made after first exposing each measuring spot to the laser light for several seconds to obtain a stable baseline.

Intracellular dye loading

Attempts to load oocytes with rhod-2 by incubating them in solutions of rhod-2 AM were unsuccessful, in part, perhaps, because of the high volume to surface area ratio of these large cells. Instead, oocytes were each injected with about 200 fmol rhod-2 (free acid), which would result in an intracellular concentration of about $0.2 \mu\text{M}$ if the dye were to distribute evenly throughout the approximately $1 \mu\text{l}$ cytosolic volume of the oocyte. In fact, the dye remained concentrated within a few hundred micrometres of the injection site, even when examined an hour or longer after loading, suggesting that its diffusion was hindered by binding to intracellular constituents. Confocal measurements were therefore confined to regions around the injection site, where the dye concentration was greater than indicated by the above calculation. Another complication in estimating dye concentration was that, as discussed above, it seemed that only a fraction of the rhod-2 was in free solution in

the cytosol and able to respond to Ca^{2+} . To better estimate the free dye concentration in the recording spot, we therefore compared the background fluorescence in the oocyte (after photobleaching to a steady value) with that from an aqueous solution of rhod-2 of known concentration. Such a comparison is possible with a confocal

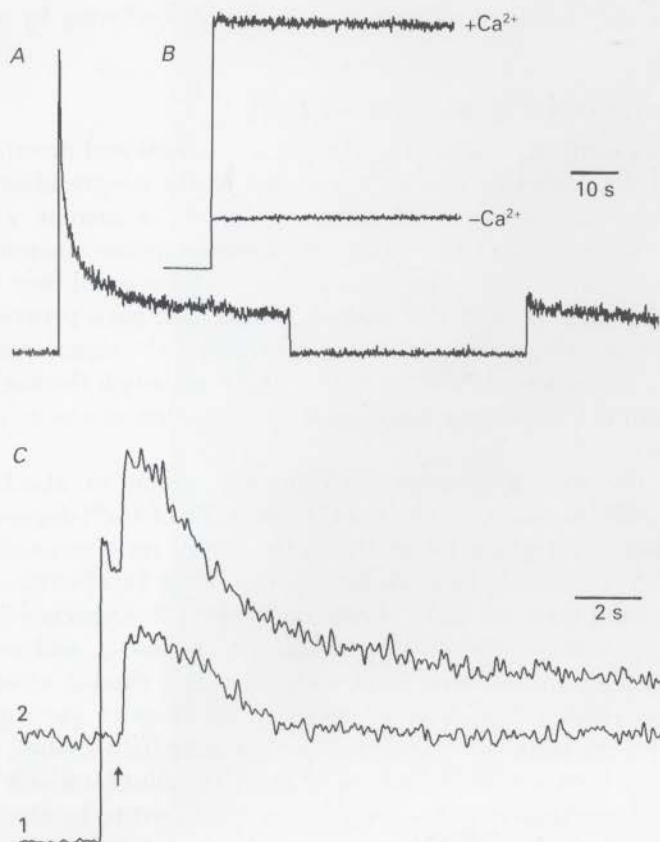


Fig. 3. Photobleaching of rhod-2. *A*, decay of fluorescence recorded from an oocyte loaded with rhod-2. The microscope was focused on the oocyte with the laser beam interrupted, and resting fluorescence was then recorded during two exposures to laser light. *B*, illumination of a solution of rhod-2 produces little photobleaching. Two superimposed traces are shown, obtained by illuminating thin films of rhodamine solution ($10\ \mu\text{M}$) in which the free Ca^{2+} level was strongly reduced by adding $200\ \mu\text{M}$ EGTA, or raised to a saturating level by addition of $2\ \text{mM}$ Ca^{2+} . *C*, the InsP_3 -evoked Ca^{2+} signal in the oocyte is not appreciably reduced by photobleaching. Two superimposed traces are shown, both obtained at the same location in the oocyte. In the first (1) the laser light was turned on for the first time shortly after the beginning of the trace, and photorelease of InsP_3 was triggered by a light flash when marked by the arrow. The second trace (2) was obtained following continuous laser illumination for about 1 min. At this time the resting fluorescence had declined to a steady level, and the oocyte was again stimulated at the arrow by a light flash identical to the first.

microscope, since the optical path length is determined by the dimensions of the confocal spot, not by the thickness of the specimen. The typical resting fluorescence in rhod-2-loaded oocytes was roughly one quarter that of $1\ \mu\text{M}$ rhod-2 in $30\ \text{nm}$ Ca^{2+}

solution, whereas the background fluorescence of non-injected oocytes was negligible ($< 5\%$ of that in injected oocytes). Thus, the intracellular free concentration of rhod-2 in most experiments was probably less than $1\ \mu\text{M}$. This is much lower than the indicator concentrations of several tens of micromolar generally used in Ca^{2+} imaging experiments (e.g. Lechleiter *et al.* 1991*a, b*; Parker & Yao, 1991), so that interference of normal cellular Ca^{2+} homeostasis resulting from Ca^{2+} buffering by the dye would be minimized.

Confocal Ca^{2+} signals evoked by photoreleased InsP_3

Figure 4*A* shows sample records of confocal Ca^{2+} signals and membrane currents evoked by photolysis flashes of various durations. In the oocyte illustrated, a flash of 5 ms duration produced no detectable changes in Ca^{2+} or current, whereas a 7 ms flash evoked a small increase in Ca^{2+} , but no current response. Increasing the flash to 8 ms gave a much larger Ca^{2+} signal, accompanied by a small membrane current response. Further lengthening of the flash duration then gave progressively larger currents. In contrast, the peak size of the confocal Ca^{2+} signal remained about constant as the flash was lengthened from 8 to 80 ms, although the initial rate of rise of the signal continued to become faster and the duration of the response became progressively longer.

Calibration of the rhod-2 fluorescence signals in terms of absolute free Ca^{2+} concentration is difficult since, unlike fura-2, the lack of Ca^{2+} -dependent shifts in excitation or emission wavelengths precludes the use of ratio measurements. None the less, a rough estimate could be made by expressing the InsP_3 -evoked fluorescence signals as a fractional increase (ΔF) above the resting fluorescence level. Oocytes showed negligible autofluorescence before loading with rhod-2, and we assume that the steady-state resting fluorescence arose only from free rhod-2, after bleaching of immobile, Ca^{2+} -insensitive dye. Measurements in six oocytes gave a mean ΔF of 2.0 ± 0.24 (S.E.M) for the peak Ca^{2+} signal following strong light flashes, and the mean peak ΔF in the same oocytes during lysis in 12 mM Ca^{2+} solution was 3.34 ± 0.74 . The resting free Ca^{2+} concentration in the oocyte was estimated to be about 35 nM from fura-2 measurements (Y. Yao & I. Parker, unpublished data), so that the resting rhod-2-fluorescence would have been about twice that in the absence of Ca^{2+} (Fig. 1*B*). From the calibration curve in Fig. 1*B*, the observed increase in fluorescence above this level following supramaximal photorelease of InsP_3 corresponds to a peak free Ca^{2+} concentration of about 140 nM.

Dose dependence of Ca^{2+} and membrane current responses

Figure 4*B* shows measurements of peak sizes of confocal Ca^{2+} signals and membrane currents in the same oocyte as Fig. 4*A*, plotted as a function of flash duration. The dose dependence of the Ca^{2+} signal was very steep. A flash of 7 ms duration was required to give any response, but the signal was already about 75% of maximum with a 10 ms flash, and further increases in duration beyond about 20 ms failed to produce an increase in the signal. Different to this, the membrane current increased as a more gradual function of the flash duration, and appeared not to have reached a maximum even with an 80 ms duration flash.

Pooled data from eight oocytes are presented in Fig. 5, showing the dependence of

peak size of the confocal Ca^{2+} signal and its rate of rise on flash duration. Because oocytes were each loaded with differing amounts of caged InsP_3 and rhod-2, it was not possible directly to compare the sizes of the Ca^{2+} signals or the threshold flash durations between different oocytes. Accordingly, the data were normalized by

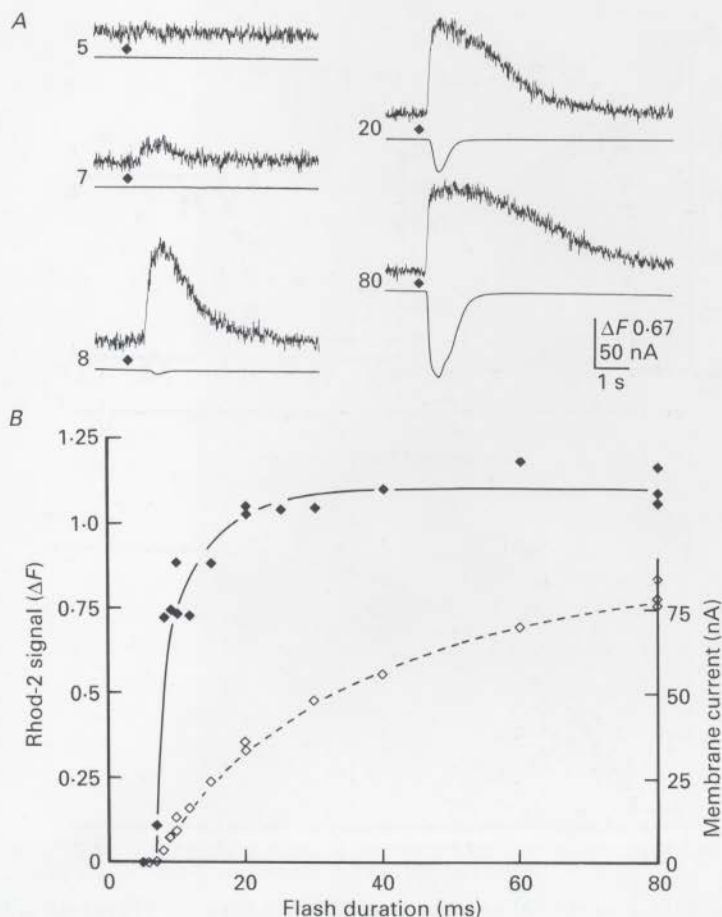


Fig. 4. Confocal Ca^{2+} signals and membrane currents evoked by light flashes of various durations. *A*, each pair of traces shows simultaneous records of confocal rhod-2 fluorescence (upper) and membrane current at a clamp potential of -60 mV (lower). Upward deflections correspond to increasing fluorescence (and increasing Ca^{2+}) and to outward membrane current. Photolysis flashes of the durations indicated (in ms) were given when marked by the diamonds. Intervals of 90 s were allowed between each trial. Records are all from a single location near the animal pole of an albino oocyte that was loaded with about 0.9 pmol caged InsP_3 and 0.45 pmol rhod-2. Optical traces were low-pass filtered at 50 Hz. Calibration bar indicates the fractional change in fluorescence from the baseline value (ΔF). *B*, measurements of peak sizes of confocal Ca^{2+} signals (\blacklozenge) and membrane currents (\diamond) from the same oocyte as *A*.

expressing the sizes of Ca^{2+} signals as a percentage of the mean maximum response in each cell, and the flash durations were expressed as multiples of that duration estimated to evoke a half-maximal response.

All oocytes showed a steep increase of the Ca^{2+} signal with increasing flash duration (Fig. 5*A* and *B*). Specifically, the responses grew from undetectable to 85 % of maximal as the flash duration was lengthened from about 90 to 110 % of that giving a half-maximal response. Attempts to fit the data assuming that co-operative

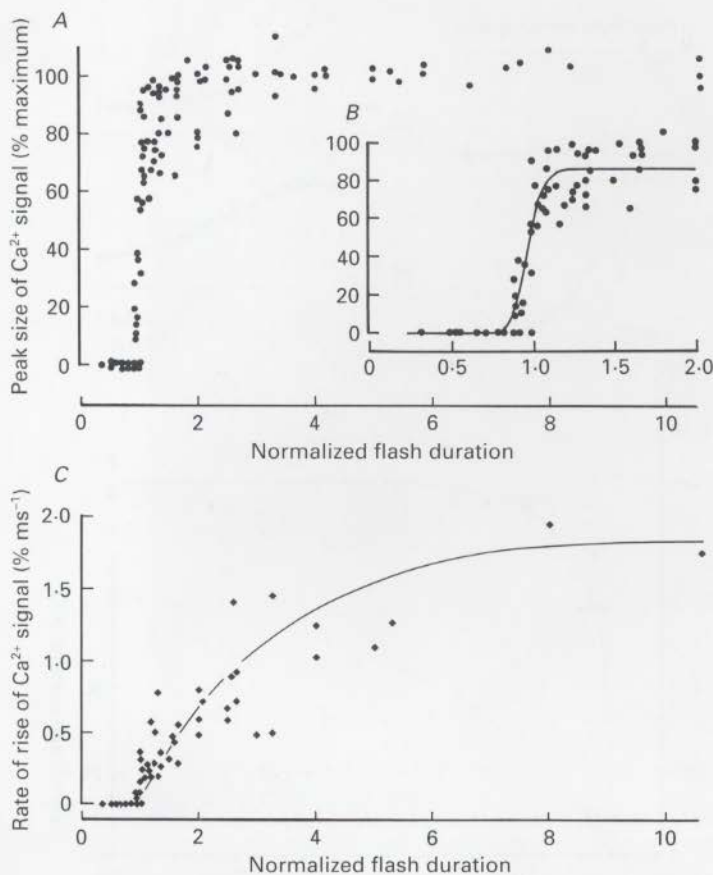


Fig. 5. Pooled data from several oocytes showing the dependence of peak amplitude and rate of rise of the confocal Ca^{2+} signal on duration of the photolysis flash. *A*, peak sizes of Ca^{2+} signals in eight oocytes, expressed as a percentage of the mean maximum response in each oocyte. The flash durations are normalized to that duration estimated to evoke a half-maximal response in each oocyte. *B*, the same data as in *A*, re-plotted on an expanded horizontal scale, the better to show measurements around the threshold. The curve shows the computer-generated best fit to the data, which yielded a Hill coefficient of 21. *C*, maximal rate of rise of Ca^{2+} signals as a function of normalized flash duration. Rates of rise are expressed as a percentage of the peak signal per ms. Measurements were obtained from the same records as in *A*, but data from three oocytes were excluded because strong filtering or poor signal-to-noise ratio precluded reliable estimation of the rate of rise.

binding of several molecules of InsP_3 causes opening of the Ca^{2+} release channel required an improbably high degree of co-operativity. Thus, in Fig. 5*B* the data points were fitted best by a model assuming a co-operativity (Hill coefficient) of

$21(\pm 4.1 \text{ S.E.M.})$. In contrast to this steep dependence of the size of the Ca^{2+} signal, its rate of rise increased more gradually with increasing flash duration, and appeared not to be maximal until the flash was 6 or more times the threshold duration (Fig. 5C).

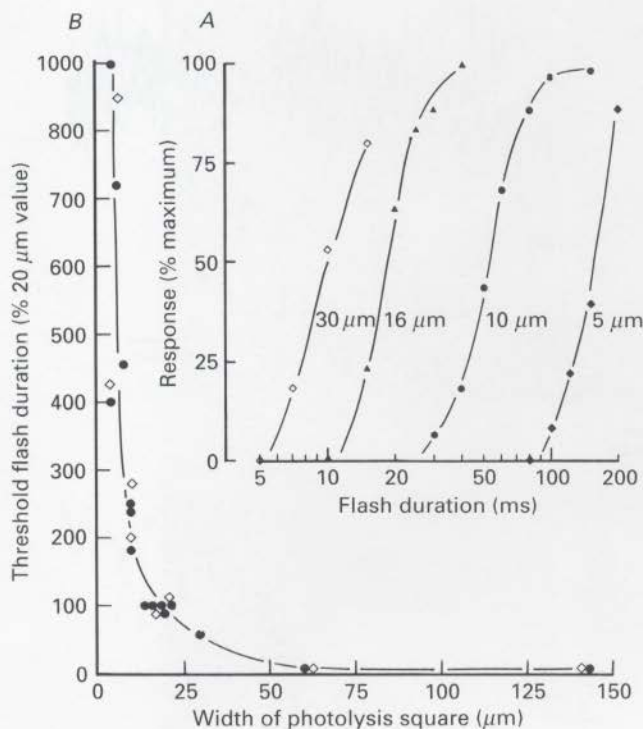


Fig. 6. The threshold flash duration required to evoke Ca^{2+} liberation depends upon the area over which InsP_3 is liberated. *A*, measurements from a single oocyte, showing peak sizes of confocal Ca^{2+} signals evoked by light flashes of various durations. The four curves were obtained with the photolysis light square set to the dimensions indicated. A $20\times$ dry objective lens was used in this experiment to allow illumination of larger areas than possible with the usual $40\times$ water objective. Because of the smaller aperture of this lens together with distortions at the fluid meniscus, the confocal effect was slight, and the dose-response relationship of the Ca^{2+} signal was less abrupt because Ca^{2+} was monitored from a greater volume of the cell. *B*, threshold flash durations required to evoke detectable signals with photolysis squares of various sizes. Data are from four oocytes, and have been normalized as a percentage of the threshold flash duration in each oocyte using a square of $20 \mu\text{m}$ side. Filled symbols indicate the threshold of the confocal Ca^{2+} signal and open symbols indicate the threshold of the membrane current response.

Threshold depends on area over which InsP_3 is photoreleased

The threshold flash duration required to evoke a detectable Ca^{2+} signal increased greatly when the aperture defining the illuminated area of the oocyte was made smaller, even though the irradiance (light energy per unit area) remained constant. For example, the oocyte in Fig. 6*A* responded to a 7 ms flash when the stimulus was a $30 \mu\text{m}$ square centred around the confocal spot, whereas the flash duration had to

be lengthened to 100 ms in order to obtain a response when the square was reduced to $5\text{ }\mu\text{m}$. The relationship between size of the photolysis square and the flash duration required to evoke a threshold response is plotted in Fig. 6*B*. Measurements were made using both the confocal Ca^{2+} signal and membrane current as indicators of

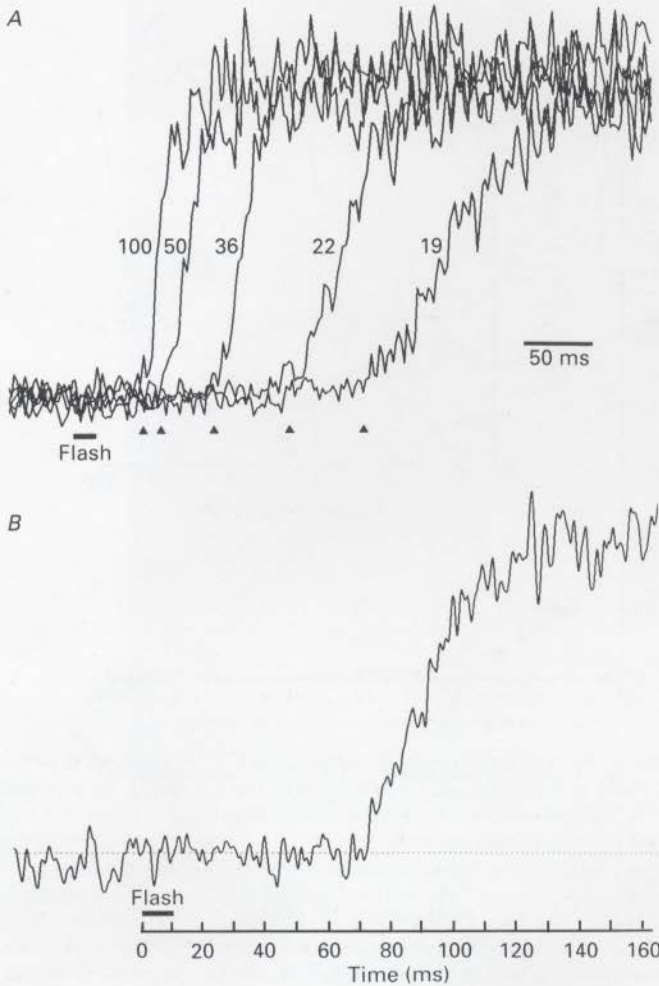


Fig. 7. Latency to onset of Ca^{2+} signals. *A*, superimposed traces showing onset of confocal Ca^{2+} signals evoked at a single location by light flashes of different intensities. The stimuli were flashes of 15 ms duration, given when marked by the bar. Neutral density filters were used to vary the flash intensities, and these are indicated as a percentage of the maximal output of the illuminator with no filters present. *B*, quiescent period preceding onset of the Ca^{2+} signal. The trace is an average of nine successive responses evoked at 60 s intervals by 10 ms flashes of maximal intensity. Different oocyte to *A*.

threshold Ca^{2+} release, since the differences between these methods were negligibly small as compared to the enormous variation in threshold with different sizes of light squares. The threshold flash duration with the smallest stimulus square tested ($4\text{ }\mu\text{m}$ side) was more than 100 times longer than with a $140\text{ }\mu\text{m}$ square.

Latency to onset of confocal Ca^{2+} signal

Measurements of the latencies to onset of Ca^{2+} signals were made using flashes of constant duration (usually 10 ms), and variable intensity (set by neutral density filters). Records were filtered at 400 Hz to improve time resolution and, to

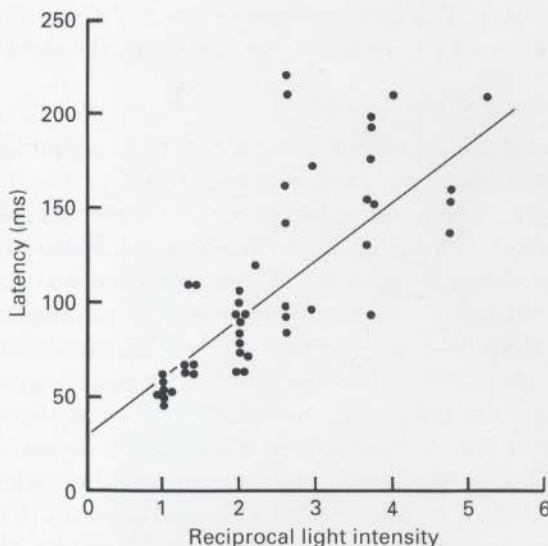


Fig. 8. Relationship between latency to onset of Ca^{2+} signal and intensity of photolysis light flash. Data were obtained in a single oocyte from records like those in Fig. 7A, by measuring the interval from the onset of the light flash to the first detectable rise in Ca^{2+} . The light intensity is plotted on a reciprocal scale, and an intensity of 1 corresponds to the maximal output of the photolysis unit. A regression line is fitted to the data.

compensate for the resulting increase in noise level, oocytes were loaded with about five times the usual amount of rhod-2.

Figure 7A shows representative traces from a single oocyte that was stimulated by light flashes varying over a fivefold range of intensities. At the maximum intensity, the Ca^{2+} signal began 50 ms after the onset of the light flash and reached 90% of its peak value within a further 20 ms. Lower intensity flashes evoked responses that began following progressively longer latencies and, whilst rising more slowly, still attained about the same peak levels. For example, the response to flash 19% of the maximal intensity began after about 200 ms, and continued to rise for a further 100 ms.

The onset of the Ca^{2+} signal was abrupt, and could be estimated by eye with little error (e.g. arrow-heads in Fig. 7A). During the latent period, the fluorescent Ca^{2+} record appeared to be quiescent. This is illustrated more clearly in Fig. 7B, which shows a computer average of nine successive responses evoked by 10 ms flashes of maximal intensity. For about 60 ms following the flash the confocal fluorescence signal did not deviate appreciably from the resting level.

Figure 8 shows measurements of response latency plotted against the reciprocal of the photolysis light intensity. Over the range explored the latency shortened

progressively with increasing intensity, indicating that even the highest available intensity did not saturate the photorelease of InsP_3 . The measurements showed appreciable scatter, but the plot could be reasonably fitted by a linear relation, implying that an InsP_3 binding step determines, at least partially, the kinetics of Ca^{2+} release channel opening. Furthermore, extrapolation of the regression line to infinitely high levels of InsP_3 release indicated that the latency of the Ca^{2+} release may include also a limiting, InsP_3 -independent delay of about 30 ms, though this value can only be approximate because of the scatter in the data.

Latencies of membrane current Ca^{2+} signals

Figure 9A shows simultaneous records of confocal Ca^{2+} signal and Ca^{2+} -activated Cl^- membrane current evoked by photorelease of InsP_3 . The Ca^{2+} signal began abruptly about 52 ms after the onset of the photolysis flash and, although the onset of the current response was more gradual, it clearly began following a longer latency of about 100 ms. This additional lag of the current response was consistently seen in all trials. For example, latencies were measured in one oocyte that was stimulated by twenty-eight identical light flashes at 90 s intervals. The currents began following a mean latency of 58 ± 4 ms (S.E.M.) after the onset of the Ca^{2+} signals. A scatter plot is shown in Fig. 9C of simultaneous measurements in five oocytes of latencies of the current *versus* latency of the corresponding fluorescence signal. A regression line fitted to the points had a slope of 0.82, and intercepted the current axis at 30 ms. Thus, the current lagged the Ca^{2+} signal with a minimal latency of about 30 ms, that lengthened slightly as the stimulus was reduced to evoke Ca^{2+} signals with increasingly longer latencies.

Experiments in which flash photolysis of caged Ca^{2+} (DM-nitrophen; McCray & Trentham, 1989) was used to evoke rapid increases of intracellular free Ca^{2+} indicated that the additional minimal delay of about 30 ms in activation of the membrane current did not arise because of the kinetics of the Cl^- channels. For example, in Fig. 9B the current began within about 5 ms of the onset of a light flash that caused photorelease of Ca^{2+} . Although the peak current in this experiment was about 10 times greater than with photoreleased InsP_3 (Fig. 9A), the entire visible hemisphere of the oocyte was exposed to photolysis light (surface area roughly $5 \times 10^5 \mu\text{m}^2$, as compared to the area of about $200 \mu\text{m}^2$ exposed in the experiment of Fig. 9A). Thus, the intracellular free Ca^{2+} concentration resulting from photolysis of caged Ca^{2+} was probably lower than that with photoreleased InsP_3 , so that the faster kinetics of Cl^- channel opening did not arise because of a higher Ca^{2+} level.

Confocal signals at different depths

A puzzling feature of the confocal Ca^{2+} records presented here, and of Ca^{2+} signals recorded from larger areas of the oocyte (Miledi & Parker, 1989; Parker & Ivorra, 1990a; Lechleiter *et al.* 1991b), is that they persist longer than the associated Ca^{2+} -activated Cl^- currents. We could envisage two explanations for this. Firstly, the chloride current declines because of inactivation or desensitization, even though the intracellular free Ca^{2+} level remains elevated. Secondly, the chloride current accurately reflects a transient increase in free Ca^{2+} close to the inner surface of the plasma membrane, whereas the fluorescent Ca^{2+} monitor senses a more prolonged

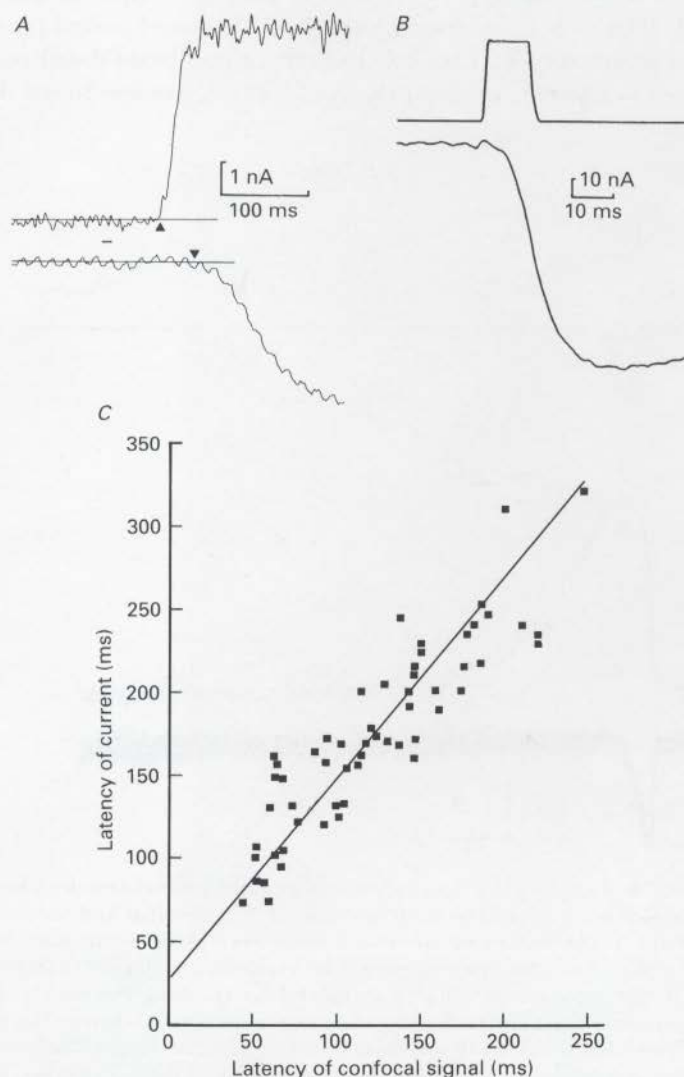


Fig. 9. Latencies of confocal Ca^{2+} signals and membrane currents. *A*, simultaneous records of confocal Ca^{2+} signal (upper trace) and membrane current (lower trace) evoked by photorelease of InsP_3 . A 10 ms light flash was given when marked by the bar, and arrow-heads indicate the estimated times of onset of the responses. Horizontal lines show the baselines. The optical and current records were filtered at 200 Hz. Some 60 Hz interference is present on the current trace. *B*, membrane current evoked by photorelease of Ca^{2+} . Upper trace indicates the duration of the light flash and lower trace shows membrane current (filtered at 400 Hz). The oocyte was loaded with about 80 pmol DM-nitrophen saturated with Ca^{2+} . Photolysis was achieved using the same optical system as used for caged InsP_3 . *C*, scatter plot showing the correlation between latencies of confocal Ca^{2+} signals and membrane currents evoked by photorelease of InsP_3 . Data are from five oocytes, which were stimulated by flashes of varying duration and intensity to give responses with widely varying latencies. A regression line is drawn through the points.

increase in Ca^{2+} deeper into the cytoplasm. Because experiments with Ca^{2+} microinjections (I. Parker & I. Ivorra, unpublished data) and paired photorelease of Ca^{2+} from a caged precursor (Y. Yao & I. Parker, unpublished data) revealed little inactivation of the Cl^- current, we used the confocal microscope to see if differences

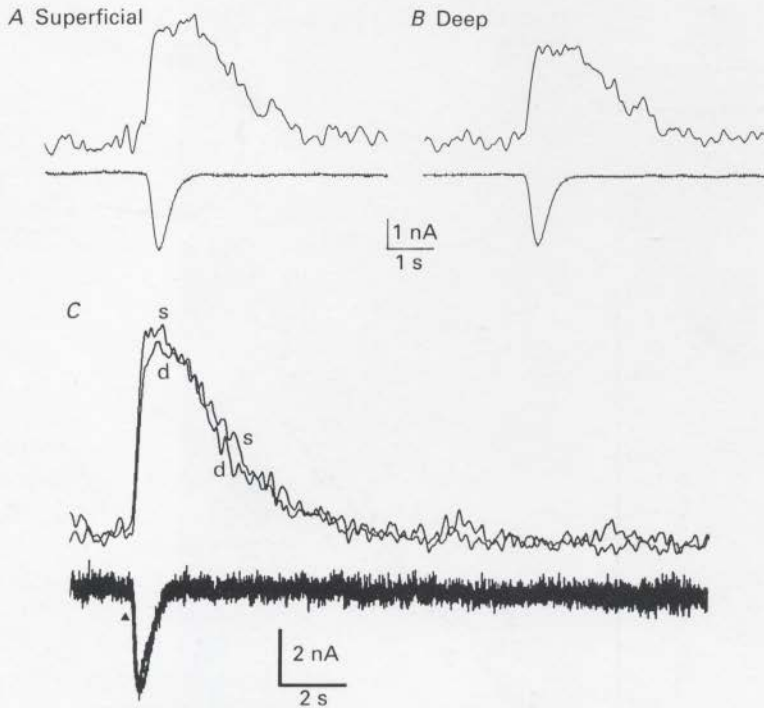


Fig. 10. Time course of confocal Ca^{2+} signals recorded at superficial and deep locations in the oocyte. In each record, upper traces show confocal Ca^{2+} monitor and lower traces are membrane current. Light flashes of 30 ms duration were given when marked by the arrow-heads. *A* and *B*, records obtained using a $20\text{ }\mu\text{m}$ detector pinhole to improve axial resolution. In *A* the microscope focus was moved away from the oocyte until the background fluorescence had declined to one-half of the maximal, whereas the records in *B* were obtained with the focus set $10\text{ }\mu\text{m}$ deeper into the oocyte. Each trace is an average of nine responses, recorded alternately at the superficial and deep locations. *C*, similar records obtained in another oocyte using a $50\text{ }\mu\text{m}$ pinhole. Superimposed traces show single responses with the microscope focused superficially (s) and $11.5\text{ }\mu\text{m}$ deeper (d) into the oocyte.

could be detected in the time course of the Ca^{2+} signals recorded superficially or more deeply into the cell.

Figure 10 shows examples of confocal Ca^{2+} signals recorded using a $20\text{ }\mu\text{m}$ pinhole, with the confocal spot focused near the surface of the oocyte (*A*) and at a depth of $10\text{ }\mu\text{m}$ (*B*). The two recording locations were selected so that InsP_3 -evoked Ca^{2+} signals were of similar size, and about one-half of the maximal signals obtained at an intermediate focus position. A $20\text{ }\mu\text{m}$ diameter pinhole was used to improve the spatial resolution of the recording, but gave noisy records. Accordingly, the Ca^{2+} signals in Fig. 10*A* and *B* are computer averages of responses to nine stimuli,

recorded alternately at the two locations. The time courses of confocal Ca^{2+} signals at the superficial and deep sites showed no obvious differences, and in both cases the Ca^{2+} signal was still nearly at the peak level at a time when the associated membrane current had decayed virtually to the baseline. A similar result was obtained in another oocyte, recording through a $50\ \mu\text{m}$ pinhole (Fig. 10C). In this case the spatial localization of the recording spot was less well defined, but the improved signal-to-noise ratio allowed better resolution of the time course of the Ca^{2+} signals.

Depression with paired flashes

We have previously shown that responses evoked by photoreleased InsP_3 were depressed following a preceding suprathreshold light flash, probably as a result of inhibition of InsP_3 -induced Ca^{2+} release by Ca^{2+} liberated during the conditioning response (Parker & Ivorra, 1990a). Ca^{2+} liberation was monitored in those experiments by membrane current recording and by fluorescence measurements over large cellular areas, and thus reflected the integrated activity of many Ca^{2+} release units. To further investigate this phenomenon at a finer subcellular level, we repeated the experiments using the confocal system to monitor highly localized Ca^{2+} liberation.

The records in Fig. 11A show simultaneous measurements of Ca^{2+} and membrane current evoked by pairs of identical light flashes delivered at various intervals. At an interval of 0.1 s, separate responses could not be discerned to each flash, and the Ca^{2+} and current signals resembled those expected from a single flash of twice the duration. That is to say, the current was roughly twice the size of that evoked by a single flash, whereas the Ca^{2+} signal was more prolonged, but not larger than that evoked by a single flash. The second flash failed to evoke any detectable Ca^{2+} or current responses when applied after intervals between about 0.5 and 2 s, but gave a small Ca^{2+} response, though no current response, after 3 s. When the interval was further lengthened to 6 s the Ca^{2+} signal had almost completely recovered, but the current remained depressed and had still not completely returned to the control size even after a 12 s interval.

Measurements are plotted in Fig. 11B of the peak sizes of Ca^{2+} signals and membrane currents as a function of inter-flash interval. The Ca^{2+} signal was completely suppressed at intervals shorter than about 2 s, but recovered rapidly with increasing interval, so that it was about one-half the control size after 3 s, and almost completely recovered after 6 s. In contrast, the dependence of the membrane current on flash interval was more complex (Fig. 11B; and see Parker & Ivorra, 1990a). At intervals shorter than about 0.5 s the total current was larger than expected from a single flash, although a component resulting from the second flash could not separately be resolved. The extra current due to the second flash declined rapidly with increasing interval, and no current was apparent at intervals between about 0.5 and 3 s. Thereafter the current increased progressively with further lengthening of the interval, but the recovery was more gradual than with the Ca^{2+} signal. For example, the current was only about one-half the control size after 6 s, when the Ca^{2+} signal had almost completely recovered.

Ca²⁺ spikes during sustained photorelease of InsP₃

In contrast to the transient increases in Ca²⁺ evoked by brief photolysis flashes, sustained photorelease of InsP₃ by light exposures lasting for several seconds usually evoked trains of repetitive Ca²⁺ spikes. Typical records are shown in Fig. 12*A*,

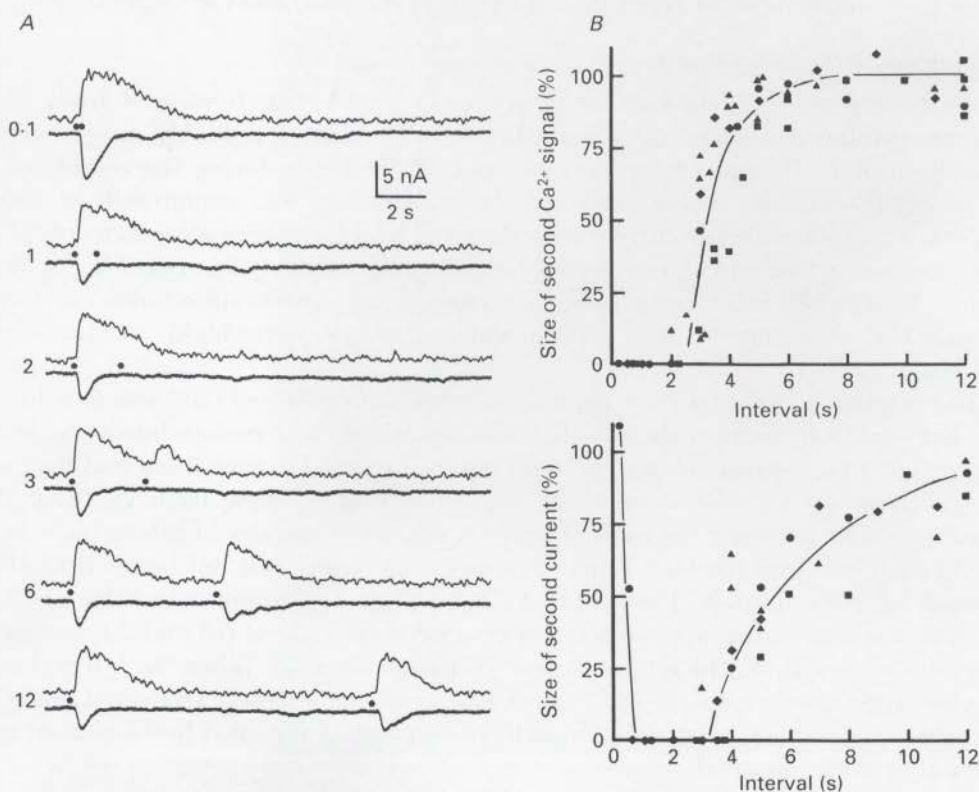


Fig. 11. Depression of InsP₃-evoked responses with paired light flashes. *A*, each frame shows confocal Ca²⁺ signals (upper) and membrane currents (lower) evoked by pairs of light flashes given with various intervals. The times of the flashes are indicated by dots, and the intervals are indicated (in seconds) next to each record. *B*, time courses of recovery from depression. Peak sizes of Ca²⁺ signals (upper graph) and membrane current (lower) are plotted against interflash interval. Responses are normalized as a percentage of that evoked by the first flash in each trial. At intervals shorter than about 0.5 s discrete current responses could not be discerned to each flash, and points at these intervals indicate the size of the response in excess of that expected in response to the first flash alone. Data are from four oocytes, indicated by different symbols.

obtained in an oocyte exposed during 30 s periods to photolysis light of various intensities. The lowest intensity that gave any response was about 0.43% of the full output, and this evoked four spikes of Ca²⁺ of increasing size, followed by a larger spike shortly after the light was extinguished. Raising the intensity to 0.65% of maximal gave a regular train of spikes, each of roughly similar size, which began following a shorter latency and occurred with higher frequency than the spikes seen

with the lower intensity. As the photolysis intensity was further increased, the latency to onset of the first spike reduced progressively, and the period of the spikes became shorter. However, although the peak sizes of the responses remained about the same at all intensities, the spikes were smaller at higher intensities and became

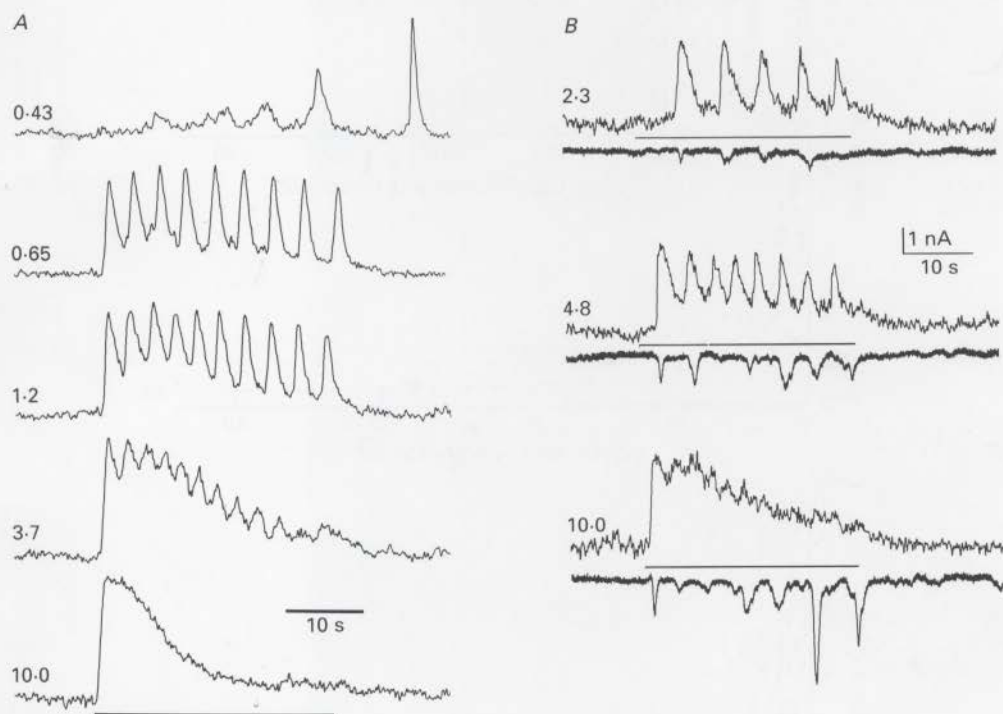


Fig. 12. Ca^{2+} spikes during prolonged photorelease of InsP_3 . Horizontal bars indicate the durations of exposure to photolysis light, and numbers next to the traces indicate the light intensity as a percentage of the full output. A, confocal Ca^{2+} signals evoked at a single recording spot by various intensities of photolysis light. B, records from a different oocyte, showing simultaneous measurements of confocal Ca^{2+} signals (upper trace in each frame) and membrane current (lower traces).

superimposed on an increasingly large background elevation of Ca^{2+} . Indeed, at intensities greater than about 10% of the maximum, spikes could no longer be discerned and, instead, the Ca^{2+} signal rose abruptly shortly after the onset of illumination and then declined monotonically.

Figure 12B shows a similar experiment in which simultaneous records were obtained of the confocal Ca^{2+} signal and membrane current. Ca^{2+} spikes were usually accompanied by corresponding spikes of Ca^{2+} -activated membrane current, but there was little correlation between their amplitudes. For example, the largest current spike (lower trace, Fig. 12B) was associated with only a very small Ca^{2+} spike. Reasons for this discrepancy are not clear, but it may have been that the current record reflected Ca^{2+} release at sites other than that monitored by the confocal recording. Another striking finding was that the current appeared to reflect only the transient increases in Ca^{2+} occurring during the spikes, whereas the more sustained

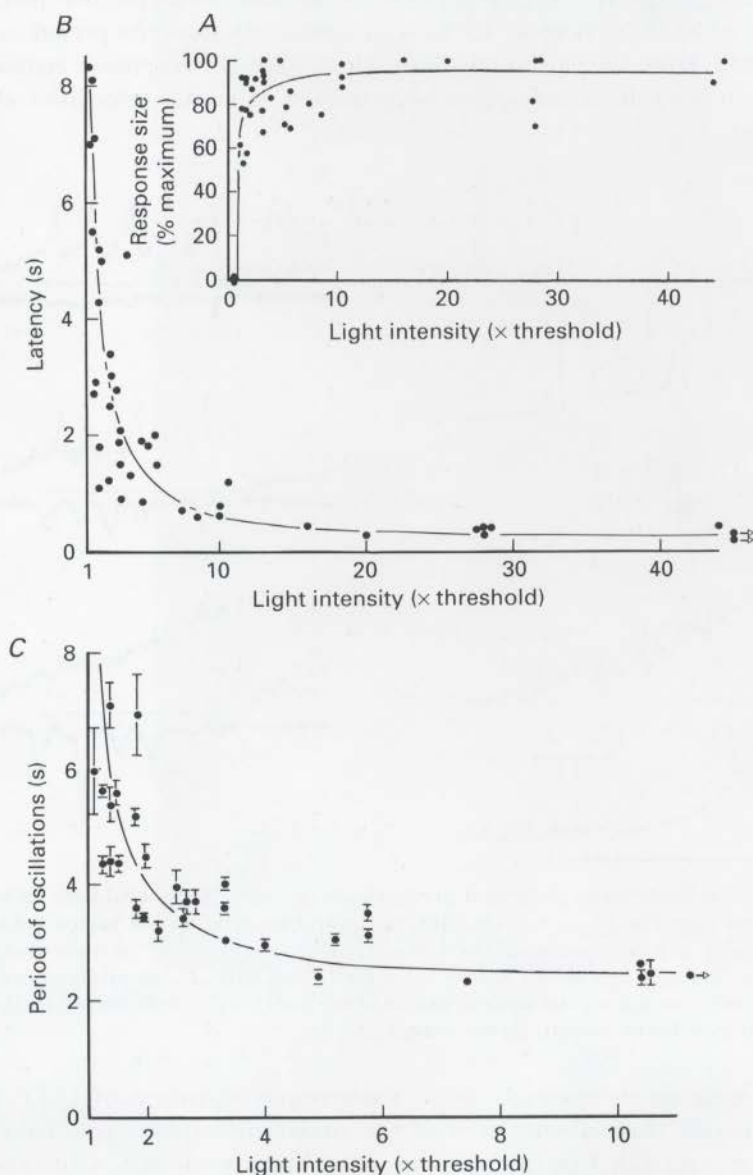


Fig. 13. Changes in characteristics of the oscillatory Ca^{2+} signals as a function of intensity of photolysis light. Measurements were made in five oocytes from records like those in Fig. 12. To allow pooling of data, light intensities are normalized to the threshold intensity required in each oocyte to evoke any response during 30 s illumination. *A*, peak size attained during each response, scaled as a percentage of the maximum obtained in that oocyte at any light intensity. *B*, latency to onset of the initial abrupt rise in Ca^{2+} . *C*, period of oscillations. Measurements were made of the intervals between peaks of the oscillations during each exposure. Points indicate mean \pm s.e.m. of measurements from individual trials. The range of light intensities is restricted for this graph, as individual oscillations could not be discerned for intensities greater than about ten times the threshold. Curves are drawn by eye.

elevation of Ca^{2+} during strong photolysis was not accompanied by any corresponding increase in current.

Ca^{2+} spikes like those in Fig. 12 were observed in six out of eight oocytes examined. In the remaining two oocytes, spikes were not evident and, instead, only the sustained elevation of Ca^{2+} was seen, the size of which was graded with photolysis intensity.

A difficulty in interpreting the Ca^{2+} signals evoked during prolonged photorelease of InsP_3 is that, although the light intensity remained constant, we do not know the resulting time course of changes in concentration of InsP_3 . If InsP_3 was photoreleased at a constant rate, its concentration would presumably rise toward a steady-state level, at which the rate of formation was matched by the loss of InsP_3 as a result of metabolism and diffusion away from the illuminated area. The rapid cessation of the Ca^{2+} signals when the light was extinguished suggests that this equilibration occurred within a few seconds. Furthermore, the relatively constant period of the Ca^{2+} spikes during each exposure also indicates that the average level of InsP_3 remained fairly constant throughout much of the photolysis period. Even though the higher light intensities were expected to consume an appreciable fraction of the caged InsP_3 , depletion during the exposure period would probably not have been important because of replenishment by caged InsP_3 diffusing from surrounding unexposed regions of the cell. Assuming the diffusion coefficient for caged InsP_3 in the cytosol is similar to that of InsP_3 ($2 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$), the mean time for diffusion over the $7.5 \mu\text{m}$ distance from the edge to the centre of the illuminated square would be only about 150 ms (Meyer & Stryer, 1991).

Dose dependence of Ca^{2+} spike parameters

Figure 13 presents measurements in five oocytes of various parameters of Ca^{2+} spike responses evoked by prolonged photorelease of InsP_3 , plotted as a function of photolysis intensity. Similar to the dose dependence of Ca^{2+} responses to brief flashes, the peak Ca^{2+} levels attained during prolonged photorelease of InsP_3 showed a nearly all-or-none characteristic (Fig. 13*A*). In particular, the peak Ca^{2+} level changed little over a more than 20-fold range of suprathreshold light intensities, even though the pattern of the responses changed from a train of discrete spikes to a smooth, though transient, elevation of Ca^{2+} . The latency to onset of the first Ca^{2+} spike shortened progressively from longer than 8 s with just suprathreshold stimuli to a few hundred milliseconds at the highest intensities used (Fig. 13*B*). Finally, the period of the Ca^{2+} spikes fell from about 7 s with just suprathreshold stimuli to a minimal value of about 2.5 s at intensities about 10 times threshold (Fig. 13*C*), beyond which individual spikes were no longer discernable.

DISCUSSION

Confocal microfluorimetry

We describe the construction and use of a confocal microfluorimeter able to monitor Ca^{2+} -dependent dye signals from minute volumes (a few femtolitres) within single cells. The device is relatively simple and inexpensive, costing less than \$1000 to modify an existing microscope fluorimeter for confocal working. It may thus be of

widespread use in many different preparations, to allow Ca^{2+} recordings to be made from restricted cellular regions (e.g. synaptic terminals), or to optically isolate signals from individual cells in multi-cellular preparations loaded using membrane permeable dye esters. Furthermore, substantial improvements in sensitivity over the present instrument should easily be realized by use of objective lenses with higher numerical aperture, together with a higher power laser and a photomultiplier with better quantum efficiency. The resulting improvement in signal-to-noise ratio would enhance the effective time resolution, since this is presently limited by filtering required to reduce noise, and could also be traded off to give a better spatial localization by using a smaller detector pinhole. A limitation of the system is that the fluorescence signals are difficult to calibrate in terms of absolute free Ca^{2+} concentration, because no currently available visible wavelength Ca^{2+} indicators show useful spectral shifts on binding Ca^{2+} , and thus do not allow ratio measurements in the way possible with fura-2 (Grynkiewicz, Poenie & Tsien, 1985). In principle, alternate dual wavelength excitation of fura-2 is possible, but may be difficult because of the need for an objective lens corrected at both UV and visible wavelengths.

The information provided by the confocal recording is analogous to that obtained with a Ca^{2+} -selective microelectrode, in that both techniques measure free Ca^{2+} concentration at a virtual point source. However, optical recording has considerable advantages in that it is less invasive, provides a much better time resolution and, except for limitations of transparency, allows the recording spot to be freely positioned anywhere within the cell. Ca^{2+} electrodes, on the other hand, can readily be calibrated to provide absolute free Ca^{2+} concentrations. Commercially available scanning confocal microscopes provide a highly localized signal like the system described here, but with the great benefit of two- or three-dimensional imaging. The argon ion lasers generally used in these systems are well suited to work with Ca^{2+} indicators including fluo-3 (Minta *et al.* 1989) and the newly developed 'calcium green' family (Molecular Probes, Eugene, OR, USA), and some applications of scanning confocal microscopy for Ca^{2+} measurement have been described (Hernandez-Cruz, Sala & Adams, 1990; Niggli & Lederer, 1990; Williams, 1990; Lechleiter *et al.* 1991*a, b*). The main drawbacks with scanning confocal microscopes are their considerable cost, and a frame acquisition time limited to between about 30 ms and several seconds, depending on operating principle and the desired spatial resolution.

Confocal recording of InsP_3 -evoked Ca^{2+} signals in the oocyte

Our initial results using confocal Ca^{2+} recording (Parker & Ivorra, 1990*b*) led us to propose that the InsP_3 -sensitive Ca^{2+} pool in the oocyte is arranged as a collection of functionally independent localized units, that each release Ca^{2+} in a nearly all-or-none manner. Subsequent experiments using video imaging of Ca^{2+} released by photolysis of caged InsP_3 supported this hypothesis, and further revealed that Ca^{2+} is released at discrete 'hot spots', spaced several micrometres apart, which are activated at varying threshold levels of InsP_3 (Parker & Yao, 1991). The work in the present paper was aimed towards characterizing the properties of Ca^{2+} release from individual units, by recording Ca^{2+} signals from highly localized regions of the cytoplasm. A difficulty was that we could not directly locate individual units, so that

in some instances the confocal spot may have fallen between units, and thus monitored the aggregate activity of several adjacent units. To mitigate this effect, we generally evoked test responses at several random locations, and selected only those spots that gave large signals for detailed study, so that the results presented here are more likely to represent single unit behaviour. Although video imaging permits unambiguous location of Ca^{2+} release sites, the spatial and temporal resolution (33 ms frame interval) are inferior to our confocal system. Ultimately, we hope that high-speed confocal imaging may combine the advantages of both approaches.

Dose dependence of localized InsP_3 liberation

Photorelease of increasing amounts of InsP_3 by brief light flashes of progressively longer duration evoked confocal signals that began abruptly at a particular threshold duration, and then grew steeply in size, so that they were about 85% of maximal with flashes only about 20% longer than the threshold. Several arguments indicate that this nearly all-or-none dose dependence arises from the mechanisms of InsP_3 -evoked Ca^{2+} release, and not from some artifactual property of the caged InsP_3 system or the Ca^{2+} monitor.

Firstly, photorelease of InsP_3 is expected to vary as a linear function of light absorption by caged InsP_3 (McCray & Trentham, 1989), a relationship that was experimentally confirmed by measuring photorelease of ATP from a caged precursor like that used to cage InsP_3 (Parker & Ivorra, 1992). Furthermore, the fact that Ca^{2+} signals continued to show a progressively faster rate of rise and slower decay when the flash was lengthened beyond threshold clearly indicates that photorelease was not saturated with suprathreshold stimuli. Secondly, the rhod-2 signal is not expected to show any threshold in Ca^{2+} -activation and, at low Ca^{2+} levels, the fluorescence rises nearly linearly with increasing free Ca^{2+} (Fig. 1B). A more serious concern is whether saturation of the Ca^{2+} signals could arise from saturation of the dye fluorescence. *In vitro* calibrations indicated that this was unlikely, as rhod-2 fluorescence did not approach saturation until the free Ca^{2+} concentration was raised to micromolar levels (e.g. fluorescence was about 75% maximal at $1\ \mu\text{M}\ \text{Ca}^{2+}$), whereas the peak InsP_3 -evoked Ca^{2+} signals in the oocyte were estimated to be about 140 nM, rising above a resting level of about 30 nM. More directly, lysis of oocytes in high Ca^{2+} solution gave fluorescence increases that were about 70% greater than the peak InsP_3 -evoked signals. However, the finding that a fraction of the rhod-2 may be bound or compartmentalized in the oocyte complicates interpretation of this result, because this immobilized dye might be liberated during lysis and artifactually increase the fluorescence signal by diffusing into the measuring spot where the immobile dye had originally been bleached before recording. We tried to circumvent this problem by using Ca^{2+} ionophores (A23187 and ionomycin) to raise intracellular Ca^{2+} to saturating levels, but these agents evoked only slight fluorescence signals, that were smaller than those evoked by InsP_3 . A final argument that the nearly all-or-none Ca^{2+} signals reflect the characteristics of InsP_3 -mediated Ca^{2+} release comes from experiments where sustained elevations of InsP_3 evoked repetitive Ca^{2+} spikes (Fig. 12). Increasing levels of InsP_3 gave a higher frequency of spikes, but their size remained about constant, and individual spikes showed no evidence of the 'clipping' that would be expected if Ca^{2+} levels were sufficient to saturate the fluorescence.

If the opening of intracellular Ca^{2+} release channels were regulated only by the

binding of InsP_3 to receptor sites, the rate of release would be expected to be a graded function of InsP_3 concentration, and the steepness of the relation would depend on the co-operativity of InsP_3 binding required to open the channel. In contrast, our results (Fig. 5A and B) suggest the existence of a distinct threshold in the release process, since the abrupt rise of the dose-response curve required a Hill coefficient of about 21 to obtain an optimal fit. This implies that twenty-one or more molecules of InsP_3 need to bind to cause channel opening, a number that is implausibly large, and is much greater than the co-operativities of between 2 and 4 derived from stop-flow measurements of Ca^{2+} release in permeabilized cells (Meyer & Stryer, 1988; Champeil, Combettes, Berthon, Doucet, Orlowski & Claret, 1989; Meyer, Wensel & Stryer, 1990). Furthermore, if the opening of Ca^{2+} release channels shows high co-operativity, the rate of release is expected to rise extremely steeply as InsP_3 levels are raised above the apparent threshold. In contrast to this, we observed a graded variation in rate of rise of the Ca^{2+} signal over a severalfold range of photolysis flash durations (Fig. 5B).

The sharp threshold of InsP_3 -evoked Ca^{2+} release suggests that this process probably involves regenerative positive feedback, and such a mechanism has also been implicated in the generation of repetitive Ca^{2+} spiking (Tsien & Tsien, 1990; Meyer & Stryer, 1991) and in the active propagation of Ca^{2+} waves (Meyer, 1991). Several models have been proposed to account for this feedback, which can be grouped into three categories. (i) Ca^{2+} released from InsP_3 -sensitive stores stimulates phospholipase C, resulting in increased formation of InsP_3 and hence a regenerative release of further Ca^{2+} (Harootunian, Kao, Paranjape & Tsien, 1991). (ii) Slow release of Ca^{2+} from InsP_3 -sensitive stores causes an overloading of secondary, InsP_3 -insensitive stores, which then explosively release their contents by a mechanism of Ca^{2+} -induced Ca^{2+} release (Berridge & Irvine, 1989; Goldbeter, Dupont & Berridge, 1990). (iii) Ca^{2+} released from InsP_3 -sensitive stores acts as a co-agonist at the InsP_3 receptor, to facilitate the opening of Ca^{2+} -release channels and thus the liberation of further Ca^{2+} (Bezprozvanny, Watras & Erlich, 1991; Finch, Turner & Goldin, 1991). We have discussed these models previously, and presented evidence favouring model (iii) to account for regenerative Ca^{2+} release in the oocyte (Parker & Yao, 1991; Yao & Parker, 1992). The present results also offer further support for this occlusion.

Firstly, the extrapolated minimal latency of about 30 ms for activation of Ca^{2+} release appears too brief to allow for the stimulated formation of InsP_3 by phospholipase C. As described later, membrane current responses lag at least 30 ms behind the Ca^{2+} signal, probably as a result of the time required for Ca^{2+} ions to diffuse from their sites of release to the Ca^{2+} -activated channels in the plasma membrane. Since InsP_3 is formed by breakdown of phosphatidylinositol bisphosphate in the plasma membrane, a similar time would be required for stimulation of InsP_3 production, together with an extra delay for InsP_3 to diffuse back to the InsP_3 -sensitive Ca^{2+} stores (though this would be shorter, due to the higher diffusion coefficient for InsP_3 in the cytosol as compared to Ca^{2+}). Thus, the total time for a single transit of Ca^{2+} and InsP_3 between the release sites and the plasma membrane already appears to be longer than the latency to onset of regenerative Ca^{2+} release, even without considering any extra delays that may be introduced by Ca^{2+} -dependent activation of phospholipase C. Furthermore, the regenerative release of

Ca^{2+} is likely to require the multiplicative effect of several cycles of Ca^{2+} release and phospholipase stimulation, rather than just a single cycle.

The short latency and abrupt onset of InsP_3 -evoked Ca^{2+} release are also difficult to reconcile with the scheme (model (ii)) in which InsP_3 -insensitive stores become 'primed' to release their contents by overfilling with Ca^{2+} liberated from InsP_3 -sensitive stores. The period available for filling of these secondary stores is brief, and we failed to detect a rise in cytosolic Ca^{2+} during the latent period, although a relatively large rise would be expected if Ca^{2+} were transferred through the cytosol between the two types of stores. Also, photorelease of Ca^{2+} from a caged precursor evoked rises in cytosolic Ca^{2+} that varied about linearly with the extent of photolysis, and decayed monotonically following a photolysis flash, rather than showing a delayed hump as might be expected from triggering of Ca^{2+} -induced Ca^{2+} release (Ivorra & Parker, 1990; I. Parker & Y. Yao, unpublished data).

Our failure in these experiments to observe a rise in Ca^{2+} level preceding the abrupt onset of the InsP_3 -evoked Ca^{2+} signal is also at odds with model (iii), in which a rise in cytosolic Ca^{2+} facilitates the action of InsP_3 to release further Ca^{2+} . However, by improving the recording sensitivity by averaging Ca^{2+} -dependent fluorescence signals over wider areas of the cell, we recently detected a small pacemaker rise in Ca^{2+} preceding the large, abrupt increase (Parker & Yao, 1991), which was probably lost in the noise level of the confocal recordings. Although both the Ca^{2+} co-agonist and the two-pool models predict a graded increase in cytosolic free Ca^{2+} preceding regenerative release, the size of this Ca^{2+} signal may be much smaller if Ca^{2+} acts as a co-agonist. Ca^{2+} is liberated from stores through channels that are part of the same molecule as the InsP_3 receptor site (Ferris, Haganir, Supattapone & Snyder, 1989), so that facilitation could arise from an elevation of cytosolic Ca^{2+} that is highly localized, even in comparison to the dimensions of the confocal recording spot. Also, a relatively small amount of Ca^{2+} will probably suffice to trigger opening of InsP_3 -sensitive release channels, whereas in the two-pool model a large part of the total Ca^{2+} involved in the regenerative response must first be transferred from InsP_3 -sensitive to InsP_3 -insensitive stores.

Because the rising phase of the confocal Ca^{2+} transients was brief as compared to their decay, the rate of rise should provide a good measure of the rate of release from InsP_3 -sensitive stores. For suprathreshold stimuli this increased in a graded manner with increasing levels of InsP_3 ; but why then did the peak amplitude of the Ca^{2+} signal show a nearly all-or-none dependence on InsP_3 level? A simple possibility is that the peak response represented the complete emptying of Ca^{2+} from the InsP_3 -sensitive store, and that the prolongation of the response at higher levels of InsP_3 arose because release channels remained open for longer, and thus immediately liberated any Ca^{2+} that was pumped back into the store. Another explanation is that the peak amplitude of the Ca^{2+} transient is regulated by a feedback mechanism. This might arise because cytosolic Ca^{2+} ions exert a delayed negative feedback on InsP_3 -mediated Ca^{2+} release (Parker & Ivorra, 1990a), and it has also been proposed (Irvine, 1990) that the intraluminal level of Ca^{2+} in the stores may regulate activity of the InsP_3 receptor.

Threshold depends on area of photoreleased InsP_3

The threshold flash duration required to evoke Ca^{2+} or membrane current signals increased by more than 100 times as the area of the oocyte exposed to photolysis light was reduced from a square with dimensions of $140\ \mu\text{m}$ to one of $5\ \mu\text{m}$. This was surprising, because the liberation of InsP_3 in the exposed region of the cell resulting from each flash was expected to be constant, since the irradiance (energy per unit area) of the photolysis light was unchanged. However, one explanation may be that because Ca^{2+} release did not begin until 200–300 ms after the flash, the concentration of InsP_3 in the exposed region might fall during this time because of diffusion into surrounding, unexposed regions of the cell. For example, the mean distance diffused by InsP_3 in two dimensions during this time would be about $20\ \mu\text{m}$, which is appreciable in comparison to the dimensions of the smaller photolysis squares. Another explanation arises from the possibility, discussed above, that a build-up of pacemaker Ca^{2+} decreases the threshold for regenerative release of a much larger amount of Ca^{2+} . Diffusional loss of Ca^{2+} from small illuminated areas might limit this build-up, and thus increase the threshold level of InsP_3 needed to trigger a regenerative release.

Using photolysis flashes illuminating large areas of the oocyte we had previously estimated the intracellular concentration of InsP_3 required to evoke a threshold response to be about 60 nM (Parker & Ivorra, 1992). Clearly, the peak concentrations immediately following threshold light flashes exposing small areas were much greater than this, but could not have exceeded about $1\ \mu\text{M}$, since oocytes were loaded with only sufficient caged InsP_3 to give this final cytosolic concentration. The 100-fold difference in threshold flash durations seen between 5 and $140\ \mu\text{m}$ photolysis squares may thus overestimate the difference in respective peak InsP_3 concentrations, as the strong stimuli required with small light squares probably consumed an appreciable fraction of the immediately available caged InsP_3 .

Regardless of the mechanism of the area dependence of the threshold, these experiments reveal a further non-linearity of the InsP_3 signalling pathway that is likely to be important for signal integration. Thus, a strong, but localized activation of InsP_3 production might fail to evoke any Ca^{2+} release, in contrast to a more widespread activation which evokes a Ca^{2+} signal even though the peak InsP_3 concentration is lower.

 Ca^{2+} spikes

Sustained photorelease of InsP_3 over several seconds led to the generation of localized repetitive spikes of intracellular Ca^{2+} (Fig. 12), resembling those described in various other cell types (for reviews see Berridge, Cobbold & Cuthbertson, 1988; Berridge & Irvine, 1989; Rink & Hallam, 1989; Tsien & Tsien, 1990; Meyer & Stryer, 1991). Early observations of 'oscillatory' membrane currents evoked by agonist activation (Kusano, Miledi & Stinnakre, 1982) and by intracellular injections of InsP_3 (Oron, Dascal, Nadler & Lupu, 1985; Parker & Miledi, 1986) already suggested that such a process occurred in the oocyte. However, the current recordings usually display irregular fluctuations, rather than discrete spikes, and it has been difficult to discern clear spikes of intracellular Ca^{2+} using various indicator

techniques (Parker & Miledi, 1986; DeLisle, Krause, Denning, Potter & Welsh, 1990; Ferguson, Han, Kao & Nuccitelli, 1991; Parker & Ivorra, 1991). The probable reason for this is that, although localized regions of the large oocyte show regular periodic Ca^{2+} spikes, different regions function independently (Parker & Yao, 1991; Lechleiter *et al.* 1991*a*), so that whole-cell recordings of membrane current and fluorescent Ca^{2+} signals monitored from wide cellular areas reflect the aggregate behaviour of many asynchronous oscillators.

The frequency of membrane potential oscillations evoked in the oocyte by activation of muscarinic receptors increases with increasing concentration of acetylcholine (Berridge, 1988), and a similar result is obtained when increasing amounts of InsP_3 are injected into the cell (DeLisle *et al.* 1990). These findings, and others like them in different cell types (Rapp & Berridge, 1981), have led to the proposal that Ca^{2+} signalling might be mediated by a frequency- or digitally encoded mechanism, rather than an amplitude-dependent mechanism (Rapp & Berridge, 1981; Berridge, 1988; Tsien & Tsien, 1990; Meyer & Stryer, 1991). The results of Figs 12 and 13 offer some support for this idea, and provide a better quantitation of the relationship between Ca^{2+} spiking frequency and level of InsP_3 than is possible from whole-cell recordings in the oocyte. However, some observations are difficult to reconcile with a mechanism of frequency encoding. Firstly, the available dynamic range appears quite limited. The interspike period varied by only a factor of about 3 (from 7 to 2.5 s) over a fourfold range of InsP_3 levels, and a further doubling of the stimulus produced virtually no change in period (Fig. 13*C*). Secondly, the spikes became progressively smaller at higher levels of InsP_3 and beyond a certain level only a monotonic rise and fall in Ca^{2+} was detected, with no discernable spikes remaining (Fig. 12*A*).

These experiments do not provide direct evidence about the mechanisms underlying spike generation, but the time course of the Ca^{2+} signals suggests that a regenerative, positive feedback process underlies the rapid upstroke of the spikes, whereas a time-dependent feedback inhibition may determine the interspike interval. Numerous models have been advanced to account for Ca^{2+} spiking (reviewed by Tsien & Tsien, 1990; Harootunian *et al.* 1990; Meyer & Stryer, 1991). Based on our findings that cytosolic Ca^{2+} can both facilitate (Yao & Parker, 1992) and inhibit (Parker & Ivorra, 1990*a*) InsP_3 -evoked Ca^{2+} liberation, we favour the idea that Ca^{2+} spike generation in the oocyte involves a rapid positive feedback of Ca^{2+} ions on the InsP_3 receptor to promote opening of the Ca^{2+} release channels (Bezprozvanny *et al.* 1991; Finch *et al.* 1991), together with a delayed feedback inhibition of the receptor (Danoff, Supattapone & Snyder, 1988; Bezprozvanny *et al.* 1991).

Ca^{2+} -activated Cl^- current

The membrane current responses mediated by InsP_3 signalling arise through activation of Ca^{2+} -dependent Cl^- channels in the oocyte membrane (Miledi & Parker, 1984; Parker & Miledi, 1989). However, simultaneous recordings of confocal intracellular Ca^{2+} signals and Ca^{2+} -activated membrane currents revealed several discrepancies between these two monitors of intracellular free Ca^{2+} .

One is apparent in the responses activated by different levels of InsP_3 . The confocal Ca^{2+} signal showed an almost all-or-none dose-response characteristic whereas,

although the current showed a roughly similar threshold for activation, it increased in a graded fashion with increasing suprathreshold stimuli. As discussed above, we feel that it is unlikely that this difference arose because the rhod-2 signal saturated with only modest increases in Ca^{2+} . Instead, we envisage several mechanisms that may all contribute to the more graded response of the current. Firstly, the Ca^{2+} signal was monitored from a highly localized spot centred in the photolysis square, in contrast to the membrane current, which reflects summated activity from the whole cell. Thus, although increasing suprathreshold levels of InsP_3 failed to evoke additional Ca^{2+} release at the measuring spot, additional current might be generated by InsP_3 which diffused out of the area exposed to photolysis light and caused Ca^{2+} release from surrounding areas of the cell. Secondly, the membrane current appears to reflect the rate of increase of free cytosolic Ca^{2+} , rather than simply the absolute Ca^{2+} level (Parker & Yao, 1992; and see below). Since the rate of rise of the Ca^{2+} signal continued to increase with increasing suprathreshold stimuli, even though the peak amplitude grew little, this may explain the continuing increase in peak size of the current response in Fig. 4B. Furthermore, the same explanation may account for the differing time courses of recovery of Ca^{2+} and current signals in the paired-flash experiments of Fig. 11.

Another difference between the confocal Ca^{2+} signals and the associated membrane currents was that the currents began following a lag of 30 ms or longer after the onset of Ca^{2+} release. This delay does not appear to arise from the Cl^- channels themselves, since rapid photorelease of Ca^{2+} from a caged precursor evoked currents that began within less than 5 ms. Instead, a simple explanation may be that InsP_3 liberates Ca^{2+} at sites in the interior of the cell, and the delay arises from the time taken for the Ca^{2+} concentration at the membrane to rise sufficiently to activate the Cl^- conductance. Although we do not know the Ca^{2+} dependence of current activation, a rough calculation indicates that the separation between the Ca^{2+} release sites and the plasma membrane may be about 1 μm . For example, in Fig. 9A the current was half-maximal about 100 ms after the rise in Ca^{2+} , and Ca^{2+} ions are expected to diffuse a mean distance in one dimension of 1.1 μm during this time. This relatively close spacing is consistent with immunohistological localization of InsP_3 receptors in the oocyte, which are concentrated within a narrow band extending about 5 μm inward from the plasma membrane (N. P. Callamare, unpublished observations). Recordings in hepatocytes (Ogden, Capiod & Carter, 1991) also showed that current responses evoked by photoreleased InsP_3 began about 75 ms after Ca^{2+} liberation, a result that was similarly interpreted as resulting from buffered diffusion of Ca^{2+} ions.

Although the Ca^{2+} -activated membrane current lags behind the Ca^{2+} signal on its rising phase, the opposite is true during the decline, when the current returns quickly to the baseline even though intracellular Ca^{2+} remains elevated for several seconds afterward (Fig. 4A; and see Miledi & Parker, 1989; Parker & Ivorra, 1990a; Lechleiter *et al.* 1991b). Thus, it appears that the current preferentially signals rapid increases in intracellular Ca^{2+} , but fails to respond to sustained elevations of Ca^{2+} . A further example of this phenomenon is shown in Fig. 12B, where photorelease of InsP_3 throughout a 30 s exposure generated spikes of Ca^{2+} superimposed on a maintained plateau. The Ca^{2+} spikes were accompanied by spikes in the current record, but the sustained plateau of Ca^{2+} failed to evoke any corresponding current.

One explanation for these discrepancies may be that the current is generated in response to transient elevations of Ca^{2+} near the inner surface of the plasma membrane, whereas the fluorescent recordings of Ca^{2+} signal more gradual changes in Ca^{2+} occurring deeper in the cell. If this is the case, our failure to detect differences in the durations of Ca^{2+} signals recorded by a small confocal spot focused close to the membrane or deeper into the cell (Fig. 10) suggests that any such transient Ca^{2+} changes must be highly localized to within less than 1 or 2 μm of the inner surface of the cell membrane. Another possibility is that the Cl^- channels inactivate rapidly, so that they open in response to a rapid rise in intracellular free Ca^{2+} but subsequently close within a few hundred milliseconds, even though the Ca^{2+} remains elevated. Against this, we found that currents evoked by Ca^{2+} injections were not reduced when Ca^{2+} was injected into oocytes following photorelease of InsP_3 , at a time when the Ca^{2+} signal evoked by InsP_3 was still high but the associated current had declined to the baseline (I. Parker & I. Ivorra, unpublished observations). However, interpretation of this result is complicated by the recent proposal that the oocyte may possess two types of Ca^{2+} -activated Cl^- conductance (Boton, Gillo, Dascal & Lass, 1989; Boton, Singer & Dascal, 1990), which are differentially activated by Ca^{2+} liberated from intracellular stores and by microinjected Ca^{2+} . A full clarification of the role of inactivation of the Cl^- conductance awaits recordings from inside-out membrane patches exposed to rapid changes in free Ca^{2+} level.

Whatever the mechanism, the properties of the Cl^- conductance allow it to act as a high-pass filter, so that spikes or oscillations of Ca^{2+} are transduced as electrical signals, while steady signals are blocked. The functional significance of this behaviour is obscure in the oocyte, but would be of obvious importance in rhythmically active cells. It is also consistent with the notion, discussed above, that information transmitted by the $\text{InsP}_3/\text{Ca}^{2+}$ pathway is encoded digitally as the frequency of spiking, rather than as a graded, analog signal.

We thank Dr Yong Yao for helpful comments. This work was supported by grant GM39831 from the US Public Health Services.

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