Fast Kinetics of Calcium Liberation Induced in *Xenopus* Oocytes by Photoreleased Inositol Trisphosphate

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ABSTRACT Inositol 1,4,5-trisphosphate ($InsP_3$) acts on intracellular receptors to cause liberation of Ca^{2+} ions into the cytosol as repetitive spikes and propagating waves. We studied the processes underlying this regenerative release of Ca^{2+} by monitoring with high resolution the kinetics of Ca^{2+} flux evoked in *Xenopus* oocytes by flash photolysis of caged $InsP_3$. Confocal microfluorimetry was used to monitor intracellular free [Ca^{2+}] from femtoliter volumes within the cell, and the underlying Ca^{2+} flux was then derived from the rate of increase of the fluorescence signals. A threshold amount of $InsP_3$ had to be photoreleased to evoke any appreciable Ca^{2+} signal, and the amount of liberated Ca^{2+} then increased only approximately fourfold with maximal stimulation, whereas the peak rate of increase of Ca^{2+} varied over a range of nearly 20-fold, reaching a maximum of ~150 μMs^{-1}. Ca^{2+} flux increased as a first-order function of [$InsP_3$], indicating a lack of cooperativity in channel opening, and was half-maximal with stimuli approximately 10 times threshold. After a brief photolysis flash, Ca^{2+} efflux began after a quiescent latent period that shortened from several hundred milliseconds with near-threshold stimuli to 25 ms with maximal flashes. This delay could not be explained by an initial "foot" of Ca^{2+} increasing toward a threshold at which regenerative release was triggered, and the onset of release seemed too abrupt to be accounted for by multiple sequential steps involved in channel opening. Ca^{2+} efflux increased to a maximum after the latent period in a time that reduced from >100 ms to ~8 ms with increasing [$InsP_3$] and subsequently declined along a two-exponential time course: a rapid fall with a time constant shortening from >100 ms to ~25 ms with increasing [$InsP_3$], followed by a much smaller tail persisting for several seconds. The results are discussed in terms of a model in which $InsP_3$ receptors must undergo a slow transition after binding $InsP_3$ before they can be activated by cytosolic Ca^{2+} acting as a co-agonist. Positive feedback by liberated Ca^{2+} ions then leads to a rapid increase in efflux to a maximal rate set by the proportion of receptors binding $InsP_3$. Subsequently, Ca^{2+} efflux terminates because of a slower inhibitory action of cytosolic Ca^{2+} on gating of $InsP_3$ receptor-channels.

INTRODUCTION

The second messenger inositol 1,4,5-trisphosphate ($InsP_3$) functions primarily by causing liberation of calcium ions from intracellular stores into the cytosol (Berridge, 1993). This process shows complex properties, which allow cells to generate transient calcium spikes that may either remain localized (Parker and Yao, 1991; Yao et al., 1995) or propagate as calcium waves (Lechleiter et al., 1991; Meyer, 1991; Parker and Yao, 1991). To fully understand calcium spikes and waves, we need detailed knowledge of the kinetics of $InsP_3$-induced calcium liberation, and this information is also likely to provide clues about the mechanisms controlling gating of $InsP_3$-activated calcium release channels. However, calcium liberation is rapid (milliseconds or tens of milliseconds) (Meyer et al., 1990; Finch et al., 1991; Parker and Ivorra, 1993), so it is difficult to evoke and measure calcium release with adequate temporal resolution in intact cells. Furthermore, to obtain information about gating of the calcium channels, it is necessary to measure initial rates of calcium mobilization rather than the total extent of calcium mobilization (Taylor, 1992).

One approach has been to use rapid mixing techniques to measure $InsP_3$-evoked calcium liberation in populations of permeabilized cells (Meyer and Stryer, 1988; Meyer et al., 1990; Champeil et al., 1989) and microsomal vesicles (Finch et al., 1991). This method allows resolution of calcium kinetics on the order of 10 ms, but it has limitations. Functioning of the $InsP_3$ receptors may be disturbed by cell permeabilization or preparation of microsomes, a time delay may be introduced by diffusional exchange within permeabilized cells (Meyer et al., 1990), and measurements are pooled from many cells or microsomes that may not act homogeneously or in synchrony. Another technique is to record ionic currents flowing through single $InsP_3$ receptor channels in excised patches or reconstituted into lipid bilayers. This provides superb time resolution of channel gating together with detailed information of the molecular functioning of the channel. However, although studies of channel gating have been made under steady-state concentrations of $InsP_3$ and calcium (Bezprozvanny et al., 1991;

Received for publication 27 February 1995 and in final form 16 October 1995.

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0006-3495/96/01/222/16 $2.00

Mayrleitner et al., 1991; Callamaras and Parker, 1994; Stehno-Bittel et al., 1995), no detailed kinetic studies have appeared. Also, the channels are far removed from their normal intracellular environment, and feedback processes that contribute to the release kinetics under physiological conditions are likely to be circumvented.

We describe here a study of the fast kinetics of calcium liberation in intact *Xenopus* oocytes, with minimal disruption of normal functioning. The method involved further development of a confocal microfluorimeter (Parker and Ivorra, 1993) to record cytosolic calcium transients evoked by photorelease of $InsP_3$. Flash photolysis of caged $InsP_3$ (Walker et al., 1987; McCray and Trentham, 1989; Parker, 1992) provided a convenient means to evoke reproducible and rapid elevations of intracellular $InsP_3$. The photolysis light was focused as a spot of ~20-μm diameter so that only a small subcellular area of the oocyte was stimulated (~300 μm^2, as compared to the roughly 1-mm^2 surface area of the whole cell), and the confocal spot recorded a representative measure of cytosolic free calcium from a virtual point source centered within the stimulated area. Because liberation of calcium into the cytosol is much faster than its subsequent removal, the rising phase of the fluorescence signal corresponds approximately to the cumulative amount of calcium released. A more useful measure is the rate of calcium liberation, which gives information about the gating of calcium release channels. This can be obtained from the first derivative of the fluorescence signal. Very 'clean' records are necessary, however, because differentiation greatly accentuates any noise in the fluorescence signals. Records obtained with our original "home-made" confocal microfluorimeter (Parker and Ivorra, 1993) were too noisy to permit this analysis, but the availability of a commercial confocal microscope made this approach possible.

MATERIALS AND METHODS

Calcium green indicators were obtained from Molecular Probes Inc. (Eugene, OR), and caged $InsP_3$ was from Calbiochem (La Jolla, CA). All other reagents were from Sigma Chemical Co., (St. Louis, MO).

Preparation of oocytes

Experiments were done on ovarian oocytes (Dumont stage V and VI) of albino *Xenopus laevis* obtained by surgical removal from donor frogs anesthetized by placing them in a 0.17% aqueous solution of MS-222 (3-aminobenzoic acid ethyl ester) for 15 min. The frogs were allowed to recover after surgery. Isolated oocytes were treated with collagenase to remove enveloping cells, and optical recordings were made while continuously superfusing oocytes with normal frog Ringer's solution (composition in mM: NaCl 120; KCl 2; $CaCl_2$ 1.8; HEPES 5; at pH ~7.0). Unless noted otherwise, all experiments were carried out at room temperature (21–25°C).

Oocytes were injected before recording with ~20 nl of an aqueous solution containing 5 mM calcium green-5N or 2.5 mM calcium green-2 (Yao and Parker, 1994) together with varying concentrations (0.5–5 mM) of caged $InsP_3$ (*myo*-inositol, 1,4,5-trisphosphate, $P^{4,(5)}$-1-(2-nitrophenyl-ethyl ester)) (McCray and Trentham, 1989). At least 30 min was allowed after injection for the caged $InsP_3$ and indicator dye to diffuse throughout the oocyte, and when high concentrations of caged $InsP_3$ were used, it was necessary to wait as long as 2 hr for contaminating $InsP_3$ to be metabolized by the cell (Parker, 1992). Because the cytosolic volume of the oocyte is ~1 μl, the resulting final intracellular concentrations would have been approximately 50 times lower than in the injection solutions. The amount of caged $InsP_3$ consumed by each flash was a negligible proportion (<1%) of the total loaded into the oocyte, even at low concentrations, allowing many reproducible responses to be evoked without run-down.

Optical recording and stimulation

Recordings of calcium transients evoked by photorelease of $InsP_3$ were made from a virtual point source within the oocyte by using confocal microfluorimetry. The principle of this method was the same as previously described (Parker and Ivorra, 1990a, 1993), but replacement of our earlier home-made confocal microscope by a commercial instrument provided a considerable improvement in signal-to-noise ratio. Oocytes were placed in a recording chamber on the stage of an Olympus IMT2 inverted microscope and viewed through a coverslip forming the base of the chamber by a 40× oil immersion objective (Nikon Neofluar, N. A. 1.3). The inverted microscope was interfaced through the phototube on a trinocular head to a Noran Odyssey laser confocal microscope (Noran Instruments, Middleton, WI). This was operated in a nonscanning mode so that the beam produced by a 100-mW argon-ion laser (488 nm) formed a stationary diffraction-limited spot that was focused ~5 μm deep into the cell, where $InsP_3$-evoked calcium signals are maximal (Yao et al., 1995). Calcium-dependent changes in fluorescence of the indicator dye were then monitored at wavelengths longer than 510 nm by a photomultiplier placed behind a confocal aperture. Most recordings were obtained by using a confocal aperture of 100 μm, which gave an axial resolution of ~1.1 μm at half width. Thus, fluorescence signals provided a measure of calcium levels within an ellipsoid measuring ~0.5 μm laterally and ~1 μm axially. This corresponds to a volume of <1 fl, one-billionth the total volume of the cell. After low-pass filtering at 200 Hz–1 kHz, fluorescence signals were recorded on floppy disc by a digital oscilloscope (12-bit resolution, 4k record length) for subsequent computer analysis.

To permit differentiation of the calcium signals, we required fluorescence recordings with the best possible signal-to-noise ratio. Two factors were primarily responsible for determining the noise level. First, the laser showed fluctuations in power output, which could be minimized by setting the feedback circuit controlling the laser power to the middle of its range and then using the accousto-optical device in the Odyssey scanner as a variable attenuator to set the desired light intensity at the specimen. The second major noise source was shot noise resulting from statistical fluctuations in photons detected by the photomultiplier. The signal-to-noise ratio was improved by increasing the laser intensity at the specimen, but continued exposure to high intensities caused local photo damage to the oocyte, which was evident as a run-down in evoked calcium signals. We therefore worked with the laser at about half power and the Odyssey power control set to ~30%. Furthermore, the laser beam was blanked during intervals between recordings. Bleaching of dye was not a significant problem because diffusional exchange of fresh dye from outside the illuminated area rapidly replenished dye bleached in the minute confocal spot (Parker and Ivorra, 1993).

Calcium green-5N was used as the calcium indicator in most experiments because its low affinity (12 μM) (Yao and Parker, 1994) reduced interference with normal calcium homeostasis and provided a fluorescence signal almost linearly proportional to free calcium over the range of concentrations found in the oocyte. As is the case with all other currently available long wavelength indicators, however, the lack of any spectral shifts on binding calcium precluded ratio calibration of signals in terms of absolute calcium concentrations. Therefore, fluorescence signals are presented as fractional changes above the resting baseline ($\Delta F/F$). A rough calibration was made by determining in vivo the maximal fluorescence change on going from zero calcium (F_{min}) to saturating calcium (F_{max}).

F_{max} was estimated by ionophoretic injection of saturating amounts of calcium through a micropipette inserted into the oocyte near the confocal spot (Ilyin and Parker, 1994). F_{min} was taken as the resting fluorescence level because the resting free calcium concentration in the oocyte (~40 nM) (Yao and Parker, 1994) is negligible compared with the dissociation constant of the dye. The mean ratio F_{max}/F_{min} was 5.3 ± 0.17 (five trials). Assuming a K_d for calcium green-5N of 12 μM (Yao and Parker, 1994), a fluorescence change of $\Delta F/F = 1$ corresponds to an increase in free calcium concentration of ~3.8 μM, and calcium concentrations would be nearly linearly proportional to fluorescence signals for values smaller than this. In some experiments calcium green-2 was used instead as the indicator. This dye has a higher affinity for calcium (K_d ~830 nM) and thus provided better resolution of small calcium increments above the resting level, at the expense of signals that approached saturation at the peak of large $InsP_3$-evoked responses.

UV light for photolysis of caged $InsP_3$ loaded into the oocyte was derived from a 100-W mercury arc epifluorescence unit fitted to the inverted microscope. An electronically controlled shutter allowed flashes of various durations to be delivered, and the light intensity was adjusted by a variable neutral density wedge. Wavelengths shorter than ~400 nm were selected by a barrier filter and dichroic mirror in a standard Olympus UV filter cube. A pinhole was used as a field stop so that light was focused on the oocyte as a spot of ~20 μm diameter, which was adjusted to be concentric around the confocal spot. In experiments in which repeated responses were recorded from the same point on the oocyte, intervals of 2–3 min were allowed between stimuli. However, in some cases the recording area was shifted between each trial by moving the microscope stage, and no rest period was then required because each stimulus was applied to a fresh region of the cell.

Expression of nicotinic acetylcholine receptors (AChRs) to allow control of calcium influx

In experiments in which the basal cytosolic free calcium level was manipulated (see Fig. 10), oocytes were injected a few days before recording with mRNAs coding for all four subunits of a mutant *Torpedo* nicotinic AChR, which displays a relatively high calcium permeability and slow desensitization (mRNAs kindly provided by Dr. K. Sumikawa, Department of Psychobiology, University of California Irvine). During recording, 1 μM ACh plus 0.1 μM atropine was added to the bathing solution, and the basal cytosolic calcium level was altered by clamping the oocyte to different potentials to vary the electrical driving force for calcium entry through the nicotinic channels.

RESULTS

Derivation of calcium flux

Fig. 1 illustrates the procedure used to derive the kinetics of calcium liberation from confocal records of cytosolic cal-

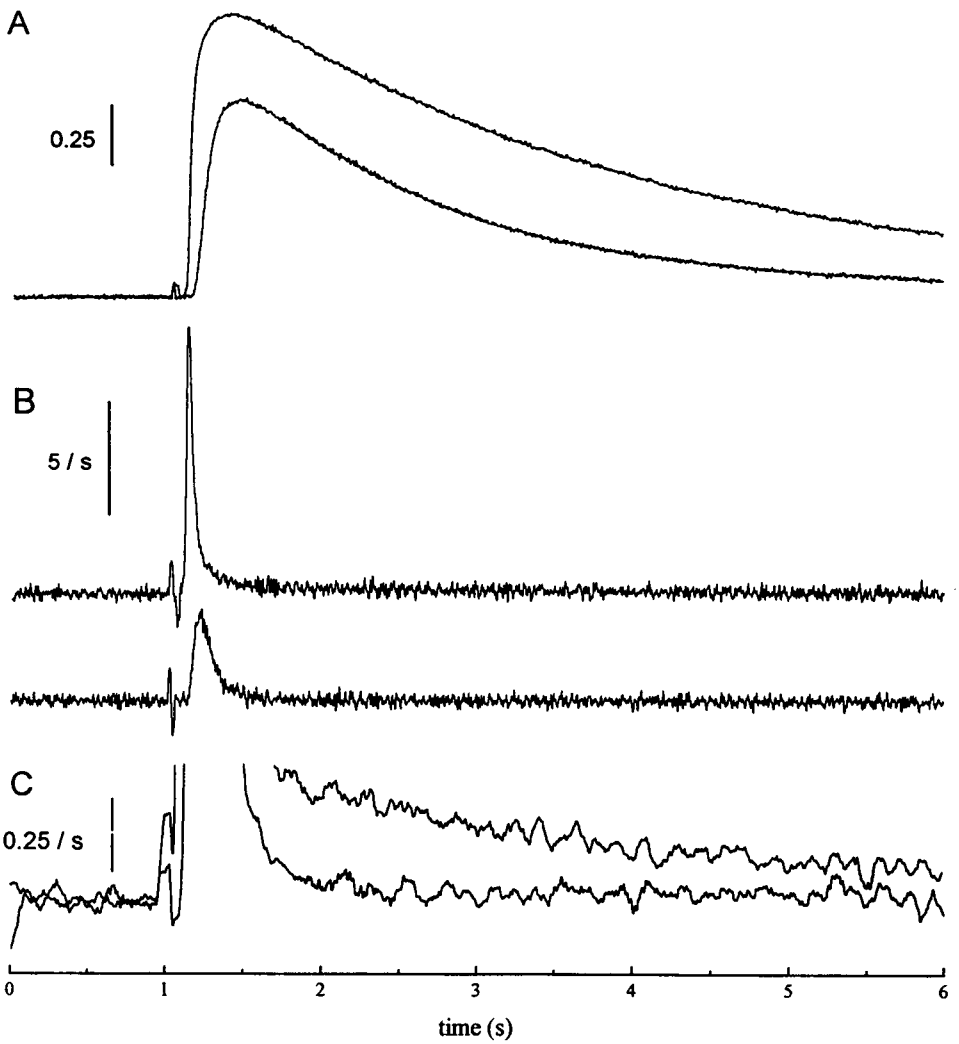

FIGURE 1 Derivation of rate of calcium liberation from confocal fluorescence records of calcium transients evoked by photorelease of $InsP_3$. (*A*) superimposed traces show fluorescence signals evoked by photolysis flashes with durations of 15 (smaller response) and 60 ms. The oocyte was loaded with 15 pmol caged $InsP_3$ together with 50 pmol calcium green-5N, and the timing of the photolysis flashes can be seen from the stimulus artifacts just preceding the responses. Calibration bar corresponds to a fractional change in fluorescence ($\Delta F/F$) of 0.25 above the resting fluorescence. A single exponential curve with a time constant of 720 ms is fitted to the decay of the response to the 15-ms flash. (*B*) Rate of calcium liberation derived from the records in *A*, as described in the text. Traces were smoothed by a 10-ms running window to reduce noise. Calibration bar corresponds to rate of change of fluorescence ($d(\Delta F/F)/dt$) of 5 s^{-1}. The biphasic "glitch" preceding each response results from differentiation of the stimulus artifact. (*C*) The same records as in *B* but shown superimposed on an expanded vertical scale and after additional smoothing to better illustrate the apparent tail of calcium liberation that persisted for several seconds after the 60-ms flash. Calibration bar corresponds to $d(\Delta F/F)/dt = 0.25$ s^{-1}.

cium transients evoked by photorelease of $InsP_3$. The traces in Fig. 1 *A* show superimposed fluorescence signals evoked by a just-suprathreshold photolysis flash (15-ms duration) and by a stronger flash (60-ms duration). Despite the four-fold increase in stimulus strength, the peak amplitude of the response to the 60-ms flash was only slightly (40%) greater than to the 15-ms flash (cf. Parker and Ivorra, 1993), but began with shorter latency, showed a faster rate of increase, and decayed more slowly. Assuming that equilibration with calcium buffers in the cell is rapid (submillisecond) (Allbritton and Meyer, 1993), the cytosolic calcium signals should reflect a balance between the rate at which calcium ions were liberated into the cytosol and their rate of removal by resequestration, extrusion across the plasma membrane, and diffusion away from the stimulated area (20-μm spot) of the cell. Although these latter processes are complex, the decay of calcium signals evoked by just-suprathreshold flashes was fitted well by single exponential curves (e.g., lower trace in Fig. 1 *A*), indicating that calcium removal could be adequately described as a first-order process. Thus, the rate of calcium efflux (E) from intracellular stores is proportional to

$$E \sim d[Ca^{2+}]/dt + k[Ca^{2+}] \quad (1)$$

where $[Ca^{2+}]$ is the free calcium level as signaled by the fluorescence and k is the rate constant for calcium removal, determined from the exponential fit to the decay of the calcium signal.

The traces in Fig. 1 *B* show the time course of calcium efflux derived in this way from the records in Fig. 1 *A*. Several features are evident that are described in more detail in subsequent sections. In brief, calcium efflux did not begin until after a latency of ~100 ms after the shorter flash, and this latency reduced after the stronger flash. The rate of calcium efflux then increased rapidly to a peak, the amplitude of which was graded with stimulus strength, and subsequently declined over several tens of milliseconds, decreasing more rapidly after the stronger stimulus.

A final point, which is illustrated more clearly in Fig. 1 *C*, is that calcium efflux seemed to persist at a low rate for several seconds after the stronger stimulus, although this was not evident after the brief flash. Although the magnitude of this tail component was small, its long duration contributed appreciably to the total amount of calcium liberated by the 60-ms flash, resulting in a slower decline of the calcium transient as compared with the response to the 15-ms flash. An alternative explanation for the slower decay of the signal after the stronger stimulus, however, is that removal of calcium from the cytosol was slower, perhaps because calcium pumps became saturated by the higher peak free calcium level. We consider this interpretation unlikely. First, the peak calcium level after the 60-ms flash was only ~40% greater than with the 15-ms flash. Second, it was already clear that relatively high concentrations of a nonmetabolizable $InsP_3$ analog can evoke a sustained liberation of calcium that persists for several minutes (Yao and Parker, 1994). Whatever the case, the assumption that calcium removal from the cytosol can be modeled as a first-order process affects only the slow tail component of calcium release to any significant extent. Our results largely concern the fast kinetics of release during the rising phase of the calcium transient, and for this purpose it made little difference whether any correction was made for calcium removal or whether calcium efflux was derived simply by differentiating the fluorescence records.

Dose-dependence of calcium liberation

Fig. 2 shows typical records of calcium transients evoked by light flashes of various durations (*upper*) together with traces in which the rate of calcium efflux from $InsP_3$-sensitive stores was derived using the procedure described above. Under the conditions used, the amount of $InsP_3$ produced by each flash would have been about linearly proportional to flash duration (Parker and Ivorra, 1992), although the absolute concentrations of $InsP_3$ in the cell were uncalibrated.

As we described previously (Parker and Ivorra, 1990a, 1993), $InsP_3$-evoked responses in the oocyte showed an abrupt threshold. For example, in Fig. 2, a flash of 8-ms duration evoked virtually no response, whereas a small increase to 10 ms gave an appreciable signal that then grew by only 80% as the flash duration was further lengthened to 50 ms. In contrast to the nearly all-or-none behavior of the peak amplitude of the fluorescence signal (which corresponds roughly to the total amount of calcium liberated), the kinetics and maximal rate of calcium liberation changed more progressively with increasing levels of $InsP_3$. These effects are illustrated in Fig. 2 and are described in detail in the following sections.

Calcium flux as a function of $InsP_3$ concentration

Fig. 3 shows measurements of peak fluorescence signal (*A*) and maximal rate of increase of fluorescence (*B*) obtained from a single oocyte, which was heavily loaded with caged $InsP_3$ (100 μM final intracellular concentration), so that the maximum available light energy from the arc lamp was able to produce high intracellular concentrations of $InsP_3$ during even a brief flash. To explore a wide range of concentrations, the light intensity was then varied by neutral density filters while the flash duration remained fixed at 40 ms.

Just-suprathreshold flashes (intensity 0.8% of maximal) evoked calcium green-5N fluorescence signals with a mean amplitude of ~0.55 $\Delta F/F$, corresponding to ~1.8 μM free calcium. The fluorescence signal then increased steeply as the flash intensity was increased further, and at intensities greater than approximately 10 times threshold it reached a maximal value of 1.7 $\Delta F/F$, corresponding to 8.1 μM free

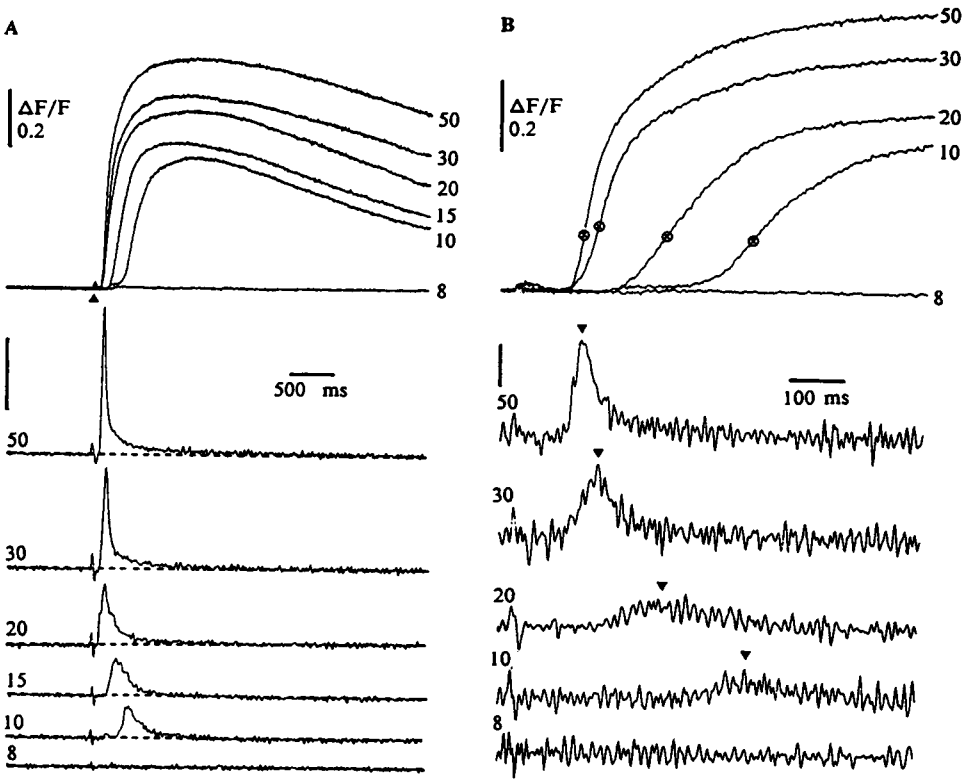

FIGURE 2 Calcium transients and kinetics of calcium efflux in response to photorelease of varying amounts of $InsP_3$. (*A*) The upper panel shows superimposed records of fluorescence signals evoked by light flashes of varying duration (indicated in milliseconds to the *right of each trace*). The arrowhead marks the time of the flashes. Individual traces show the kinetics of calcium efflux evoked by each flash, derived by using the same procedure as in Fig. 1. (*B*) Selected records from *A*, shown on an expanded time scale. Traces of calcium efflux were smoothed less than in *A*, so as to better resolve the fast kinetics of calcium liberation. Arrowheads and corresponding symbols in the upper panel indicate times at which the rate of calcium liberation was maximal during each response. Calibration bars in the upper panels of *A* and *B* correspond to $\Delta F/F = 0.2$; bars in the lower panels correspond to $d(\Delta F/F)/dt = 5$.

calcium or approximately four times larger than the threshold response. The data were fit well by a hyperbolic relationship (Fig. 3 *A*), and half-maximal calcium liberation was achieved with a stimulus of about twice the threshold intensity.

In contrast to the limited variation in amount of calcium liberated by differing suprathreshold concentrations of $InsP_3$, the maximal rate of liberation varied over a greater range and as a more shallow function of $InsP_3$ levels (Fig. 3 *B*). Just-suprathreshold stimuli evoked fluorescence signals with a maximal rate of increase of ~4 $\Delta F/F\ s^{-1}$, and this rate then continued to increase progressively to a value approximately 10 times greater as the stimulus intensity was increased to more than 100 times the threshold. Because maximal calcium efflux occurred when the cytosolic calcium had increased to about the same level during each response (Fig. 2 *B*), the concentration gradient driving the movement of calcium ions was presumably similar at that time for all stimuli. Measurements of maximal efflux rate are expected, therefore, to provide a relative indication of the number of open calcium channels.

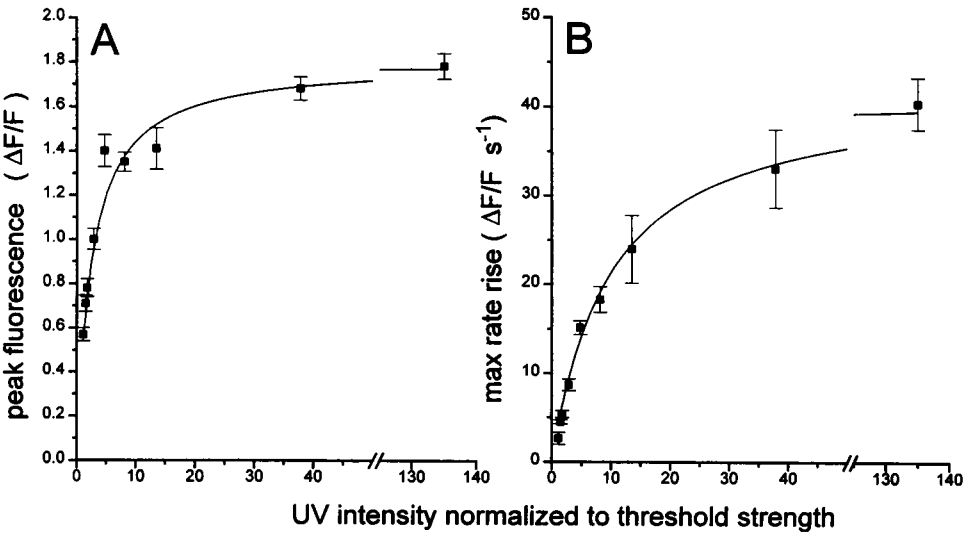

FIGURE 3 Dependence of peak size (*A*) and maximal rate of increase (*B*) of calcium signal on intensity of stimulus flash. Data are from a single oocyte loaded with calcium green-5N and caged $InsP_3$. Intensity is expressed relative to the threshold intensity required to evoke calcium release. Points represent mean ± SE of four replicates at each intensity, and each measurement was obtained from a different spot within the animal hemisphere of the oocyte. Curves were fitted as described in the text.

We did not find evidence for any cooperativity of $InsP_3$ in activating calcium liberation for stimuli above threshold. The curve in Fig. 3 *B* was drawn according to the hyperbolic relationship,

$$dF/dt = (dF/dt)_{max} \times I/(I + I_{50}) \quad (2)$$

where dF/dt is the steepest rate of rise of fluorescence during a calcium transient, $(dF/dt)_{max}$ is the maximal rate of fluorescence increase at saturating levels of $InsP_3$, I is the photolysis flash intensity, and I_{50} is the intensity giving half-maximal rate of release. A good fit to the data in Fig. 3 *B* was obtained with I_{50} equal to 7.5 times the threshold stimulus. We had estimated previously the threshold concentration of $InsP_3$ in the oocyte to be ~60 nM (Parker and Ivorra, 1992), so that I_{50} would then correspond to a concentration of ~450 nM $InsP_3$. The maximal rate of fluorescence increase approached a saturating value with strong stimuli of ~40 $\Delta F/F$ s^{-1}, corresponding to a rate of increase of free calcium concentration of ~120 μM s^{-1}. Similar data are shown in Fig. 4 *B*, pooling measurements in eight oocytes using calcium green-2 as the indicator. The data were again fit well by the relationship of Eq. 2, and in this case I_{50} was 9.9 times threshold (~600 nM $InsP_3$).

A possible concern was whether the saturation of rate of calcium release might have arisen because strong stimuli photolyzed all the available caged $InsP_3$, rather than through saturation of the calcium release process itself. This was not the case, because oocytes that were loaded with small amounts of caged $InsP_3$ failed to show saturation even with maximal UV stimuli, whereas other oocytes loaded with up to 100 times more caged $InsP_3$ gave saturating responses with much weaker flashes.

For stimulus intensities up to about six times threshold, the peak rate of calcium efflux approximated a linear function of flash duration (Fig. 4 *A*), further confirming the lack of cooperativity in $InsP_3$ action. An interesting point is that a regression line fitted to measurements with suprathreshold stimuli intercepted the abscissa close to the origin and not at the threshold flash duration. Thus, the

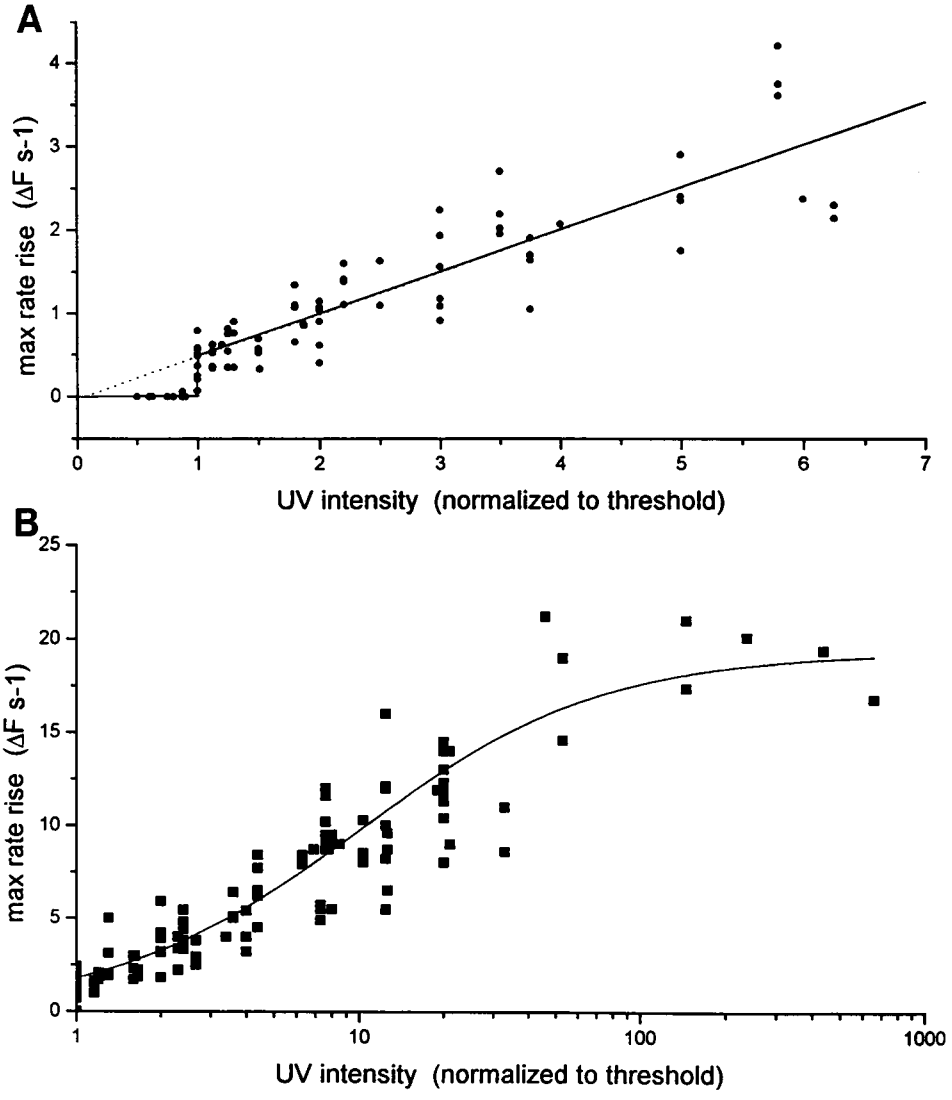

FIGURE 4 Calcium flux increases as the first order of [InsP3] for suprathreshold stimuli. (*A*) Pooled data from five oocytes showing maximum rate of increase of the fluorescence signal (expressed as intensity units s^{-1}) as a function of photolysis flash intensity. Oocytes were loaded with calcium green-5N, and the mean resting fluorescence level (F) was 0.285 + 0.084 (SD). Flash intensities are normalized to the threshold in each oocyte. A regression line is shown fitted to all data points except those below threshold. (*B*) Variation of maximal rate of increase of fluorescence over a wide range of photolysis intensities. Data are shown from seven oocytes loaded with calcium green-2, in which the mean resting fluorescence (F) was 0.158 + 0.062 (SD). Flash intensities are normalized to the threshold in each oocyte and are plotted on a logarithmic scale to better display the wide range of stimuli explored. The curve was drawn according to Eq. 2, with I_{50} = 9.9 times threshold.

maximal rate of calcium release did not increase progressively from zero as stimuli were increased above threshold, but rather the rate of release jumped abruptly from zero to a certain minimal value as the flash duration was increased above the threshold.

Kinetics of activation and inactivation of calcium flux

As is evident in Fig. 2, the duration of calcium efflux became shorter as increasing amounts of $InsP_3$ were photoreleased, thus explaining why the total amount of calcium release grew little as the stimulus was increased beyond a certain point, even though the maximal rate of release continued to increase. To quantify these changes in kinetics we measured two parameters, as illustrated in Fig. 5 *A*. After a latent period following the flash (see below), the calcium flux increased rapidly to a peak value. Because noise in the recordings precluded a more detailed analysis, we simply measured this activation time as the interval between first detectable calcium flux and the time at which peak flux was attained. The rapid subsequent decline in calcium flux was fitted by a single exponential (neglecting the slower and much smaller component described earlier), and measurements were made of this inactivation time constant.

The mean activation time after just-suprathreshold stimuli was ~120 ms, and it shortened progressively with increasing extent of photorelease of $InsP_3$ to approach a minimal limiting value of ~8 ms (Fig. 5 *B*). Similarly, the inactivation time constant shortened progressively with increasing stimulus strength from ~125 ms after just-suprathreshold flashes to approach a limiting value of ~25 ms (Fig. 5 *C*).

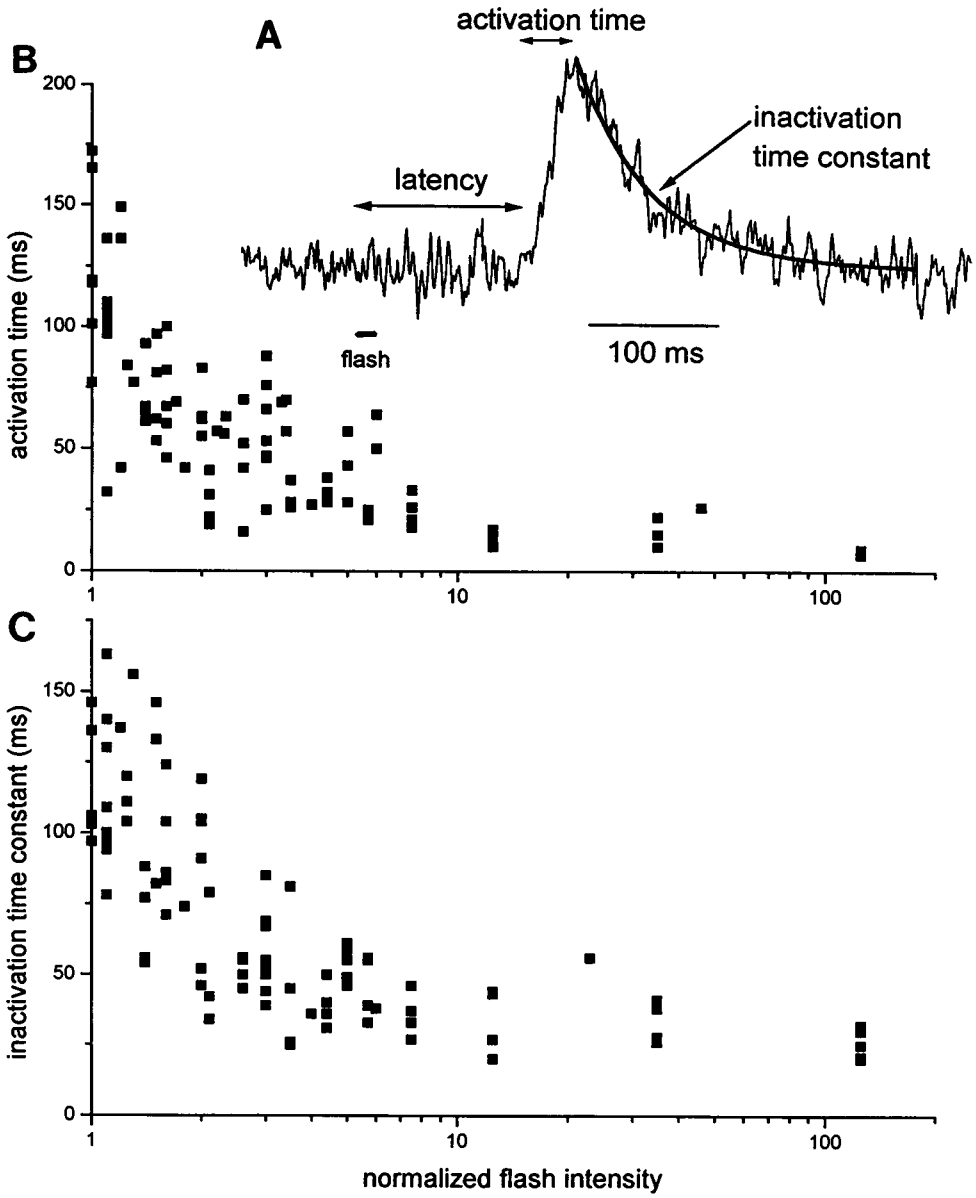

FIGURE 5 Kinetics of activation and inactivation of calcium flux after photorelease of $InsP_3$. (*A*) Inset shows typical calcium green-5N signal illustrating how measurements plotted in *B* and *C* were obtained. (*B*) Measurement of activation times and (*C*) measurement of inactivation time constants as functions of flash intensity normalized to threshold. Data are from four oocytes, all loaded with calcium green-5N.

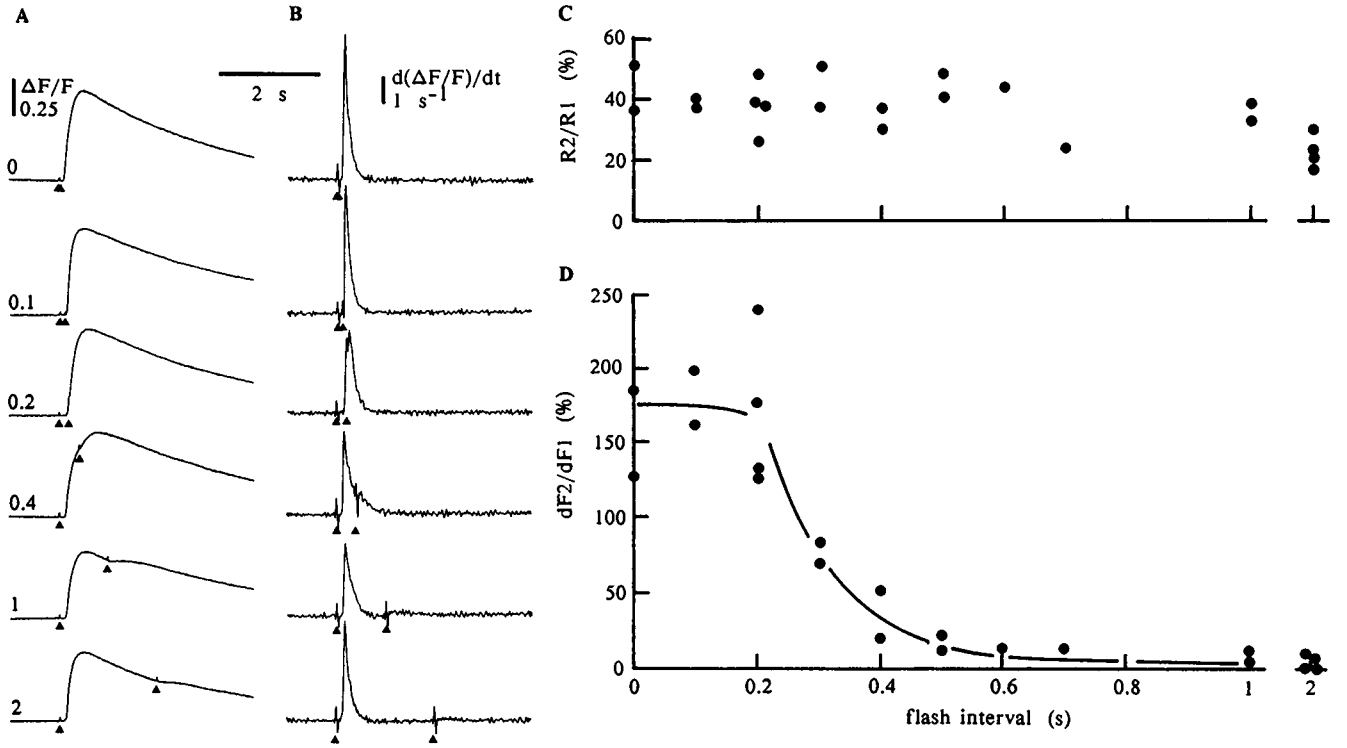

FIGURE 6 Inactivation of calcium flux monitored by paired photolysis flashes. Traces show calcium green-5N fluorescence signals (*A*) and derived calcium flux rates (*B*) in response to paired photolysis flashes (each ~120% threshold) delivered at intervals as indicated (in seconds). Graphs plot the incremental amount of calcium (*C*) and rate of calcium efflux (*D*) evoked by the second flash as a function of interflash interval. Responses to single flashes were recorded several times throughout the experiment, and responses to the second flash in each pair are expressed as a percentage of these control values. At short intervals, when separate responses could not be discerned to each flash, the contribution due to the second flash was estimated by subtracting single flash responses from the combined response. Data in *C* and *D* are from an oocyte different from that in *A* and *B*, and similar results were obtained in a total of three oocytes.

Inhibition of calcium release monitored by paired flashes

As an alternative approach to study this inhibitory process, we used a paired-flash protocol (Fig. 6). Two identical flashes were delivered at various intervals, with their duration set to be slightly (~20%) above threshold. As expected from the dose-response relationships in Fig. 3, two flashes at zero interval (i.e., equivalent to a single flash of twice the duration) gave a fluorescence signal with a peak size ~40% greater than that evoked by a single flash and with a maximum rate of increase about double that of the single response. Little change was then seen as the interval was increased, provided that the second flash occurred within the latent period after the first flash (~100 ms for the oocyte in Fig. 6, *A* and *B*, and ~200 ms for the oocyte in Fig. 6, *C* and *D*). However, once the interval was lengthened sufficiently so that the second flash was delivered after the calcium level had already begun to increase, the rate of calcium efflux evoked by the second flash abruptly declined to a low level. For example, the peak rate of calcium efflux evoked by paired flashes at a 200-ms interval in Fig. 6 *B* was not greater than that evoked by a single flash and at longer intervals, where the components due to each flash could be more clearly discerned, the efflux rate was less than one-tenth of that evoked by the first flash. In contrast to this marked slowing of the rate of calcium liberation (Fig. 6, *B* and *D*), the additional amount of calcium liberated by the second flash declined to a lesser extent as the interval was lengthened to 1 or 2 s (Fig. 6, *A* and *C*).

Recovery from inhibition

As the interval between paired flashes was progressively lengthened, the size of the fluorescence signal evoked by the second flash reduced to a minimum after ~2 s and thereafter recovered over several seconds (Fig. 7, *A* and *C*) (see Parker and Ivorra, 1990b; Ilyin and Parker, 1994). As with the onset of inhibition, however, the time course of recovery of the calcium efflux rate differed markedly from the recovery of the amount of calcium liberated by the second flash. The amount of calcium release (peak size of fluorescence signal) was half-recovered at an interval of ~3 s, whereas the corresponding time for recovery of the maximal rate of calcium efflux was ~5 s (Fig. 7).

Latency to onset of calcium liberation

Photolysis flashes were expected to produce a rapid increase of intracellular $InsP_3$ level, lagging behind the stimulus

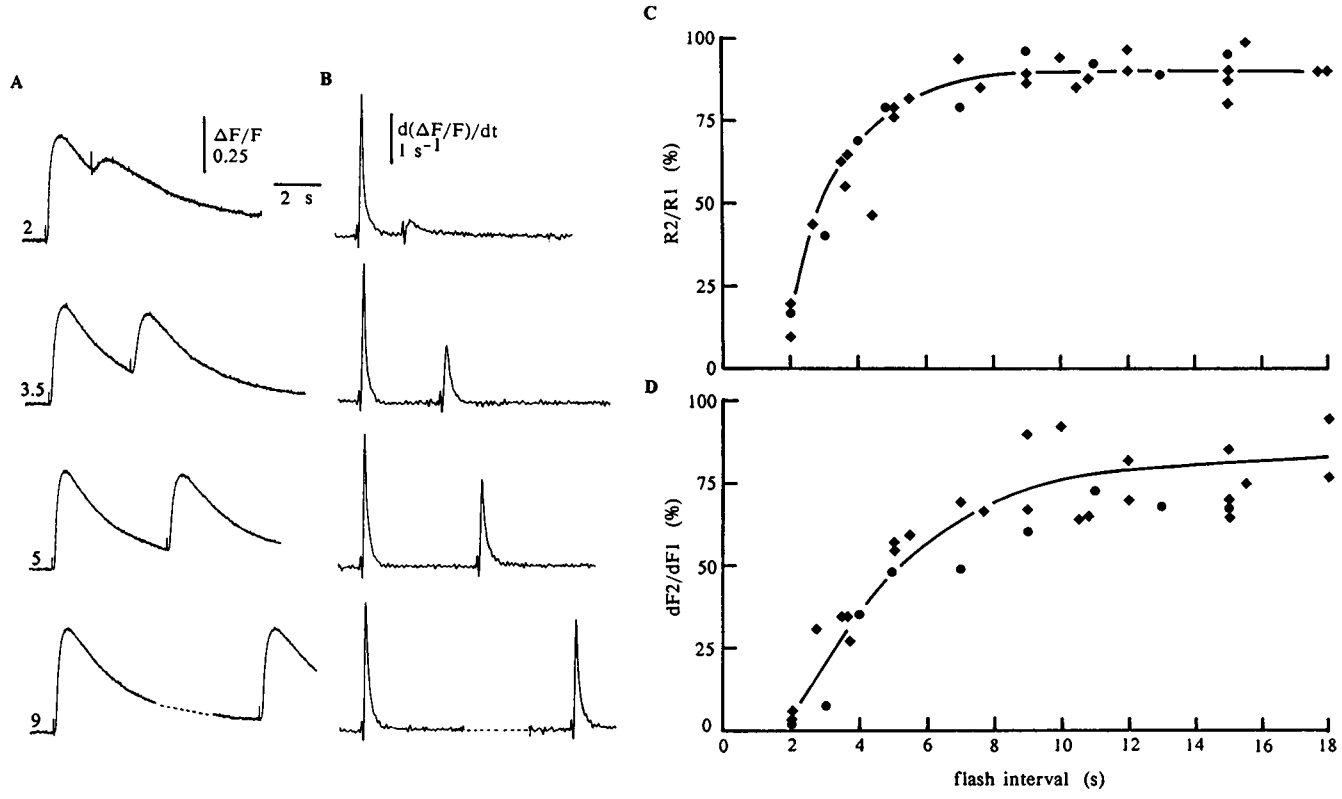

FIGURE 7 Recovery of calcium efflux rate is slower than recovery of the amount of calcium liberated by a test flash. Traces show calcium green-5N fluorescence signals (*A*) and derived calcium flux rates (*B*) in response to paired identical light flashes at various intervals longer than 2 s. The timing of stimulus flashes can be seen from the stimulus artifacts, and intervals are indicated (in seconds) next to each pair of traces. Graphs show recovery of peak size of the fluorescence signal (*C*) and maximal rate of rise of the signal (*D*) as a function of interflash interval. Data are from two oocytes (*different symbols*) and are expressed as percentages of the response to the first flash in each trial. Curves were drawn by eye.

flash by only ~1 ms due to the kinetics of the photolysis reaction (Walker et al., 1987). The calcium signals, however, abruptly began following a much longer latency of several tens or hundreds of milliseconds (Fig. 8 *A*). Because imaging experiments have revealed that $InsP_3$-evoked calcium liberation is not homogeneous but begins at particular "hot spots" spaced a few micrometers apart (Parker and Yao, 1991), we were concerned that the latency observed with confocal point recording might arise artifactually from the time taken for calcium release to propagate from the nearest hot spot to a randomly located recording site. To test this, calcium signals were imaged over an extensive region of the cell after uniform exposure to a photolysis flash. Calcium liberation began at multiple discrete hot spots (Parker and Yao, 1991), but the first of these spots did not appear until ~100 ms after the flash. Other spots began to appear rapidly thereafter, so that an increase in calcium was seen at virtually all areas within an additional 50 ms.

As an alternative approach, we used confocal spot recording to monitor responses evoked by identical test flashes at 43 randomly chosen locations within the animal hemisphere of an oocyte. Fig. 9 *A* shows examples of eight consecutive responses, and the cumulative distribution of latencies is plotted in Fig. 9 *B*. The measured latencies were clustered quite tightly around a mean value. For example, in Fig. 9 *B* the mean was 106 ± 16 ms (SD), and we failed to observe any responses beginning earlier than 79 ms after the flash. Similar results were obtained in two other oocytes.

Thus, although spatial inhomogeneities in calcium release introduce some variability in latencies recorded by randomly located confocal spots, there remains an appreciable minimal latency at even the most rapidly responding sites.

Effect of cytosolic calcium level on latency

One explanation proposed to account for the latency is that there is a pacemaker-like increase in calcium toward a threshold level, at which a positive feedback mechanism is triggered to evoke regenerative release (Parker and Yao, 1991; Iino et al., 1993). We failed previously to detect such a "foot" in calcium signals recorded by confocal microfluorometry in the oocyte (Parker and Ivorra, 1993), but the greatly improved resolution available with our new system made reexamination of this point worthwhile.

The record in Fig. 8 *A* was obtained using calcium green-2 as the indicator, because the higher affinity of this

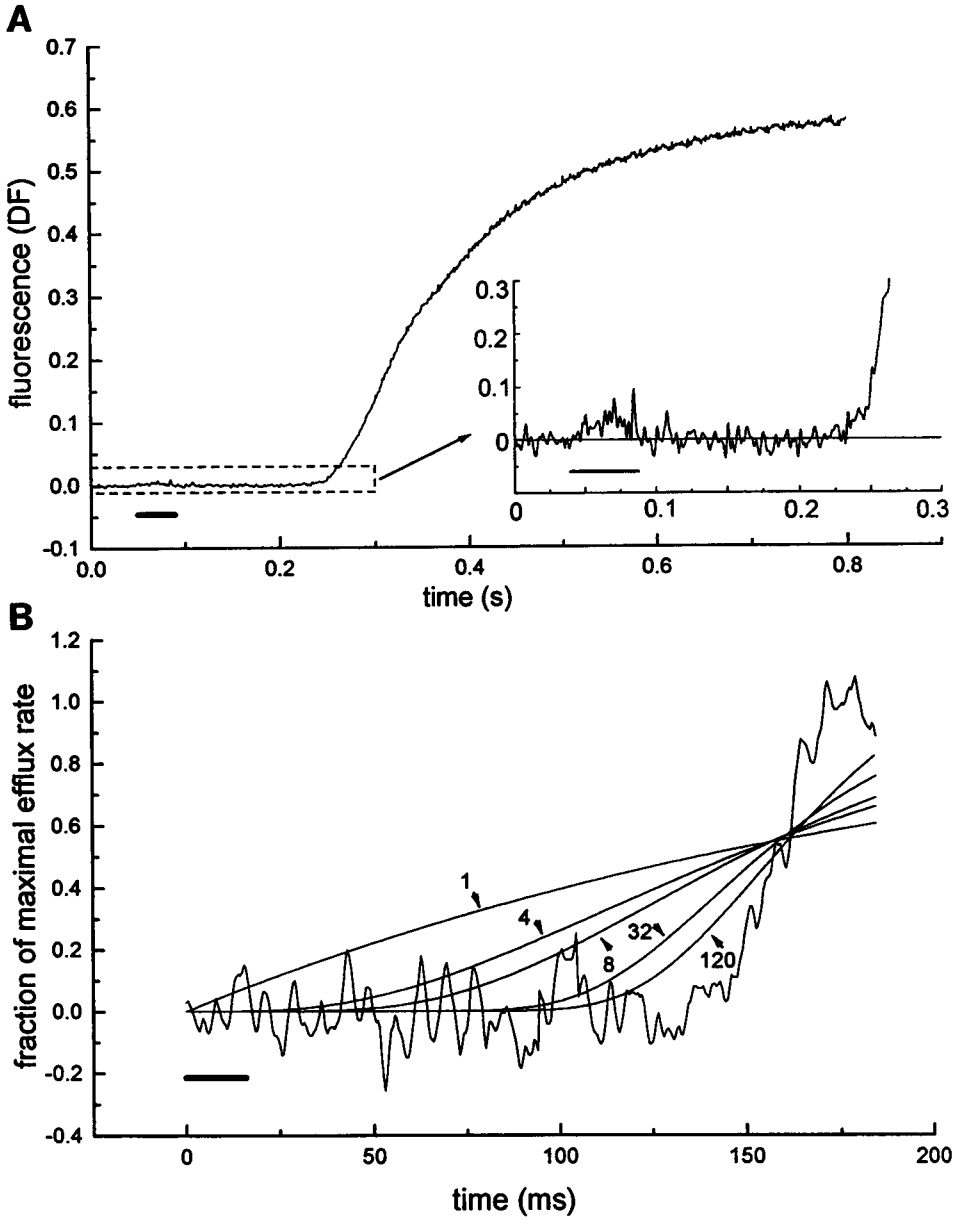

FIGURE 8 Abrupt onset of calcium liberation after a quiescent latent period. (*A*) Calcium green-2 fluorescence signal evoked by a photolysis flash delivered when marked by the bar. The inset shows the portion of the record within the dashed box on enlarged scales. (*B*) Experimental trace (*noisy record*) shows the onset of calcium efflux obtained by differentiation of a calcium green-2 fluorescence record. A photolysis flash was delivered when marked by the bar. Smooth curves were drawn according to the kinetic model described in the text with 1, 4, 8, 32, and 120 delay time constants. Records in *A* and *B* were from different oocytes.

dye as compared with calcium green-5N allowed better resolution of small calcium increments above the resting level. The fluorescence signal showed no detectable increase above the baseline for ~150 ms after the flash but then increased abruptly. Given the noise level in the recording, an increase in fluorescence of $\Delta F/F$ of ~0.01 should have been resolved clearly, corresponding to only 0.3% of the peak $InsP_3$-evoked response and an increase in free calcium concentration of 4 nM.

As a further test of whether the latency might arise through a buildup of cytosolic calcium that was too small for us to detect, we examined whether exogenous elevations of basal calcium level could shorten the latency. In Fig. 10 *A*, the cytosolic free calcium level was elevated by allowing influx of extracellular calcium through nicotinic AChR/channels expressed in the plasma membrane (see Materials and Methods). Relatively large elevations of Ca^{2+} were used to approximate the concentration producing maximal activity of oocyte $InsP_3$ receptor/channels (~1 μM) (Stehno-Bittel et al., 1995) and thus to accentuate any effect on the latency. With use of a photolysis flash ~50% greater than threshold, the latency shortened from 165 ms to 69 ms when free calcium was raised by ~1.1 μM ($\Delta F/F$ 0.34) above the basal level for several seconds before a test flash. Similar results were obtained in a total of 10 trials (six oocytes), using comparable flash intensities and covering a range of calcium elevations of 0.6–2.2 μM ($\Delta F/F$ 0.18–0.65) above the basal level. The mean control latency was 131 ± 12 ms, and this shortened to 74 ± 4 ms after calcium elevation.

In contrast to this shortening of the latency of signals evoked by relatively weak flashes, calcium elevations pro-

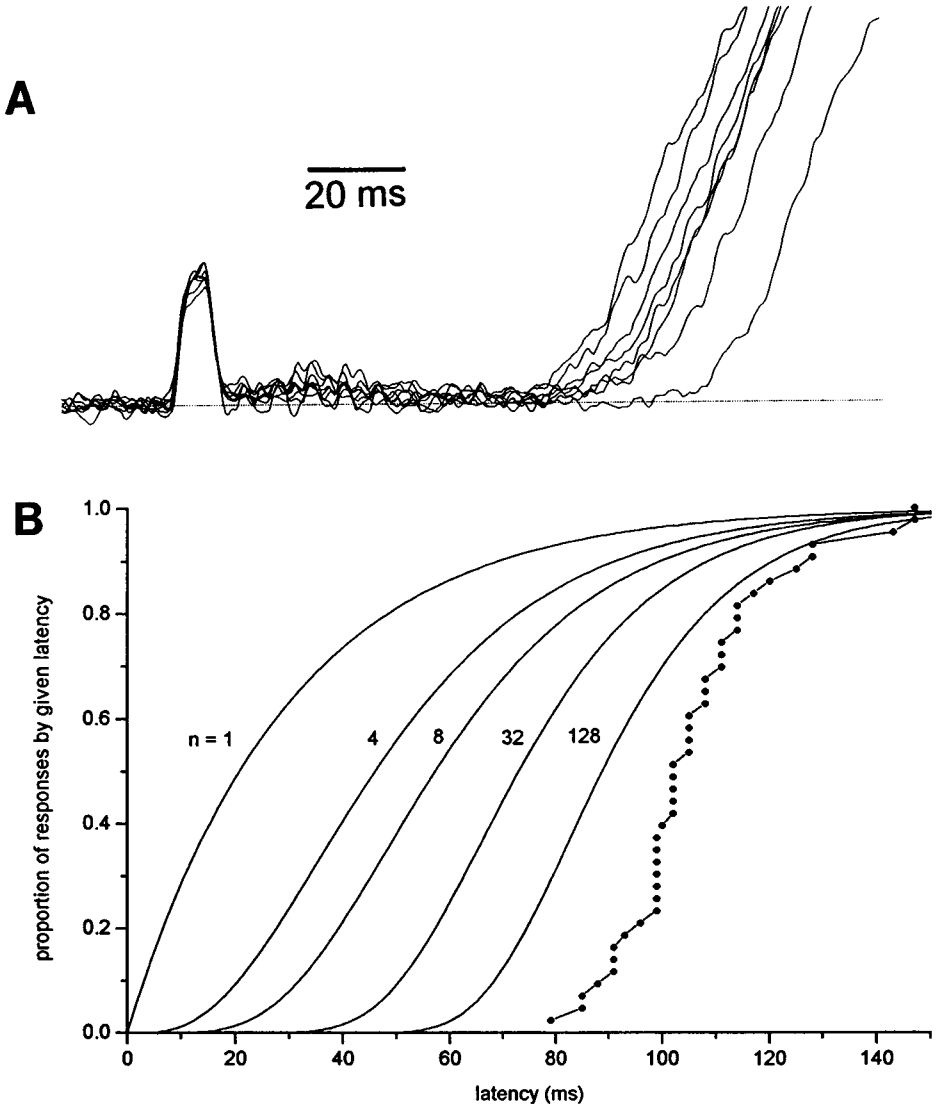

FIGURE 9 Distribution of response latencies. (*A*) Superimposed traces show eight successive fluorescence responses evoked at different locations in an oocyte by a constant photolysis flash. The timing of the flash is apparent from the stimulus artifact, and the subsequent small "hump" in the fluorescence records arose artifactually as a result of mechanical vibration from the shutter. (*B*) Distribution of latencies to first detectable increase of calcium signal evoked by 43 identical photolysis flashes at differing, random locations. Data points indicate the cumulative fraction of trials in which calcium liberation had begun by a given latency. Smooth curves were derived as described in the text.

duced no further reduction of the already short latency of responses evoked by strong stimuli. For example, in Fig. 10 *B*, a flash with an intensity about 20 times threshold evoked a response beginning after 32 ms, but this latency actually lengthened slightly to 36 ms when an identical flash was repeated after raising the basal calcium level by ~0.7 μM. In four trials with similar strong stimuli, the mean latency after elevating calcium by 0.3–1.1 μM ($\Delta F/F$ 0.08–0.35) was 106 ± 6% of the control latency. It is also evident in Fig. 10 *B* that the rate of increase of the calcium signal

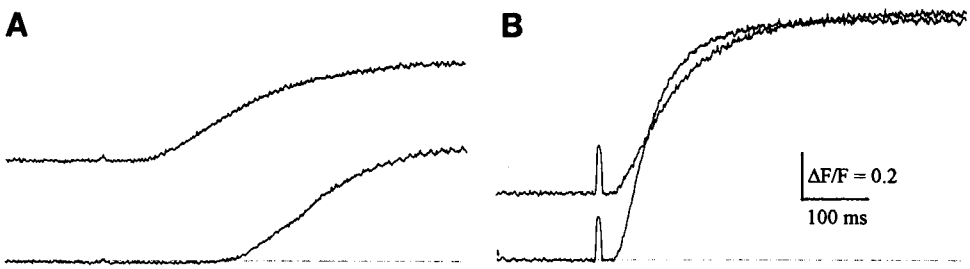

FIGURE 10 Effect of elevated cytosolic free calcium on latency of $InsP_3$-evoked signal. Superimposed traces within each frame show fluorescence signals evoked by two identical photolysis flashes. Control responses were first recorded while calcium influx through expressed nicotinic channels was minimized by voltage clamping the cell at +20 mV, and the membrane potential was then polarized to −100 mV; the response to an identical flash was recorded 30 s or longer after the basal fluorescence had increased to a new steady level. (*A*) Responses to a light flash with an intensity ~50% above threshold; (*B*) responses in a second oocyte to flashes 20 times greater than threshold.

evoked by strong flashes was slowed by previous calcium elevation, whereas this effect was less pronounced with stimuli only just above threshold.

From these results it is clear that although the latency of $InsP_3$-evoked calcium release is influenced by the cytosolic free calcium level, it is unlikely to arise as a result of time required for pacemaker calcium to build toward a triggering threshold. An additional point illustrated in Fig. 10 is that the amplitudes of $InsP_3$-evoked Ca^{2+} transient were only slightly reduced by large preceding elevations of cytosolic $[Ca^{2+}]$ (see also Ilyin and Parker, 1994), suggesting that $InsP_3$ must be bound to receptor sites before Ca^{2+} ions are effective in causing inhibition of release.

Sequential binding of multiple $InsP_3$ molecules cannot explain activation kinetics

A different mechanism proposed to account for the time course of opening of $InsP_3$-gated calcium channels is that their activation may depend on the binding of multiple $InsP_3$ molecules to independent sites (Meyer et al., 1988). The fraction of open channels then increases according to the function $[1 - \exp(-t/\tau)]^n$ (Meyer et al., 1990), where t is time, τ is the time constant for binding to each site, and n is the number of sites. Because we found that the maximal rate of calcium liberation in the oocyte varies as a first order function of $InsP_3$ level (Fig. 4 *A*), it already seemed that such a mechanism was unlikely, and this conclusion is strengthened by the experiment in Fig. 8 *B*. The noisy trace shows the observed increase in calcium efflux rate after a photolysis flash, and the smooth curves are fit to the initial part of the data according to the function described above, with $n = 1$, 4, 8, 32, and 120. Resulting τ values were 200, 80, 60, 39.5, and 32 ms, respectively. Unlike the results of Meyer et al. (1990), a value of $n = 4$ could not account for the abrupt increase of calcium flux after a long latency, and a poor fit was obtained even with $n = 120$. Because it is improbable that activation of calcium release could involve so many binding steps, the data suggest that a different mechanism must be sought to account for the latency.

Latency is not readily explained by stochastic opening of a single calcium channel

We also considered whether the latency might arise if the random opening of a single $InsP_3$ receptor/channel released sufficient calcium to trigger regenerative activation of other channels (Finch et al., 1991; Bezprozvanny et al., 1991; Yao and Parker, 1992), so that the latency of the calcium signal would be determined by the stochastic distribution of latencies to first opening of a channel.

To explore this idea, we compared the distributions expected from stochastic channel openings with the cumulative distribution of response latencies shown in Fig. 10 *B*. In the simplest case, in which channel opening requires only a single reaction step, the cumulative probability that a channel will have opened by a given time (t) after a flash increases exponentially, a relationship that clearly does not fit the data (curve labeled $n = 1$ in Fig. 9 *B*). If multiple sequential steps are involved in channel activation, the cumulative probability of opening increases with t as $(1\text{-}e^{-t/\tau})^n$, where n is the number of reaction steps, each with a time constant τ. This scheme predicts that the cumulative latency distribution should increase as a sigmoid function of time after the flash and become increasingly steep with higher values of n. The smooth curves in Fig. 9 *B* illustrate predicted distributions for $n = 1$, 4, 8, 32, and 128, with corresponding values of $\tau = 30$, 25, 23, 19, and 17 ms. However, a good fit was not obtained to the data even with $n = 128$, an implied number of sequential steps so great as to make it implausible that the latency can be accounted for by this model.

Dependence of mean latency on $InsP_3$ concentration

Fig. 11 shows the dependence of mean latency to first onset of calcium release on the intensity of a photolysis flash (30-ms duration). Flashes with an intensity just above threshold gave latencies longer than 300 ms, but this delay shortened progressively to a minimum of ~28 ms at the maximum available intensity of the illuminator. A plot of latency versus reciprocal photolysis intensity (*inset*, Fig. 11) gave a reasonable fit to a straight line, implying that an $InsP_3$ binding step determines, at least partially, the kinetics of calcium-release channel opening (Parker and Ivorra, 1993). Extrapolation of this line to infinite $[InsP_3]$ indicates the existence of a minimal, $InsP_3$-independent delay. A regression line fitted to all the data gave an intercept of 35 ms, but this is clearly an overestimate because a minimum latency of 28 ms was actually observed, and a line fitted to the five data points at highest intensities (which had smaller standard errors) gave an intercept of ~27 ms.

DISCUSSION

In these experiments we used flash photolysis of caged $InsP_3$ in *Xenopus* oocytes to evoke nearly step-wise increments of intracellular $[InsP_3]$ and derived the resulting rates of calcium flux from $InsP_3$-sensitive intracellular stores from the rate of increase of cytosolic free calcium, as monitored by confocal microfluorimetry from a virtual point source. This provides a description of calcium liberation in an intact cell with excellent temporal resolution and yields kinetic parameters useful for modeling the dynamics of calcium spikes and waves. However, because of the complexities arising from the functional properties of $InsP_3$ receptors and the morphological distribution of $InsP_3$-sensitive stores in the oocyte, interpretation in terms of under-

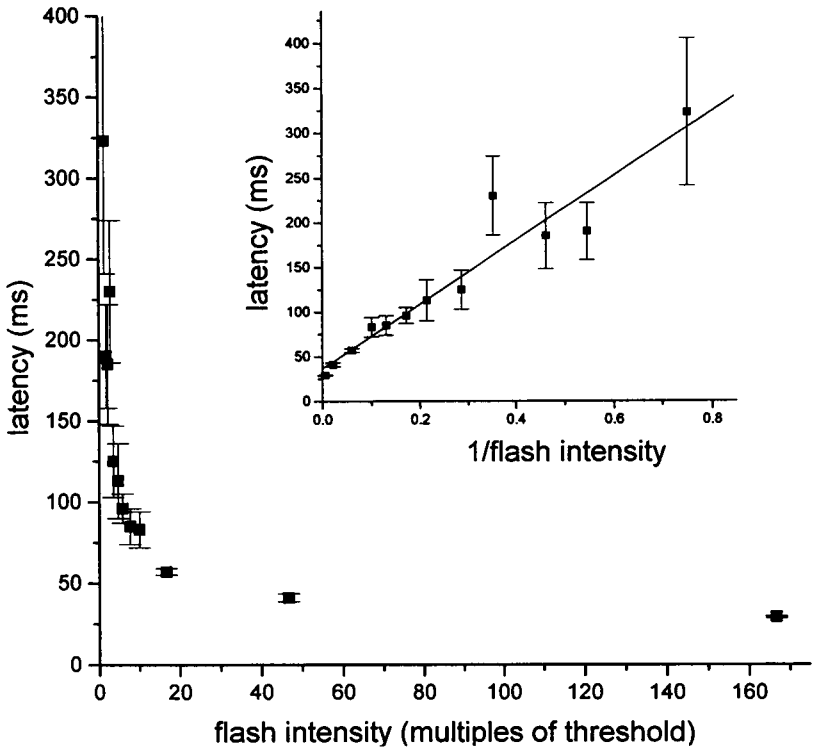

FIGURE 11 Dependence of latency on intensity of photolysis flash. Main graph plots the latency to onset of calcium liberation as a function of intensity of the stimulus flash. Data are from a single oocyte loaded with calcium green-5N, and points represent mean + SE of four to five measurements at each stimulus intensity. Latencies were measured from the beginning of the flash (duration 30 ms) from traces like those in Fig. 8 *A*. Flash intensity is expressed relative to the threshold intensity required to just evoke a response. The inset graph shows the same data, replotted as a function of reciprocal flash intensity. A regression line is fitted to the data.

lying mechanisms is more complex than with reduced microsomal or single channel systems.

In the following discussions we have taken as a starting point that gating of $InsP_3$ receptor/channels is regulated by both $InsP_3$ and calcium acting as co-agonists (Finch et al., 1991; Bezprozvanny et al., 1991), so that positive feedback by calcium ions released into the cytosol will lead to a rapid, regenerative activation of calcium liberation (Yao and Parker, 1992; Iino and Endo, 1992). This key ability of free calcium as an activator of $InsP_3$-induced calcium liberation has recently been questioned (Combettes and Champeil, 1994; Combettes et al., 1994) on the grounds that buffers such as BAPTA that are used experimentally to set low free calcium levels may themselves antagonize binding of $InsP_3$ to its receptor (Richardson and Taylor, 1993; Combettes et al., 1994). Other studies on calcium release from cerebellar microsomes, however, show a marked calcium-dependent facilitation of calcium liberation that cannot be attributed to artifactual effects of calcium chelators (Finch and Goldin, 1994). It is also clear that in the oocyte, $InsP_3$-evoked calcium liberation is facilitated by raised cytosolic free calcium when the only exogenous chelator is 50 μM indicator dye or even when exogenous calcium chelators are entirely absent and calcium liberation is monitored by an endogenous calcium-activated chloride conductance (Yao and Parker, 1992, 1994).

Latency to onset of calcium liberation

The latent period preceding any detectable liberation of calcium is a puzzling feature of our results and differs from the behavior of various other ligand-gated channels such as the nicotinic AChR, which open within microseconds of binding agonist (Katz and Miledi, 1965).

Two explanations have been proposed previously to account for delays seen in the activation of rapid calcium liberation by $InsP_3$. In smooth muscle cells, agonist-stimulated formation of $InsP_3$ leads to a slowly rising foot of calcium, which triggers regenerative release after a certain threshold calcium concentration is attained (Iino et al., 1993). We had also described a similar pacemaker increase in calcium preceding calcium spikes in oocytes in which $InsP_3$ was photoreleased over wide (thousands of μm^2) areas of the oocyte (Parker and Yao, 1991). However, under the present experimental conditions using small (20 μm diameter) photolysis spots, no pacemaker signal could be detected, probably because of rapid diffusion of $InsP_3$ and calcium away from the stimulated region. Therefore, it seems that a slow increase of cytosolic free calcium toward a trigger threshold cannot account for the latent period observed here, and this conclusion is supported by the finding that large elevations of basal calcium level produced only a modest shortening of the latency. Another proposal is that calcium release involves multiple sequential steps, for example the binding of multiple $InsP_3$ molecules to independent sites on the $InsP_3$ receptor (Meyer et al., 1990). This would give rise to a sigmoidal increase in calcium flux after a flash, but the observed abrupt increase in flux after a quiescent period requires such a high degree of cooperativity to provide an adequate fit that this mechanism also seems unlikely.

A variation on the latter model is that regenerative calcium release might be triggered by the opening of a single calcium channel so that calcium liberation is initiated by stochastic channel behavior rather than representing the summated activity of a population of channels. Again, we feel this explanation is unlikely because channel activation would have to involve an implausibly high number of sequential reaction steps to account for the observed tight clustering of latencies around a mean value.

Instead, we propose an alternative model, the basis of which is that a certain proportion of receptor sites must become occupied by $InsP_3$ molecules and subsequently adopt a state in which channel opening can be triggered by cytosolic calcium before a regenerative wave of calcium efflux is triggered. The latency then represents the time course by which the proportion of sensitized receptors increases toward this threshold level. A simplified kinetic model for this process is

$$\underset{\text{resting}}{InsP_3} + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{\substack{\text{bound but}\\ \text{calcium-}\\ \text{insensitive}}}{InsP_3R} \xrightarrow{k_2} \underset{\substack{\text{calcium}\\ \text{sensitized}}}{InsP_3R^*}$$

where R = $InsP_3$ receptor, $InsP_3R$ = receptor with bound $InsP_3$, $InsP_3R^*$ = receptor in state where channel opening can be activated by calcium, and k_1, k_{-1}, and k_2 are rate constants for the various transitions.

Note that none of the indicated states corresponds to an open receptor/channel, but regenerative opening of a population of channels is triggered by calcium feedback once the proportion of receptors in the $InsP_3R^*$ state has increased to a given fraction f_θ of R_{total}. On the basis of previous observations that low $InsP_3$ concentrations evoke transient, localized "puffs" of calcium release and higher concentrations evoke propagating waves (Parker and Yao, 1991; Yao et al., 1995; Parker and Yao, 1995), we believe that $InsP_3$ receptors are clustered in the oocyte at hot spots spaced a few micrometers apart. For a regenerative calcium wave to be sustained, the sensitivity of hot spots (i.e., proportion of receptors binding $InsP_3$) must then be sufficiently high that calcium liberation can be triggered by calcium ions diffusing from a neighboring active site. Although we have not explicitly considered this spatial arrangement of receptors in the present model, it is clear that f_θ must be determined by factors including the diffusivity of calcium in the cytosol and the spacing between hot spots, as well as by the inherent properties of $InsP_3$ receptors.

Making a preequilibrium approximation that k_1 and k_{-1} are $\gg k_2$ and defining $K_d = k_{-1}/k_1$, then $InsP_3R^*$ at any given time t after a step of $[InsP_3]$ is given by

$$[InsP_3R^*] = k_2 \times [InsP_3R] \times t$$

$$= [R_{total}] \times \frac{k_2 \times [InsP_3] \times t}{K_d + [InsP_3] + k_2 \times [InsP_3] \times t} \quad (3)$$

and hence the latency t_L to attain a given f_θ is

$$t_L = \frac{f_\theta}{1 - f_\theta} \times \frac{1}{k_2} \times \left(1 + \frac{K_d}{[InsP_3]}\right) \quad (4)$$

In this relation, the latency t_L varies linearly with $1/[InsP_3]$, and in agreement the observed latencies in Fig. 11 fit well to a straight line when plotted against the reciprocal of the photolysis flash intensity. The slope of the line given by Eq. 4 is $(f_\theta \times K_d)/((1 - f_\theta) \times k_2)$, and the intercept at infinite $[InsP_3]$ is $f_\theta/((1 - f_\theta) \times k_2)$. K_d is hence given by the slope/intercept and is about 13 times the threshold concentration of $InsP_3$ (Fig. 11), in good agreement with the value for the EC_{50} (10–12 times threshold) derived from measurements of calcium efflux rate. From those data we can also estimate f_θ to be ~5%, because the maximal rate of calcium efflux evoked by a just-threshold flash was about 20 times smaller than that with a saturating flash. Finally, k_2 is equal to $f_\theta/((1\text{-}f_\theta) \times$ intercept), giving a value of ~1.8 s^{-1}. The attainment of calcium sensitivity by $InsP_3$ receptors after binding $InsP_3$ is thus expected to be quite slow (several hundred milliseconds), a prediction that should be open to experimental test using the procedures of Finch et al. (1991) to monitor calcium flux induced by calcium pulses delivered at various intervals after applying $InsP_3$.

Kinetics of calcium liberation

After the initial latent period, calcium release began abruptly and attained a maximal rate in as little as 8 ms at high $InsP_3$ concentrations, during which time the flux increased with a doubling time no more than ~2 ms. This rapid activation after a latent period is consistent with the triggering of a regenerative process and sets some constraints on the nature of the feedback mechanism. Two models have been proposed whereby cytosolic calcium ions might promote the further liberation of calcium. 1) Calcium stimulates phospholipase C, resulting in formation of $InsP_3$ and hence a regenerative release of additional calcium (Meyer and Stryer, 1988; Harootunian et al., 1991), and 2) calcium ions act as a co-agonist at the $InsP_3$ receptor to facilitate the opening of release channels (Finch et al., 1991; Bezprozvanny et al., 1991; Yao and Parker, 1992). We had suggested previously that the first possibility was unlikely because the minimal latency of 30 ms for calcium release seemed too short for calcium ions to diffuse from their release sites and activate the phospholipase at the plasma membrane (Parker and Ivorra, 1993). The present finding that calcium flux can increase with a doubling time of only 2 ms considerably strengthens that argument, because calcium ions are expected to diffuse <1 μm within this time (Allbritton and Meyer, 1993). Instead, the rapid regeneration time is consistent with the positive regulatory sites residing on the $InsP_3$ receptor itself, where the rate of activation of calcium release would be determined not by diffusion but rather by the kinetics of calcium-activation of $InsP_3$ receptors.

After attaining a peak rate, calcium efflux following a photolysis flash subsequently declined to a low level along a roughly exponential time course with time constants of 150–25 ms, depending on the dose of $InsP_3$. This is unlikely to be due to any appreciable fall in $InsP_3$ concentration, since that occurs over several seconds (Parker and Ivorra, 1992), and because a second flash delivered while the calcium flux was slowing failed to restore the rate of efflux. Remaining possibilities are that efflux declined as $InsP_3$-sensitive stores became depleted, or that the $InsP_3$-gated channels closed. Several observations suggest that the latter is the dominant factor. (i) Calcium efflux induced by weak stimuli largely ceased within a few hundred ms, even though stronger stimuli could evoke release of up to 4-times more calcium. (ii) Paired flash experiments showed that the rate of calcium flux declined and recovered along different time courses to the amount of calcium released by a test flash (Figs 6, 7), consistent with a modulation of channel gating. However, a definite conclusion cannot yet be made, as it is difficult to exclude the possibility that different populations of stores may be activated by stronger flashes, and by the second flash in a pair. A widely held view is that feedback inhibition by relatively high calcium levels is responsible for terminating calcium flux (Parker and Ivorra, 1990b; Payne et al., 1990; Bezprozvanny et al., 1991; Finch et al., 1991), though some additional factor, such as occupancy of $InsP_3$ binding sites, must also be involved since the time course of inhibition does not correspond to that of the free calcium transient (Figs. 6, 7; and see Ilyin and Parker, 1994).

Dose-dependence of calcium liberation

The present results confirm previous findings of a threshold in $InsP_3$ action (Parker and Miledi, 1989; Parker and Ivorra, 1990a, 1992, 1993), in that photorelease of $InsP_3$ had to exceed a certain level before any calcium liberation was detected. We had previously described the calcium liberation as a nearly all-or-none phenomenon, because fluorescence signals monitored by rhod-2 grew by <20% as the stimulus flash was increased severalfold above that giving a just-suprathreshold response (Parker and Ivorra, 1990a, 1993). However, with use of calcium green-5N we now find that maximal stimuli evoke calcium signals nearly four times greater than threshold responses, a difference that is probably explained because the relatively high affinity of rhod-2 caused the fluorescence signals to approach saturation even with threshold responses. Nevertheless, it remains the case that cytosolic free calcium transients show a highly nonlinear relation with $[InsP_3]$, increasing abruptly from nearly zero to a peak amplitude of ~1.6 μM at threshold, and then to a maximum of ~6–7 μM at concentrations of $InsP_3$ 5 to 10 times greater. The origin of the threshold probably develops from the requirement for both $InsP_3$ and calcium as co-agonists to open the $InsP_3$ receptor/channel (Finch et al., 1991; Bezprozvanny et al., 1991; Yao and Parker, 1992; Iino and Endo, 1992), because a certain level of $InsP_3$ will be needed before a regenerative feedback by released calcium ions is established.

In contrast to the relatively small range of free calcium between threshold and maximal responses, the rate of calcium efflux varied over a greater range, from ~10 $\mu M\ s^{-1}$ at threshold to a maximal value of ~150 $\mu M\ s^{-1}$. The peak rate of calcium flux increased as a first-order function of $[InsP_3]$, suggesting that under physiological conditions in the intact oocyte there is little or no cooperativity in the action of $InsP_3$ molecules to open calcium release channels. Although interpretation is complicated because both the gating of the channels and the chemical gradient for calcium flux would have been affected by changing calcium levels during the rising phase of responses, the maximal calcium efflux rate always occurred when the cytosolic free $[Ca^{2+}]$ was at about the same level during the rising phase of calcium transients. Our results are thus in agreement with previous studies showing little or no cooperativity under conditions in which the cytosolic free calcium level was clamped (Watras et al., 1991; Finch et al., 1991; Iino and Endo, 1992). Although some groups have reported cooperative $InsP_3$-stimulated calcium release (Champeil et al., 1989; Meyer et al., 1990), it was probably a consequence of the stimulatory effects of cytosolic calcium (Marshall and Taylor, 1993). Thus, the steeply nonlinear threshold dependence of calcium liberation on $InsP_3$ concentration seems to arise as a result of the regulatory effect of cytosolic calcium rather than through cooperativity of $InsP_3$ binding.

The absolute concentration of $InsP_3$ required to evoke threshold calcium liberation in the oocyte was not determined in the present experiments, but an earlier calibration of the photolysis system gave a value of ~60 nM (Parker and Ivorra, 1992). In approximate agreement, regenerative calcium waves were evoked by injections of 3-F-$InsP_3$ (a poorly metabolized analog having a potency about equal to $InsP_3$ itself) (Kozikowski et al., 1990) to a threshold intracellular concentration of ~90 nM (Yao et al., 1995). Because the rate of calcium liberation was half-maximal with photolysis flashes approximately 10 times greater than threshold, the EC_{50} for $InsP_3$ is therefore ~600–900 nM, a value similar to that reported in various other peripheral tissues (for references see Ribeiro-Do-Valle et al., 1994). On the other hand, binding studies on isolated oocyte $InsP_3$ receptors show a much higher affinity, with a K_d of 5 nM (Callamaras and Parker, 1994) or 46 nM (Parys et al., 1992), and similar discrepancies between K_ds and EC_{50}s have been noted previously in other tissues (Taylor and Richardson, 1991; Ribeiro-Do-Valle et al., 1994). The reasons for this difference are not yet clear, but it may be that equilibrium binding measurements do not identify the functional state of the receptor responsible for calcium release or, if channel activation is relatively slow, that a high concentration of $InsP_3$ is needed to open half of the channels in the short time (tens or hundreds of milliseconds) before release is terminated by feedback inhibition.

We thank Dr. K. Sumikawa for supplying nAChR mRNAs. This work was supported by grant GM48071 from the United States Public Health Service.

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