

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

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### SUMMARY

1. The subcellular characteristics of inositol 1,4,5-trisphosphate ( $\text{InsP}_3$ )-induced  $\text{Ca}^{2+}$  liberation were studied in *Xenopus* oocytes by the use of confocal microfluorimetry to monitor  $\text{Ca}^{2+}$  signals from minutely localized region of the cell in response to photorelease of  $\text{InsP}_3$  from a caged precursor.

2. Photorelease of increasing amounts of  $\text{InsP}_3$  by progressively longer light flashes evoked transient  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  signals, but their rate of rise continued to increase and their duration became longer. Simultaneous measurements of  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  currents showed a slightly higher threshold than the  $\text{Ca}^{2+}$  signal, and a more graded dependence upon flash duration.

3. The threshold flash durations required to evoke  $\text{Ca}^{2+}$  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.  $\text{Ca}^{2+}$  signals evoked by photoreleased  $\text{InsP}_3$  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  $\text{InsP}_3$  was about 30 ms.

5.  $\text{InsP}_3$ -evoked membrane currents began 30 ms or longer after the corresponding  $\text{Ca}^{2+}$  signals, whereas currents evoked by photorelease of  $\text{Ca}^{2+}$  from a caged precursor began within 5 ms of the onset of the light flash.

6. No differences in duration of  $\text{InsP}_3$ -evoked  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  signals were more prolonged than the associated membrane current signals.

7.  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  signals.

8. Photorelease of  $\text{InsP}_3$  during 30 s exposures of low intensity evoked trains of repetitive  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$ . At high light intensities spikes could not be discerned and, after an initial abrupt increase, the  $\text{Ca}^{2+}$  level declined monotonically over several seconds. The  $\text{Ca}^{2+}$ -activated membrane current reflected the  $\text{Ca}^{2+}$  spikes, but not the maintained elevation of  $\text{Ca}^{2+}$ .

9. We conclude that  $\text{InsP}_3$ -sensitive  $\text{Ca}^{2+}$  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  $\text{InsP}_3$ , producing single spikes of  $\text{Ca}^{2+}$  following transient elevation of  $\text{InsP}_3$  and repetitive spikes during maintained elevation of  $\text{InsP}_3$ .

#### INTRODUCTION

Inositol, 1,4,5-trisphosphate ( $\text{InsP}_3$ ) 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  $\text{Ca}^{2+}$  ions from intracellular stores (Berridge & Irvine, 1989). Studies of  $\text{InsP}_3$  action in intact cells have been greatly facilitated by the availability of a photolabile caged  $\text{InsP}_3$  (McCray & Trentham, 1989), that allows rapid and precisely controlled elevations of intracellular  $\text{InsP}_3$  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  $\text{Ca}^{2+}$  level by recording a  $\text{Ca}^{2+}$ -activated chloride membrane conductance, and by measuring the fluorescence of intracellularly loaded  $\text{Ca}^{2+}$  indicator dyes (Parker & Miledi, 1989; Parker & Ivorra, 1990*a*, 1991, 1992; Ivorra & Parker, 1990). However, there is increasing evidence that  $\text{InsP}_3$ -mediated  $\text{Ca}^{2+}$  liberation does not occur homogeneously throughout the oocyte. Specifically, intracellular injections of  $\text{InsP}_3$  produce a localized depression of responses to subsequent injections (Berridge, 1989), agonist activation evokes complex patterns of  $\text{Ca}^{2+}$  waves travelling across the oocyte (Lechleiter, Girard, Peralta & Clapham, 1991*a, b*; Yao & Parker, 1991), and video imaging of  $\text{Ca}^{2+}$  signals evoked by uniform photorelease of  $\text{InsP}_3$  reveals that  $\text{Ca}^{2+}$  is released at discrete 'hot spots' spaced several micrometres apart, which each function autonomously (Parker & Yao, 1991). Thus, interpretation of  $\text{Ca}^{2+}$ -evoked chloride currents is difficult, because they arise across the entire membrane area of the cell, and probably reflect the summation of asynchronous  $\text{Ca}^{2+}$  transients from many different regions. Furthermore,  $\text{Ca}^{2+}$  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  $\text{InsP}_3$ -induced  $\text{Ca}^{2+}$  release at a subcellular level, we developed a point confocal optical system (Pawley, 1990) that allows fluorescence signals from  $\text{Ca}^{2+}$  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  $\text{InsP}_3$  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,  $\text{Ca}^{2+}$  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  $\text{InsP}_3$ -sensitive stores in the oocyte and, in particular, the dependence of the size and time course of cytoplasmic  $\text{Ca}^{2+}$  signals on the level of  $\text{InsP}_3$ . 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;  $\text{CaCl}_2$ , 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  $\text{InsP}_3$  together with the fluorescent  $\text{Ca}^{2+}$  indicator dye rhod-2 (Minta *et al.* 1989). This pipette was filled with an aqueous solution including 1 mM caged  $\text{InsP}_3$  (*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  $\text{InsP}_3$  and DM-nitrophen (dimethoxynitrophenamine; caged  $\text{Ca}^{2+}$ ) 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  $\text{InsP}_3$ , 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 ( $4\text{ mW mm}^{-2}$ ) was estimated to photolyse  $< 1\%$  of the caged  $\text{InsP}_3$  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

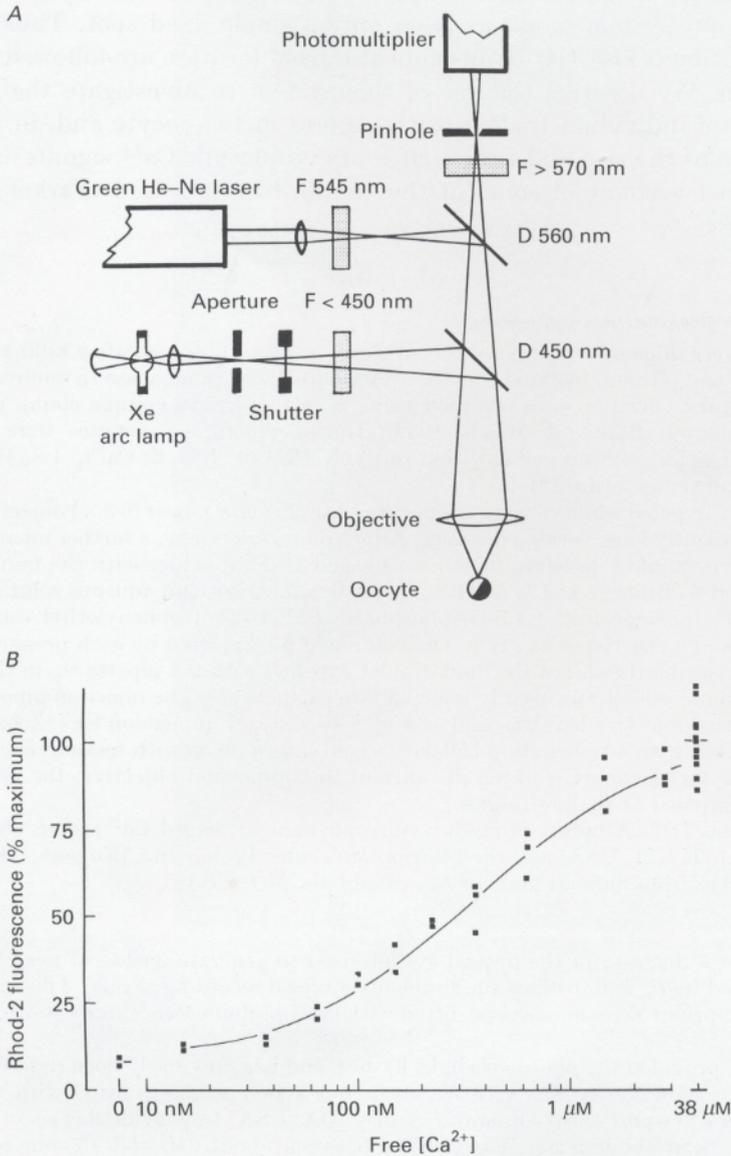


Fig. 1. *A*, schematic diagram of the optical system for simultaneous flash photolysis and confocal monitoring of  $Ca^{2+}$  transients. See text for details. F = filter, D = dichroic mirror; with wavelengths indicated in nm. *B*, rhod-2 fluorescence measured by the confocal microfluorimeter as a function 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  $\mu\text{m}$  hole, in order to have sufficient light through-put to give a good signal-to-noise ratio. However, this was replaced by a 20  $\mu\text{m}$  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  $\text{InsP}_3$ , 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  $\text{InsP}_3$  and  $\text{Ca}^{2+}$  in the oocyte cytoplasm were made assuming respective diffusion coefficients of  $2 \times 10^{-6}$  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  $\text{InsP}_3$ -evoked  $\text{Ca}^{2+}$  release made with this system.

### *$\text{Ca}^{2+}$ dependence of rhod-2 fluorescence*

The  $\text{Ca}^{2+}$  dependence of rhod-2 was measured using calibration solutions containing 20  $\mu\text{M}$  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  $\text{Ca}^{2+}$  concentrations were calculated assuming a dissociation constant of 150 nM for  $\text{Ca}^{2+}$  binding to EGTA. Fluorescence measurements were made using the confocal system to record from 5  $\mu\text{l}$  droplets of these solutions, and are plotted in Fig. 1B as a function of free  $\text{Ca}^{2+}$  concentration. The fluorescence in saturating  $\text{Ca}^{2+}$  was 12-times greater than that in  $\text{Ca}^{2+}$ -free solution, and the curve in Fig. 1B was calculated using an effective  $K_d$  of about 340 nM for  $\text{Ca}^{2+}$  binding to rhod-2. These values differ substantially from those (dissociation constant,  $K_d$ , = 1  $\mu\text{M}$ , fluorescence ratio excess *vs.* zero  $\text{Ca}^{2+}$  = 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

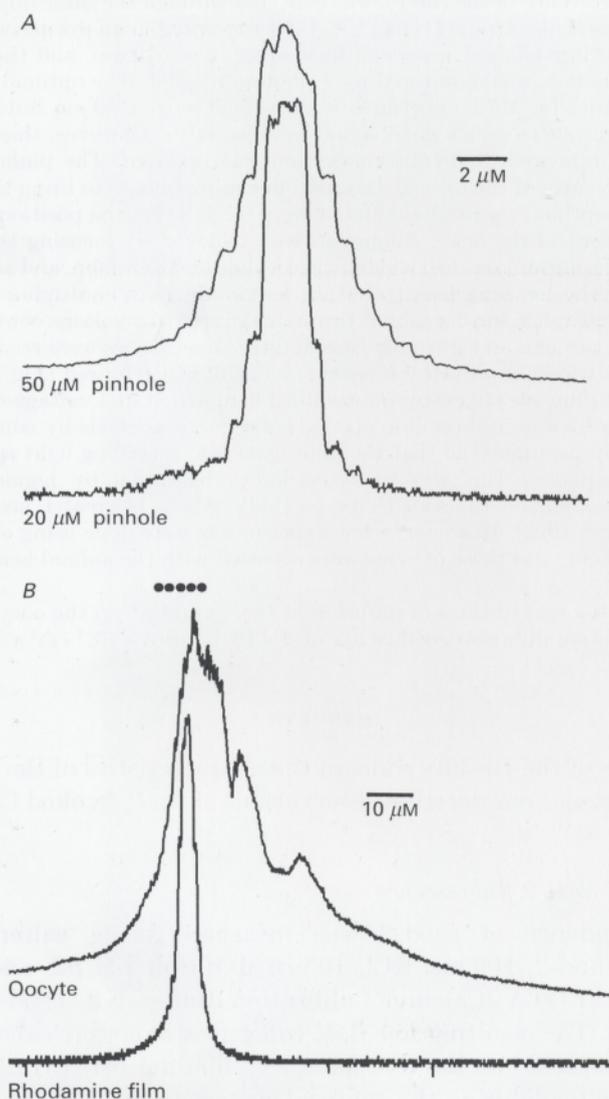


Fig. 2. Depth resolution of the confocal system. *A*, traces show the photomultiplier signal as the microscope was focused in increments through a thin film of rhodamine solution sandwiched between a slide and coverslip. The upper trace was obtained using a  $50\ \mu\text{m}$  pinhole and the lower with a  $20\ \mu\text{m}$  pinhole. For ease of comparison, both traces were scaled to similar peak heights; the signal with the  $20\ \mu\text{m}$  pinhole was 5.4 times smaller than with the  $50\ \mu\text{m}$  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  $50\ \mu\text{m}$  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  $50\ \mu\text{m}$  diameter pinhole, resulting in a lateral spot diameter of  $1.25\ \mu\text{m}$ . With a  $20\ \mu\text{m}$  pinhole the corresponding value was  $0.5\ \mu\text{m}$ . 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  $50\ \mu\text{m}$  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  $2\ \mu\text{m}$ , as compared to about  $4\ \mu\text{m}$  for the  $50\ \mu\text{m}$  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  $20\ \mu\text{m}$  pinhole would therefore arise mainly within an ellipsoid measuring less than  $1\ \mu\text{m}$  laterally and about  $2\ \mu\text{m}$  axially. This corresponds to a volume of roughly 1 fl. By comparison the volume of an oocyte is about  $1\ \mu\text{l}$ ; 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  $15\ \mu\text{m}$ . 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  $30\ \mu\text{m}$ , and to one-tenth after about  $100\ \mu\text{m}$ . 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  $3 \times 10^{22}$  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  $\text{Ca}^{2+}$  (Fig. 3*B*). Recordings from oocytes loaded with rhod-2, on the other hand, gave very different results. As shown in Fig. 3*A*, 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 probable explanation for the bleaching effect in Fig. 3*A* 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  $\text{Ca}^{2+}$ . This was tested by evoking  $\text{Ca}^{2+}$  liberation in response to photorelease of  $\text{InsP}_3$ . Figure 3*C* 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  $\text{Ca}^{2+}$ -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  $\text{Ca}^{2+}$ -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  $\text{Ca}^{2+}$ . Recordings of  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$ . 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

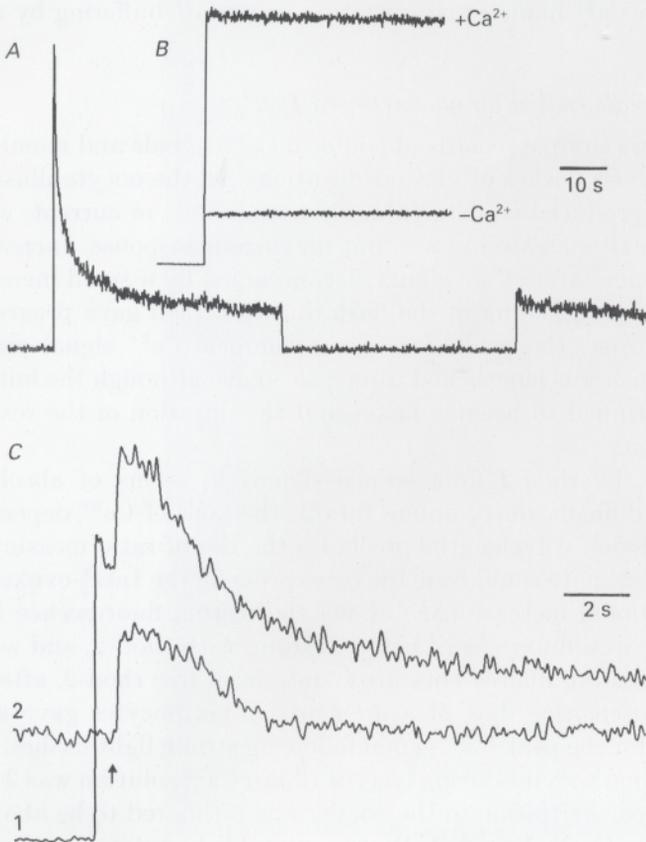


Fig. 3. Photobleaching of rhod-2. *A*, decay of fluorescence recorded from an oocyte loaded with rhod-2. The microscope was focused on the oocyte with the laser beam interrupted, and resting fluorescence was then recorded during two exposures to laser light. *B*, illumination of a solution of rhod-2 produces little photobleaching. Two superimposed traces are shown, obtained by illuminating thin films of rhodamine solution ( $10 \mu\text{M}$ ) in which the free  $\text{Ca}^{2+}$  level was strongly reduced by adding  $200 \mu\text{M}$  EGTA, or raised to a saturating level by addition of  $2 \text{ mM}$   $\text{Ca}^{2+}$ . *C*, the  $\text{InsP}_3$ -evoked  $\text{Ca}^{2+}$  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  $\text{InsP}_3$  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  $30 \text{ nM}$   $\text{Ca}^{2+}$

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  $\text{Ca}^{2+}$  imaging experiments (e.g. Lechleiter *et al.* 1991*a, b*; Parker & Yao, 1991), so that interference of normal cellular  $\text{Ca}^{2+}$  homeostasis resulting from  $\text{Ca}^{2+}$  buffering by the dye would be minimized.

#### *Confocal $\text{Ca}^{2+}$ signals evoked by photoreleased $\text{InsP}_3$*

Figure 4*A* shows sample records of confocal  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  or current, whereas a 7 ms flash evoked a small increase in  $\text{Ca}^{2+}$ , but no current response. Increasing the flash to 8 ms gave a much larger  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  concentration is difficult since, unlike fura-2, the lack of  $\text{Ca}^{2+}$ -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  $\text{InsP}_3$ -evoked fluorescence signals as a fractional increase ( $\Delta F$ ) 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,  $\text{Ca}^{2+}$ -insensitive dye. Measurements in six oocytes gave a mean  $\Delta F$  of  $2.0 \pm 0.24$  (s.e.m.) for the peak  $\text{Ca}^{2+}$  signal following strong light flashes, and the mean peak  $\Delta F$  in the same oocytes during lysis in 12 mM  $\text{Ca}^{2+}$  solution was  $3.34 \pm 0.74$ . The resting free  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  (Fig. 1*B*). From the calibration curve in Fig. 1*B*, the observed increase in fluorescence above this level following supramaximal photorelease of  $\text{InsP}_3$  corresponds to a peak free  $\text{Ca}^{2+}$  concentration of about 140 nM.

#### *Dose dependence of $\text{Ca}^{2+}$ and membrane current responses*

Figure 4*B* shows measurements of peak sizes of confocal  $\text{Ca}^{2+}$  signals and membrane currents in the same oocyte as Fig. 4*A*, plotted as a function of flash duration. The dose dependence of the  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  signal and its rate of rise on flash duration. Because oocytes were each loaded with differing amounts of caged  $\text{InsP}_3$  and rhod-2, it was not possible directly to compare the sizes of the  $\text{Ca}^{2+}$  signals or the threshold flash durations between different oocytes. Accordingly, the data were normalized by

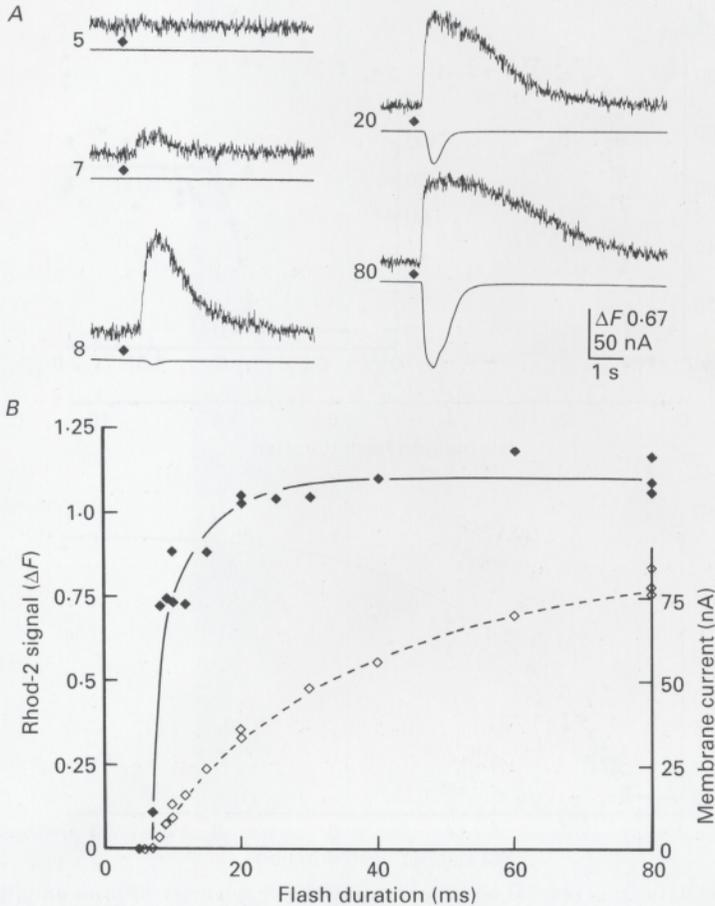


Fig. 4. Confocal  $\text{Ca}^{2+}$  signals and membrane currents evoked by light flashes of various durations. *A*, each pair of traces shows simultaneous records of confocal rhod-2 fluorescence (upper) and membrane current at a clamp potential of  $-60$  mV (lower). Upward deflections correspond to increasing fluorescence (and increasing  $\text{Ca}^{2+}$ ) 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  $\text{InsP}_3$  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 ( $\Delta F$ ). *B*, measurements of peak sizes of confocal  $\text{Ca}^{2+}$  signals (◆) and membrane currents (◇) from the same oocyte as *A*.

expressing the sizes of  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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

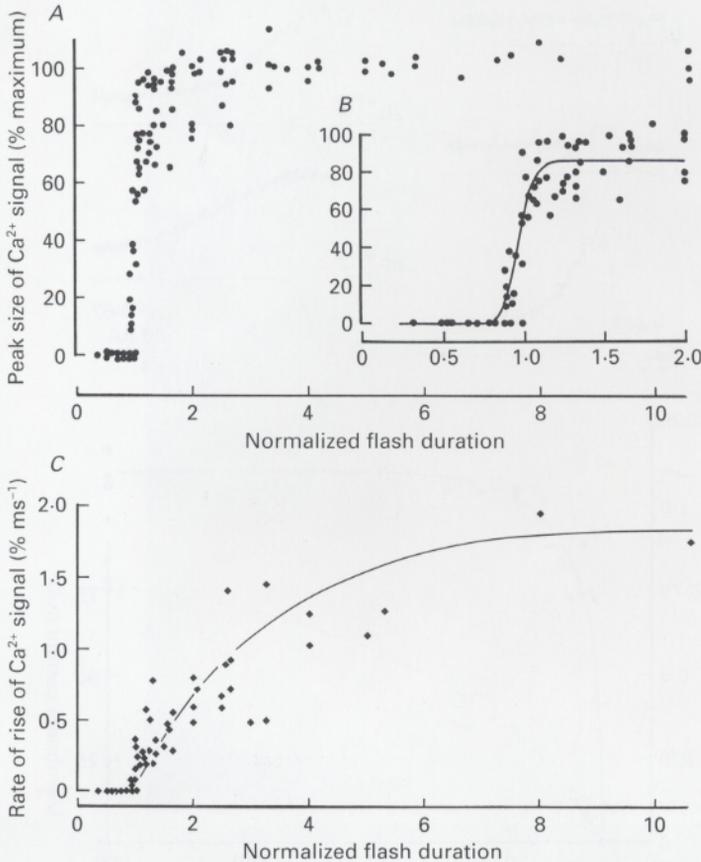


Fig. 5. Pooled data from several oocytes showing the dependence of peak amplitude and rate of rise of the confocal  $\text{Ca}^{2+}$  signal on duration of the photolysis flash. *A*, peak sizes of  $\text{Ca}^{2+}$  signals in eight oocytes, expressed as a percentage of the mean maximum response in each 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  $\text{Ca}^{2+}$  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  $\text{InsP}_3$  causes opening of the  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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).

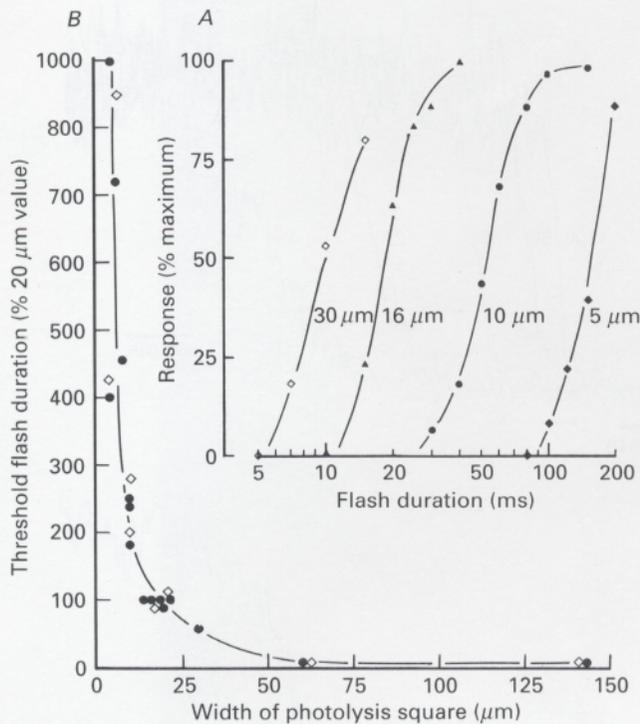


Fig. 6. The threshold flash duration required to evoke  $\text{Ca}^{2+}$  liberation depends upon the area over which  $\text{InsP}_3$  is liberated. *A*, measurements from a single oocyte, showing peak sizes of confocal  $\text{Ca}^{2+}$  signals evoked by light flashes of various durations. The four 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  $\text{Ca}^{2+}$  signal was less abrupt because  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  signal and open symbols indicate the threshold of the membrane current response.

#### *Threshold depends on area over which $\text{InsP}_3$ is photoreleased*

The threshold flash duration required to evoke a detectable  $\text{Ca}^{2+}$  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  $5 \mu\text{m}$ . 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  $\text{Ca}^{2+}$  signal and membrane current as indicators of

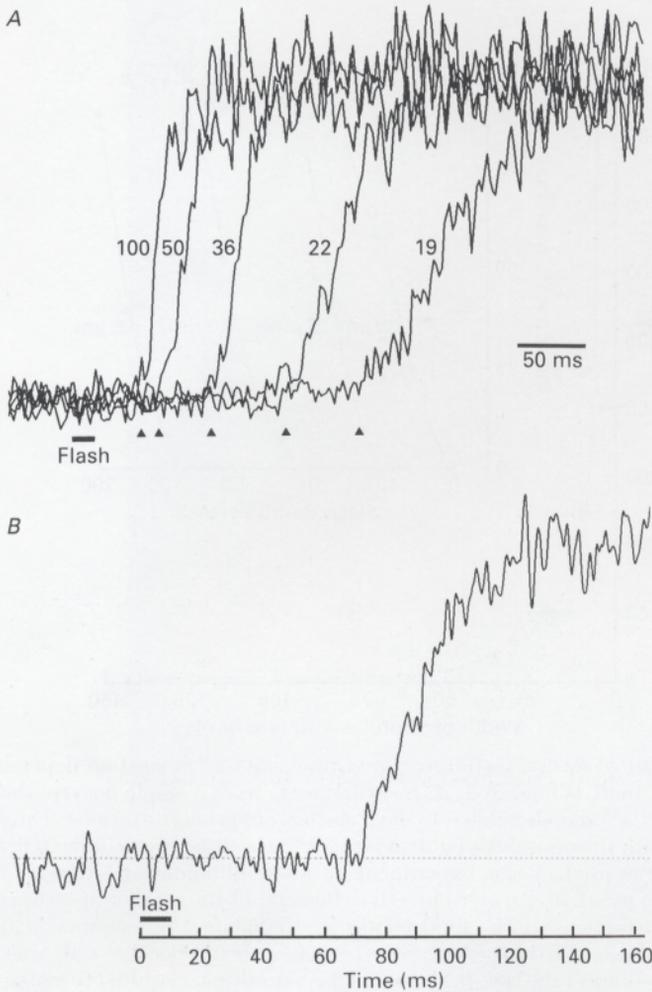


Fig. 7. Latency to onset of  $\text{Ca}^{2+}$  signals. *A*, superimposed traces showing onset of confocal  $\text{Ca}^{2+}$  signals evoked at a single location by light flashes of different intensities. The stimuli were flashes of 15 ms duration, given when marked by the bar. Neutral density filters 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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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 ( $4 \mu\text{m}$  side) was more than 100 times longer than with a  $140 \mu\text{m}$  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

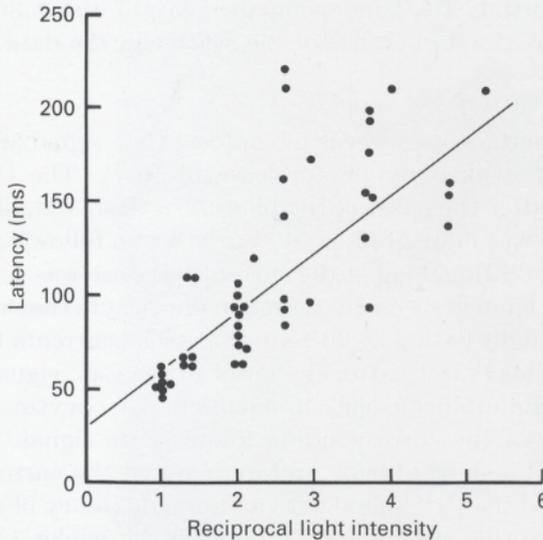


Fig. 8. Relationship between latency to onset of  $Ca^{2+}$  signal and intensity of photolysis light flash. Data were obtained in a single oocyte from records like those in Fig. 7A, by measuring the interval from the onset of the light flash to the first detectable rise in  $Ca^{2+}$ . The light intensity 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  $\text{InsP}_3$ . The measurements showed appreciable scatter, but the plot could be reasonably fitted by a linear relation, implying that an  $\text{InsP}_3$  binding step determines, at least partially, the kinetics of  $\text{Ca}^{2+}$  release channel opening. Furthermore, extrapolation of the regression line to infinitely high levels of  $\text{InsP}_3$  release indicated that the latency of the  $\text{Ca}^{2+}$  release may include also a limiting,  $\text{InsP}_3$ -independent delay of about 30 ms, though this value can only be approximate because of the scatter in the data.

#### *Latencies of membrane current $\text{Ca}^{2+}$ signals*

Figure 9A shows simultaneous records of confocal  $\text{Ca}^{2+}$  signal and  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  membrane current evoked by photorelease of  $\text{InsP}_3$ . The  $\text{Ca}^{2+}$  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  $58 \pm 4$  ms (S.E.M.) after the onset of the  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  signal with a minimal latency of about 30 ms, that lengthened slightly as the stimulus was reduced to evoke  $\text{Ca}^{2+}$  signals with increasingly longer latencies.

Experiments in which flash photolysis of caged  $\text{Ca}^{2+}$  (DM-nitrophen; McCray & Trentham, 1989) was used to evoke rapid increases of intracellular free  $\text{Ca}^{2+}$  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  $\text{Cl}^-$  channels. For example, in Fig. 9B the current began within about 5 ms of the onset of a light flash that caused photorelease of  $\text{Ca}^{2+}$ . Although the peak current in this experiment was about 10 times greater than with photoreleased  $\text{InsP}_3$  (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  $\text{Ca}^{2+}$  concentration resulting from photolysis of caged  $\text{Ca}^{2+}$  was probably lower than that with photoreleased  $\text{InsP}_3$ , so that the faster kinetics of  $\text{Cl}^-$  channel opening did not arise because of a higher  $\text{Ca}^{2+}$  level.

#### *Confocal signals at different depths*

A puzzling feature of the confocal  $\text{Ca}^{2+}$  records presented here, and of  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  currents. We could envisage two explanations for this. Firstly, the chloride current declines because of inactivation or desensitization, even though the intracellular free  $\text{Ca}^{2+}$  level remains elevated. Secondly, the chloride current accurately reflects a transient increase in free  $\text{Ca}^{2+}$  close to the inner surface of the plasma membrane, whereas the fluorescent  $\text{Ca}^{2+}$  monitor senses a more prolonged

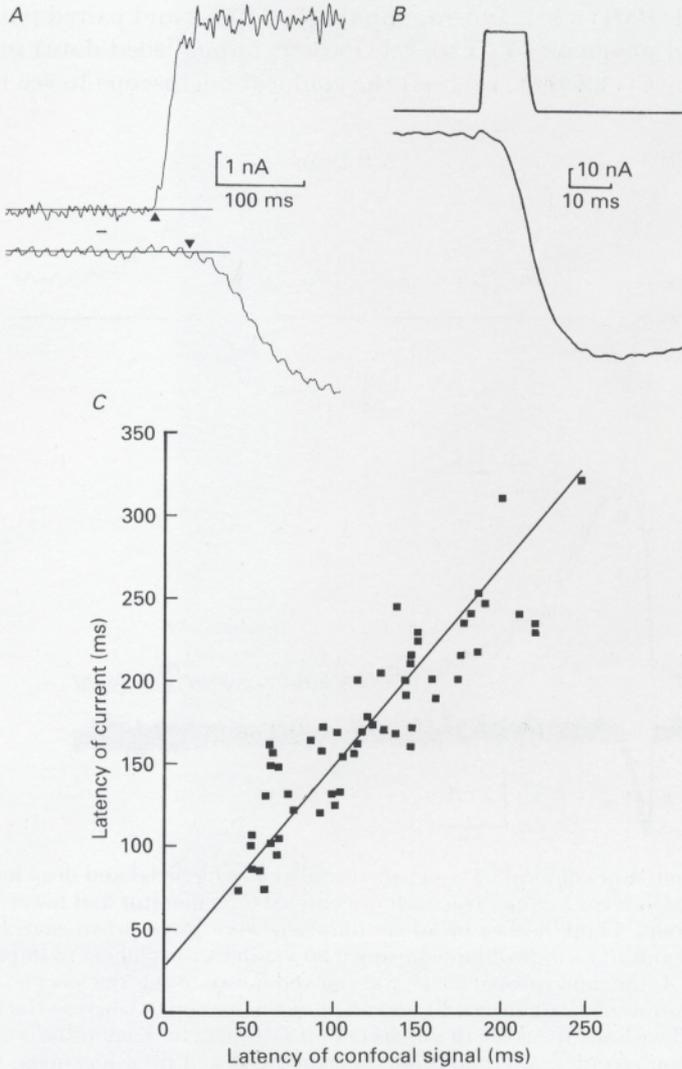


Fig. 9. Latencies of confocal  $Ca^{2+}$  signals and membrane currents. *A*, simultaneous records of confocal  $Ca^{2+}$  signal (upper trace) and membrane current (lower trace) evoked by photorelease of  $InsP_3$ . A 10 ms light flash was given when marked by the bar, and arrowheads indicate the estimated 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  $\text{Ca}^{2+}$  deeper into the cytoplasm. Because experiments with  $\text{Ca}^{2+}$  microinjections (I. Parker & I. Ivorra, unpublished data) and paired photorelease of  $\text{Ca}^{2+}$  from a caged precursor (Y. Yao & I. Parker, unpublished data) revealed little inactivation of the  $\text{Cl}^-$  current, we used the confocal microscope to see if differences

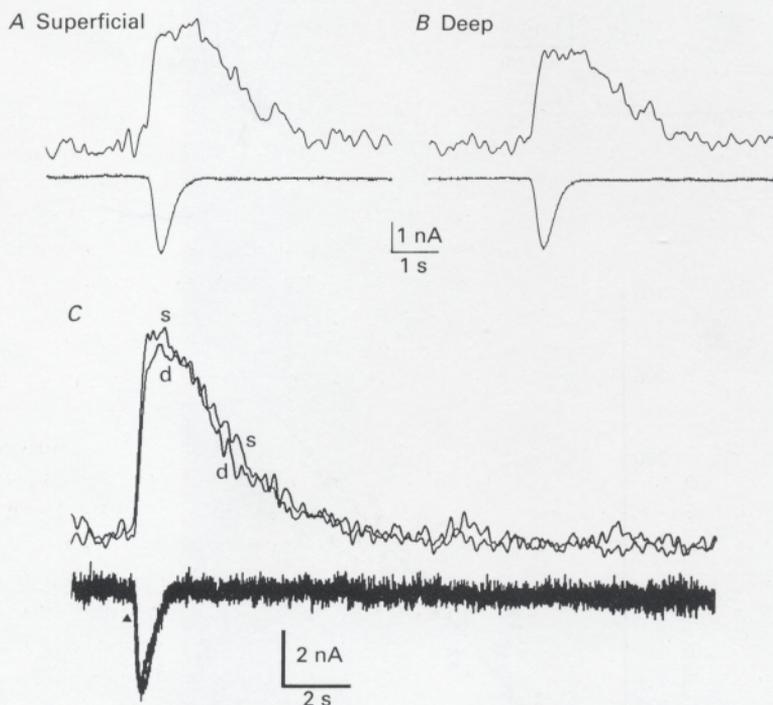


Fig. 10. Time course of confocal  $\text{Ca}^{2+}$  signals recorded at superficial and deep locations in the oocyte. In each record, upper traces show confocal  $\text{Ca}^{2+}$  monitor and lower traces are membrane current. Light flashes of 30 ms duration were given when marked by the arrow-heads. *A* and *B*, records obtained using a  $20\ \mu\text{m}$  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  $10\ \mu\text{m}$  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  $50\ \mu\text{m}$  pinhole. Superimposed traces show single responses with the microscope focused superficially (*s*) and  $11.5\ \mu\text{m}$  deeper (*d*) into the oocyte.

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

Figure 10 shows examples of confocal  $\text{Ca}^{2+}$  signals recorded using a  $20\ \mu\text{m}$  pinhole, with the confocal spot focused near the surface of the oocyte (*A*) and at a depth of  $10\ \mu\text{m}$  (*B*). The two recording locations were selected so that  $\text{InsP}_3$ -evoked  $\text{Ca}^{2+}$  signals were of similar size, and about one-half of the maximal signals obtained at an intermediate focus position. A  $20\ \mu\text{m}$  diameter pinhole was used to improve the spatial resolution of the recording, but gave noisy records. Accordingly, the  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  signals at the superficial and deep sites showed no obvious differences, and in both cases the  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  signals.

#### *Depression with paired flashes*

We have previously shown that responses evoked by photoreleased  $\text{InsP}_3$  were depressed following a preceding suprathreshold light flash, probably as a result of inhibition of  $\text{InsP}_3$ -induced  $\text{Ca}^{2+}$  release by  $\text{Ca}^{2+}$  liberated during the conditioning response (Parker & Ivorra, 1990a).  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  release units. To further investigate this phenomenon at a finer subcellular level, we repeated the experiments using the confocal system to monitor highly localized  $\text{Ca}^{2+}$  liberation.

The records in Fig. 11A show simultaneous measurements of  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  signal was more prolonged, but not larger than that evoked by a single flash. The second flash failed to evoke any detectable  $\text{Ca}^{2+}$  or current responses when applied after intervals between about 0.5 and 2 s, but gave a small  $\text{Ca}^{2+}$  response, though no current response, after 3 s. When the interval was further lengthened to 6 s the  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  signals and membrane currents as a function of inter-flash interval. The  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  signal. For example, the current was only about one-half the control size after 6 s, when the  $\text{Ca}^{2+}$  signal had almost completely recovered.

*Ca<sup>2+</sup> spikes during sustained photorelease of InsP<sub>3</sub>*

In contrast to the transient increases in  $\text{Ca}^{2+}$  evoked by brief photolysis flashes, sustained photorelease of  $\text{InsP}_3$  by light exposures lasting for several seconds usually evoked trains of repetitive  $\text{Ca}^{2+}$  spikes. Typical records are shown in Fig. 12*A*,

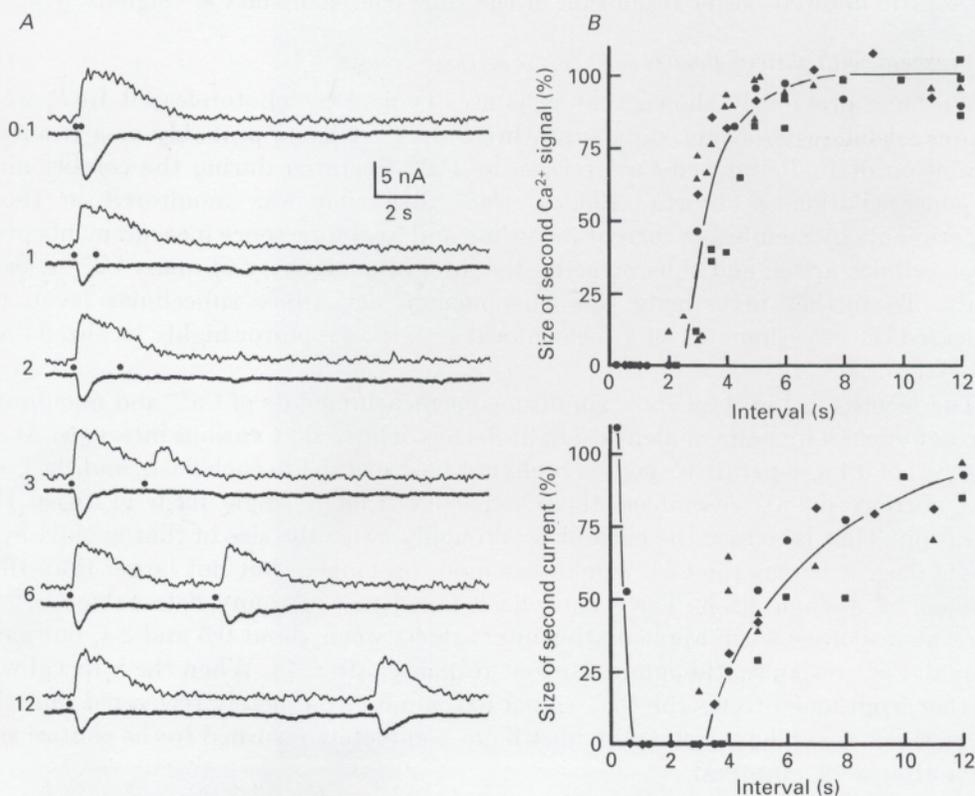


Fig. 11. Depression of  $\text{InsP}_3$ -evoked responses with paired light flashes. *A*, each frame shows confocal  $\text{Ca}^{2+}$  signals (upper) and membrane currents (lower) evoked by pairs of light flashes given with various intervals. The times of the flashes are indicated by dots, and the intervals are indicated (in seconds) next to each record. *B*, time courses of recovery from depression. Peak sizes of  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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

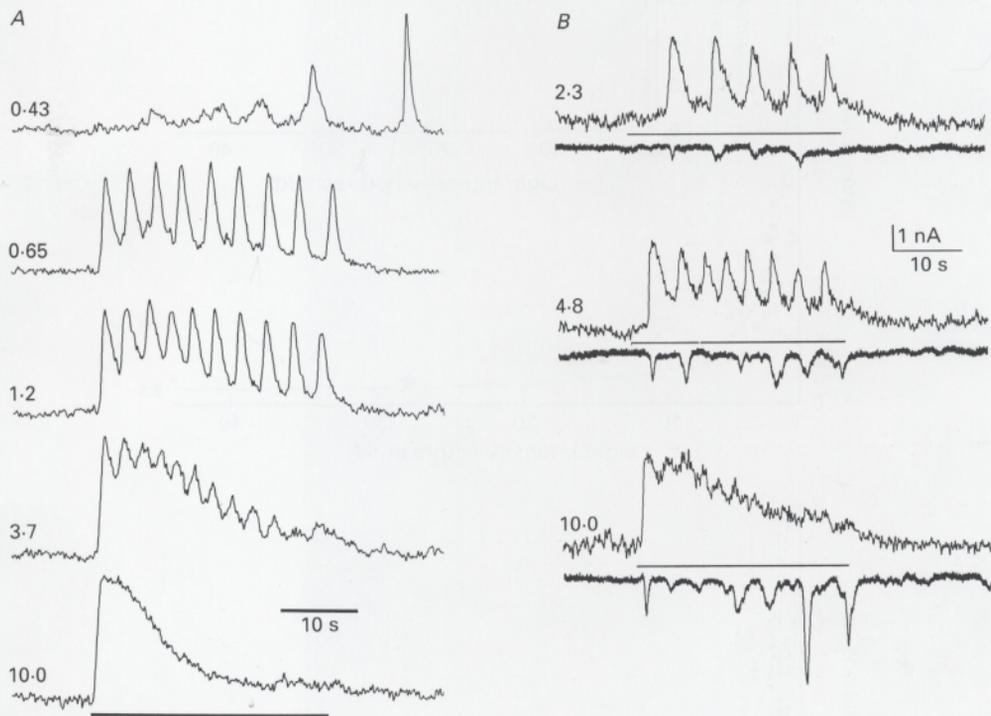


Fig. 12.  $Ca^{2+}$  spikes during prolonged photorelease of  $InsP_3$ . Horizontal bars indicate the durations of exposure to photolysis light, and numbers next to the traces indicate the light intensity as a percentage of the full output. *A*, confocal  $Ca^{2+}$  signals evoked at a single recording 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 12*B* 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. 12*B*) 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

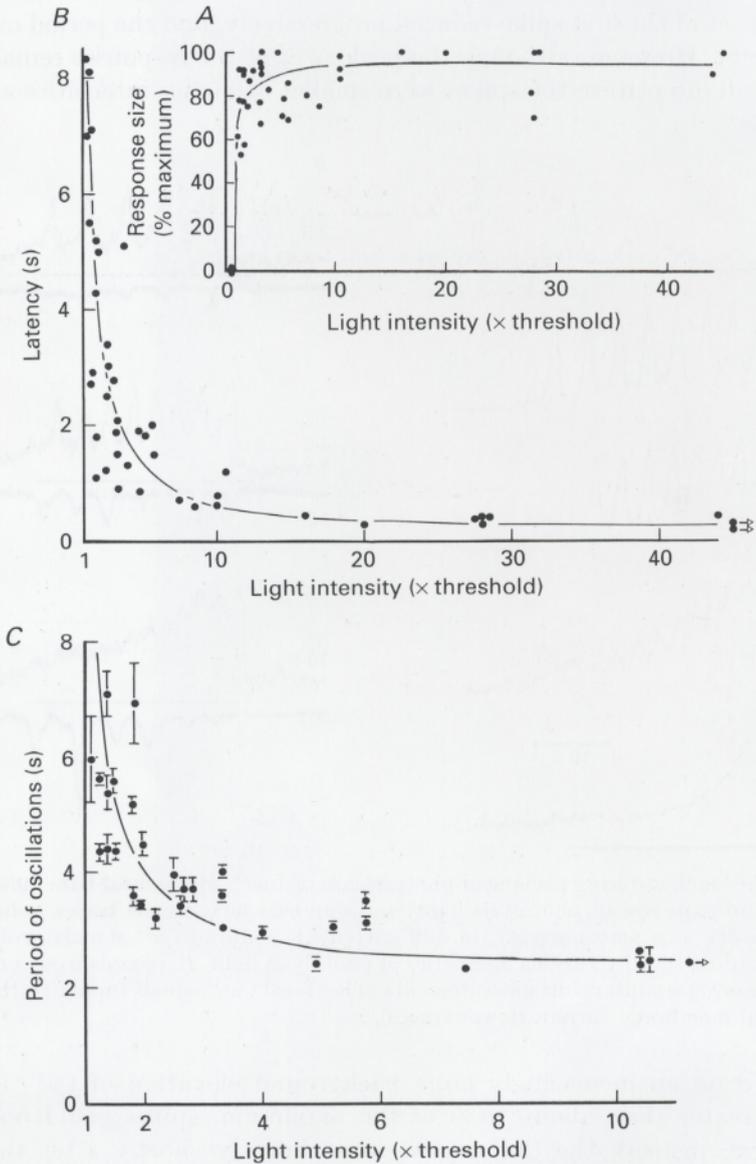


Fig. 13. Changes in characteristics of the oscillatory  $\text{Ca}^{2+}$  signals as a function of intensity of photolysis light. Measurements were made in five oocytes from records like those in Fig. 12. To allow pooling of data, light intensities are normalized to the threshold intensity required in 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  $\text{Ca}^{2+}$ . *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  $\text{Ca}^{2+}$  during strong photolysis was not accompanied by any corresponding increase in current.

$\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  was seen, the size of which was graded with photolysis intensity.

A difficulty in interpreting the  $\text{Ca}^{2+}$  signals evoked during prolonged photorelease of  $\text{InsP}_3$  is that, although the light intensity remained constant, we do not know the resulting time course of changes in concentration of  $\text{InsP}_3$ . If  $\text{InsP}_3$  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  $\text{InsP}_3$  as a result of metabolism and diffusion away from the illuminated area. The rapid cessation of the  $\text{Ca}^{2+}$  signals when the light was extinguished suggests that this equilibration occurred within a few seconds. Furthermore, the relatively constant period of the  $\text{Ca}^{2+}$  spikes during each exposure also indicates that the average level of  $\text{InsP}_3$  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  $\text{InsP}_3$ , depletion during the exposure period would probably not have been important because of replenishment by caged  $\text{InsP}_3$  diffusing from surrounding unexposed regions of the cell. Assuming the diffusion coefficient for caged  $\text{InsP}_3$  in the cytosol is similar to that of  $\text{InsP}_3$  ( $2 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ ), 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 $\text{Ca}^{2+}$ spike parameters*

Figure 13 presents measurements in five oocytes of various parameters of  $\text{Ca}^{2+}$  spike responses evoked by prolonged photorelease of  $\text{InsP}_3$ , plotted as a function of photolysis intensity. Similar to the dose dependence of  $\text{Ca}^{2+}$  responses to brief flashes, the peak  $\text{Ca}^{2+}$  levels attained during prolonged photorelease of  $\text{InsP}_3$  showed a nearly all-or-none characteristic (Fig. 13A). In particular, the peak  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$ . The latency to onset of the first  $\text{Ca}^{2+}$  spike shortened progressively from longer than 8 s with just suprathreshold stimuli to a few hundred milliseconds at the highest intensities used (Fig. 13B). Finally, the period of the  $\text{Ca}^{2+}$  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. 13C), beyond which individual spikes were no longer discernable.

## DISCUSSION

### *Confocal microfluorimetry*

We describe the construction and use of a confocal microfluorimeter able to monitor  $\text{Ca}^{2+}$ -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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  concentration, because no currently available visible wavelength  $\text{Ca}^{2+}$  indicators show useful spectral shifts on binding  $\text{Ca}^{2+}$ , 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  $\text{Ca}^{2+}$ -selective microelectrode, in that both techniques measure free  $\text{Ca}^{2+}$  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.  $\text{Ca}^{2+}$  electrodes, on the other hand, can readily be calibrated to provide absolute free  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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 $\text{InsP}_3$ -evoked $\text{Ca}^{2+}$ signals in the oocyte*

Our initial results using confocal  $\text{Ca}^{2+}$  recording (Parker & Ivorra, 1990*b*) led us to propose that the  $\text{InsP}_3$ -sensitive  $\text{Ca}^{2+}$  pool in the oocyte is arranged as a collection of functionally independent localized units, that each release  $\text{Ca}^{2+}$  in a nearly all-or-none manner. Subsequent experiments using video imaging of  $\text{Ca}^{2+}$  released by photolysis of caged  $\text{InsP}_3$  supported this hypothesis, and further revealed that  $\text{Ca}^{2+}$  is released at discrete 'hot spots', spaced several micrometres apart, which are activated at varying threshold levels of  $\text{InsP}_3$  (Parker & Yao, 1991). The work in the present paper was aimed towards characterizing the properties of  $\text{Ca}^{2+}$  release from individual units, by recording  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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 $\text{InsP}_3$ liberation*

Photorelease of increasing amounts of  $\text{InsP}_3$  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  $\text{InsP}_3$ -evoked  $\text{Ca}^{2+}$  release, and not from some artifactual property of the caged  $\text{InsP}_3$  system or the  $\text{Ca}^{2+}$  monitor.

Firstly, photorelease of  $\text{InsP}_3$  is expected to vary as a linear function of light absorption by caged  $\text{InsP}_3$  (McCray & Trentham, 1989), a relationship that was experimentally confirmed by measuring photorelease of ATP from a caged precursor like that used to cage  $\text{InsP}_3$  (Parker & Ivorra, 1992). Furthermore, the fact that  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$ -activation and, at low  $\text{Ca}^{2+}$  levels, the fluorescence rises nearly linearly with increasing free  $\text{Ca}^{2+}$  (Fig. 1B). A more serious concern is whether saturation of the  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  concentration was raised to micromolar levels (e.g. fluorescence was about 75% maximal at  $1 \mu\text{M}$   $\text{Ca}^{2+}$ ), whereas the peak  $\text{InsP}_3$ -evoked  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  solution gave fluorescence increases that were about 70% greater than the peak  $\text{InsP}_3$ -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  $\text{Ca}^{2+}$  ionophores (A23187 and ionomycin) to raise intracellular  $\text{Ca}^{2+}$  to saturating levels, but these agents evoked only slight fluorescence signals, that were smaller than those evoked by  $\text{InsP}_3$ . A final argument that the nearly all-or-none  $\text{Ca}^{2+}$  signals reflect the characteristics of  $\text{InsP}_3$ -mediated  $\text{Ca}^{2+}$  release comes from experiments where sustained elevations of  $\text{InsP}_3$  evoked repetitive  $\text{Ca}^{2+}$  spikes (Fig. 12). Increasing levels of  $\text{InsP}_3$  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  $\text{Ca}^{2+}$  levels were sufficient to saturate the fluorescence.

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

binding of  $\text{InsP}_3$  to receptor sites, the rate of release would be expected to be a graded function of  $\text{InsP}_3$  concentration, and the steepness of the relation would depend on the co-operativity of  $\text{InsP}_3$  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  $\text{InsP}_3$  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  $\text{Ca}^{2+}$  release in permeabilized cells (Meyer & Stryer, 1988; Champeil, Combettes, Berthon, Doucet, Orlowski & Claret, 1989; Meyer, Wensel & Stryer, 1990). Furthermore, if the opening of  $\text{Ca}^{2+}$  release channels shows high co-operativity, the rate of release is expected to rise extremely steeply as  $\text{InsP}_3$  levels are raised above the apparent threshold. In contrast to this, we observed a graded variation in rate of rise of the  $\text{Ca}^{2+}$  signal over a severalfold range of photolysis flash durations (Fig. 5B).

The sharp threshold of  $\text{InsP}_3$ -evoked  $\text{Ca}^{2+}$  release suggests that this process probably involves regenerative positive feedback, and such a mechanism has also been implicated in the generation of repetitive  $\text{Ca}^{2+}$  spiking (Tsien & Tsien, 1990; Meyer & Stryer, 1991) and in the active propagation of  $\text{Ca}^{2+}$  waves (Meyer, 1991). Several models have been proposed to account for this feedback, which can be grouped into three categories. (i)  $\text{Ca}^{2+}$  released from  $\text{InsP}_3$ -sensitive stores stimulates phospholipase C, resulting in increased formation of  $\text{InsP}_3$  and hence a regenerative release of further  $\text{Ca}^{2+}$  (Harootunian, Kao, Paranjape & Tsien, 1991). (ii) Slow release of  $\text{Ca}^{2+}$  from  $\text{InsP}_3$ -sensitive stores causes an overloading of secondary,  $\text{InsP}_3$ -insensitive stores, which then explosively release their contents by a mechanism of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (Berridge & Irvine, 1989; Goldbeter, Dupont & Berridge, 1990). (iii)  $\text{Ca}^{2+}$  released from  $\text{InsP}_3$ -sensitive stores acts as a co-agonist at the  $\text{InsP}_3$  receptor, to facilitate the opening of  $\text{Ca}^{2+}$ -release channels and thus the liberation of further  $\text{Ca}^{2+}$  (Bezprozvanny, Watras & Erlich, 1991; Finch, Turner & Goldin, 1991). We have discussed these models previously, and presented evidence favouring model (iii) to account for regenerative  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  release appears too brief to allow for the stimulated formation of  $\text{InsP}_3$  by phospholipase C. As described later, membrane current responses lag at least 30 ms behind the  $\text{Ca}^{2+}$  signal, probably as a result of the time required for  $\text{Ca}^{2+}$  ions to diffuse from their sites of release to the  $\text{Ca}^{2+}$ -activated channels in the plasma membrane. Since  $\text{InsP}_3$  is formed by breakdown of phosphatidylinositol biphosphate in the plasma membrane, a similar time would be required for stimulation of  $\text{InsP}_3$  production, together with an extra delay for  $\text{InsP}_3$  to diffuse back to the  $\text{InsP}_3$ -sensitive  $\text{Ca}^{2+}$  stores (though this would be shorter, due to the higher diffusion coefficient for  $\text{InsP}_3$  in the cytosol as compared to  $\text{Ca}^{2+}$ ). Thus, the total time for a single transit of  $\text{Ca}^{2+}$  and  $\text{InsP}_3$  between the release sites and the plasma membrane already appears to be longer than the latency to onset of regenerative  $\text{Ca}^{2+}$  release, even without considering any extra delays that may be introduced by  $\text{Ca}^{2+}$ -dependent activation of phospholipase C. Furthermore, the regenerative release of

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

The short latency and abrupt onset of  $\text{InsP}_3$ -evoked  $\text{Ca}^{2+}$  release are also difficult to reconcile with the scheme (model (ii)) in which  $\text{InsP}_3$ -insensitive stores become 'primed' to release their contents by overfilling with  $\text{Ca}^{2+}$  liberated from  $\text{InsP}_3$ -sensitive stores. The period available for filling of these secondary stores is brief, and we failed to detect a rise in cytosolic  $\text{Ca}^{2+}$  during the latent period, although a relatively large rise would be expected if  $\text{Ca}^{2+}$  were transferred through the cytosol between the two types of stores. Also, photorelease of  $\text{Ca}^{2+}$  from a caged precursor evoked rises in cytosolic  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (Ivorra & Parker, 1990; I. Parker & Y. Yao, unpublished data).

Our failure in these experiments to observe a rise in  $\text{Ca}^{2+}$  level preceding the abrupt onset of the  $\text{InsP}_3$ -evoked  $\text{Ca}^{2+}$  signal is also at odds with model (iii), in which a rise in cytosolic  $\text{Ca}^{2+}$  facilitates the action of  $\text{InsP}_3$  to release further  $\text{Ca}^{2+}$ . However, by improving the recording sensitivity by averaging  $\text{Ca}^{2+}$ -dependent fluorescence signals over wider areas of the cell, we recently detected a small pacemaker rise in  $\text{Ca}^{2+}$  preceding the large, abrupt increase (Parker & Yao, 1991), which was probably lost in the noise level of the confocal recordings. Although both the  $\text{Ca}^{2+}$  co-agonist and the two-pool models predict a graded increase in cytosolic free  $\text{Ca}^{2+}$  preceding regenerative release, the size of this  $\text{Ca}^{2+}$  signal may be much smaller if  $\text{Ca}^{2+}$  acts as a co-agonist.  $\text{Ca}^{2+}$  is liberated from stores through channels that are part of the same molecule as the  $\text{InsP}_3$  receptor site (Ferris, Haganir, Supattapone & Snyder, 1989), so that facilitation could arise from an elevation of cytosolic  $\text{Ca}^{2+}$  that is highly localized, even in comparison to the dimensions of the confocal recording spot. Also, a relatively small amount of  $\text{Ca}^{2+}$  will probably suffice to trigger opening of  $\text{InsP}_3$ -sensitive release channels, whereas in the two-pool model a large part of the total  $\text{Ca}^{2+}$  involved in the regenerative response must first be transferred from  $\text{InsP}_3$ -sensitive to  $\text{InsP}_3$ -insensitive stores.

Because the rising phase of the confocal  $\text{Ca}^{2+}$  transients was brief as compared to their decay, the rate of rise should provide a good measure of the rate of release from  $\text{InsP}_3$ -sensitive stores. For suprathreshold stimuli this increased in a graded manner with increasing levels of  $\text{InsP}_3$ ; but why then did the peak amplitude of the  $\text{Ca}^{2+}$  signal show a nearly all-or-none dependence on  $\text{InsP}_3$  level? A simple possibility is that the peak response represented the complete emptying of  $\text{Ca}^{2+}$  from the  $\text{InsP}_3$ -sensitive store, and that the prolongation of the response at higher levels of  $\text{InsP}_3$  arose because release channels remained open for longer, and thus immediately liberated any  $\text{Ca}^{2+}$  that was pumped back into the store. Another explanation is that the peak amplitude of the  $\text{Ca}^{2+}$  transient is regulated by a feedback mechanism. This might arise because cytosolic  $\text{Ca}^{2+}$  ions exert a delayed negative feedback on  $\text{InsP}_3$ -mediated  $\text{Ca}^{2+}$  release (Parker & Ivorra, 1990*a*), and it has also been proposed (Irvine, 1990) that the intraluminal level of  $\text{Ca}^{2+}$  in the stores may regulate activity of the  $\text{InsP}_3$  receptor.

*Threshold depends on area of photoreleased InsP<sub>3</sub>*

The threshold flash duration required to evoke Ca<sup>2+</sup> 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 μm to one of 5 μm. This was surprising, because the liberation of InsP<sub>3</sub> 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<sup>2+</sup> release did not begin until 200–300 ms after the flash, the concentration of InsP<sub>3</sub> 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<sub>3</sub> in two dimensions during this time would be about 20 μm, 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<sup>2+</sup> decreases the threshold for regenerative release of a much larger amount of Ca<sup>2+</sup>. Diffusional loss of Ca<sup>2+</sup> from small illuminated areas might limit this build-up, and thus increase the threshold level of InsP<sub>3</sub> needed to trigger a regenerative release.

Using photolysis flashes illuminating large areas of the oocyte we had previously estimated the intracellular concentration of InsP<sub>3</sub> 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 μM, since oocytes were loaded with only sufficient caged InsP<sub>3</sub> to give this final cytosolic concentration. The 100-fold difference in threshold flash durations seen between 5 and 140 μm photolysis squares may thus overestimate the difference in respective peak InsP<sub>3</sub> concentrations, as the strong stimuli required with small light squares probably consumed an appreciable fraction of the immediately available caged InsP<sub>3</sub>.

Regardless of the mechanism of the area dependence of the threshold, these experiments reveal a further non-linearity of the InsP<sub>3</sub> signalling pathway that is likely to be important for signal integration. Thus, a strong, but localized activation of InsP<sub>3</sub> production might fail to evoke any Ca<sup>2+</sup> release, in contrast to a more widespread activation which evokes a Ca<sup>2+</sup> signal even though the peak InsP<sub>3</sub> concentration is lower.

*Ca<sup>2+</sup> spikes*

Sustained photorelease of InsP<sub>3</sub> over several seconds led to the generation of localized repetitive spikes of intracellular Ca<sup>2+</sup> (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<sub>3</sub> (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<sup>2+</sup> 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  $\text{Ca}^{2+}$  spikes, different regions function independently (Parker & Yao, 1991; Lechleiter *et al.* 1991a), so that whole-cell recordings of membrane current and fluorescent  $\text{Ca}^{2+}$  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  $\text{InsP}_3$  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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  spiking frequency and level of  $\text{InsP}_3$  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  $\text{InsP}_3$  levels, and a further doubling of the stimulus produced virtually no change in period (Fig. 13C). Secondly, the spikes became progressively smaller at higher levels of  $\text{InsP}_3$  and beyond a certain level only a monotonic rise and fall in  $\text{Ca}^{2+}$  was detected, with no discernable spikes remaining (Fig. 12A).

These experiments do not provide direct evidence about the mechanisms underlying spike generation, but the time course of the  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  spiking (reviewed by Tsien & Tsien, 1990; Harootunian *et al.* 1990; Meyer & Stryer, 1991). Based on our findings that cytosolic  $\text{Ca}^{2+}$  can both facilitate (Yao & Parker, 1992) and inhibit (Parker & Ivorra, 1990a)  $\text{InsP}_3$ -evoked  $\text{Ca}^{2+}$  liberation, we favour the idea that  $\text{Ca}^{2+}$  spike generation in the oocyte involves a rapid positive feedback of  $\text{Ca}^{2+}$  ions on the  $\text{InsP}_3$  receptor to promote opening of the  $\text{Ca}^{2+}$  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).

#### $\text{Ca}^{2+}$ -activated $\text{Cl}^-$ current

The membrane current responses mediated by  $\text{InsP}_3$  signalling arise through activation of  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  channels in the oocyte membrane (Miledi & Parker, 1984; Parker & Miledi, 1989). However, simultaneous recordings of confocal intracellular  $\text{Ca}^{2+}$  signals and  $\text{Ca}^{2+}$ -activated membrane currents revealed several discrepancies between these two monitors of intracellular free  $\text{Ca}^{2+}$ .

One is apparent in the responses activated by different levels of  $\text{InsP}_3$ . The confocal  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$ . Instead, we envisage several mechanisms that may all contribute to the more graded response of the current. Firstly, the  $\text{Ca}^{2+}$  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  $\text{InsP}_3$  failed to evoke additional  $\text{Ca}^{2+}$  release at the measuring spot, additional current might be generated by  $\text{InsP}_3$  which diffused out of the area exposed to photolysis light and caused  $\text{Ca}^{2+}$  release from surrounding areas of the cell. Secondly, the membrane current appears to reflect the rate of increase of free cytosolic  $\text{Ca}^{2+}$ , rather than simply the absolute  $\text{Ca}^{2+}$  level (Parker & Yao, 1992; and see below). Since the rate of rise of the  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  and current signals in the paired-flash experiments of Fig. 11.

Another difference between the confocal  $\text{Ca}^{2+}$  signals and the associated membrane currents was that the currents began following a lag of 30 ms or longer after the onset of  $\text{Ca}^{2+}$  release. This delay does not appear to arise from the  $\text{Cl}^-$  channels themselves, since rapid photorelease of  $\text{Ca}^{2+}$  from a caged precursor evoked currents that began within less than 5 ms. Instead, a simple explanation may be that  $\text{InsP}_3$  liberates  $\text{Ca}^{2+}$  at sites in the interior of the cell, and the delay arises from the time taken for the  $\text{Ca}^{2+}$  concentration at the membrane to rise sufficiently to activate the  $\text{Cl}^-$  conductance. Although we do not know the  $\text{Ca}^{2+}$  dependence of current activation, a rough calculation indicates that the separation between the  $\text{Ca}^{2+}$  release sites and the plasma membrane may be about 1  $\mu\text{m}$ . For example, in Fig. 9A the current was half-maximal about 100 ms after the rise in  $\text{Ca}^{2+}$ , and  $\text{Ca}^{2+}$  ions are expected to diffuse a mean distance in one dimension of 1.1  $\mu\text{m}$  during this time. This relatively close spacing is consistent with immunohistological localization of  $\text{InsP}_3$  receptors in the oocyte, which are concentrated within a narrow band extending about 5  $\mu\text{m}$  inward from the plasma membrane (N. P. Callamares, unpublished observations). Recordings in hepatocytes (Ogden, Capiod & Carter, 1991) also showed that current responses evoked by photoreleased  $\text{InsP}_3$  began about 75 ms after  $\text{Ca}^{2+}$  liberation, a result that was similarly interpreted as resulting from buffered diffusion of  $\text{Ca}^{2+}$  ions.

Although the  $\text{Ca}^{2+}$ -activated membrane current lags behind the  $\text{Ca}^{2+}$  signal on its rising phase, the opposite is true during the decline, when the current returns quickly to the baseline even though intracellular  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$ , but fails to respond to sustained elevations of  $\text{Ca}^{2+}$ . A further example of this phenomenon is shown in Fig. 12B, where photorelease of  $\text{InsP}_3$  throughout a 30 s exposure generated spikes of  $\text{Ca}^{2+}$  superimposed on a maintained plateau. The  $\text{Ca}^{2+}$  spikes were accompanied by spikes in the current record, but the sustained plateau of  $\text{Ca}^{2+}$  failed to evoke any corresponding current.

One explanation for these discrepancies may be that the current is generated in response to transient elevations of  $\text{Ca}^{2+}$  near the inner surface of the plasma membrane, whereas the fluorescent recordings of  $\text{Ca}^{2+}$  signal more gradual changes in  $\text{Ca}^{2+}$  occurring deeper in the cell. If this is the case, our failure to detect differences in the durations of  $\text{Ca}^{2+}$  signals recorded by a small confocal spot focused close to the membrane or deeper into the cell (Fig. 10) suggests that any such transient  $\text{Ca}^{2+}$  changes must be highly localized to within less than 1 or 2  $\mu\text{m}$  of the inner surface of the cell membrane. Another possibility is that the  $\text{Cl}^-$  channels inactivate rapidly, so that they open in response to a rapid rise in intracellular free  $\text{Ca}^{2+}$  but subsequently close within a few hundred milliseconds, even though the  $\text{Ca}^{2+}$  remains elevated. Against this, we found that currents evoked by  $\text{Ca}^{2+}$  injections were not reduced when  $\text{Ca}^{2+}$  was injected into oocytes following photorelease of  $\text{InsP}_3$ , at a time when the  $\text{Ca}^{2+}$  signal evoked by  $\text{InsP}_3$  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  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  conductance (Boton, Gillo, Dascal & Lass, 1989; Boton, Singer & Dascal, 1990), which are differentially activated by  $\text{Ca}^{2+}$  liberated from intracellular stores and by microinjected  $\text{Ca}^{2+}$ . A full clarification of the role of inactivation of the  $\text{Cl}^-$  conductance awaits recordings from inside-out membrane patches exposed to rapid changes in free  $\text{Ca}^{2+}$  level.

Whatever the mechanism, the properties of the  $\text{Cl}^-$  conductance allow it to act as a high-pass filter, so that spikes or oscillations of  $\text{Ca}^{2+}$  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.

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