Role of cytosolic Ca^{2+} in inhibition of $Ins P_3$-evoked Ca^{2+} release in *Xenopus* oocytes

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1. Calcium liberation induced in *Xenopus* oocytes by flash photorelease of inositol 1,4,5-trisphosphate ($Ins P_3$) from a caged precursor was monitored by confocal microfluorimetry. The object was to determine whether inhibition of Ca^{2+} release seen with paired flashes arose as a direct consequence of elevated cytosolic free [Ca^{2+}].
2. Responses evoked by just-suprathreshold test flashes were not inhibited by subthreshold conditioning flashes, but were strongly suppressed when conditioning flashes were raised above threshold.
3. Inhibition at first increased progressively as the inter-flash interval was lengthened to about 2 s and thereafter declined, with a half-recovery at about 4 s.
4. Intracellular injections of Ca^{2+} caused relatively slight inhibition of $Ins P_3$-evoked signals, even when cytosolic free [Ca^{2+}] was elevated to levels similar to those at which strong inhibition was seen in paired-flash experiments.
5. Recovery from inhibition was not appreciably slowed when Ca^{2+} was injected to raise the free Ca^{2+} level between paired flashes.
6. We conclude that inhibition of $Ins P_3$-evoked Ca^{2+} liberation is not directly proportional to cytosolic free Ca^{2+} level and that recovery from inhibition in paired-pulse experiments involves factors other than the decline of cytosolic [Ca^{2+}] following a conditioning response.

A characteristic feature of inositol 1,4,5-trisphosphate ($Ins P_3$)-mediated signalling is that this second messenger induces cyclical release of Ca^{2+} ions from intracellular stores in the form of repetitive Ca^{2+} spikes and waves (Berridge, 1993). Since Ca^{2+} spikes persist in the presence of stable concentrations of non-metabolizable $Ins P_3$ analogues, $Ins P_3$-gated Ca^{2+} release channels must show a transient inactivation or adaptation in the maintained presence of agonist (Wakui, Potter & Petersen, 1989; Payne & Potter, 1991; Yao & Parker, 1994). Such a refractory period has been well characterized in various cells including *Xenopus* oocytes (Berridge, 1988; Parker & Ivorra, 1990, 1993), *Limulus* ventral photoreceptors (Payne, Walz, Levy & Fein, 1988; Payne, Flores & Fein, 1990; Levitan, Hillman & Payne, 1993; Levy & Payne, 1993) and hepatocytes (Ogden, Capiod, Walker & Trentham, 1990). Intracellular elevations of $Ins P_3$, produced by microinjection or photorelease from a caged precursor, evoke a transient release of intracellular Ca^{2+} which terminates within a few hundred milliseconds or shorter, and is followed by a refractory period of several seconds during which responses to a second pulse of $Ins P_3$ are depressed.

Several mechanisms have been proposed to account for this depression and its subsequent recovery. A widely held view is that feedback inhibition on the $Ins P_3$ receptor by cytosolic Ca^{2+} ions limits further release of Ca^{2+}, and that recovery of sensitivity to $Ins P_3$ follows the subsequent decline of cytosolic Ca^{2+} as it is re-sequestered. In support of this idea, $Ins P_3$-mediated Ca^{2+} release is inhibited by intracellular injections of Ca^{2+} (Parker & Ivorra, 1990; Payne *et al.* 1990), recovery from inhibition is accelerated by injections of EGTA (Levy & Payne, 1993) and high cytosolic Ca^{2+} levels inhibit Ca^{2+} flux through $Ins P_3$-gated channels (Bezprozvanny, Watras & Erlich, 1991; Finch, Turner & Goldin, 1991). Another possibility is that depression of Ca^{2+} release may arise from the depletion of intralumenal Ca^{2+} within the stores, either because little Ca^{2+} remains or because the $Ins P_3$ receptor is regulated by intralumenal Ca^{2+} (Missiaen, Taylor & Berridge, 1992). Finally, the depression might be an inherent property of the $Ins P_3$ receptor, triggered by its activation independently of any resulting Ca^{2+} flux.

The present experiments were designed primarily to test the role of cytosolic free Ca^{2+} in the onset and recovery of

depression of InsP_3-evoked Ca^{2+} release in *Xenopus* oocytes. As before (Parker & Ivorra, 1990), we used flash photolysis of caged InsP_3 to evoke reproducible and precisely timed elevations of intracellular InsP_3. Those experiments, however, largely used Ca^{2+}-activated Cl^- currents as an intrinsic reporter of cytosolic free Ca^{2+} level, whereas a fluorescent Ca^{2+} indicator (calcium green-5N) now provided a more direct measure. Furthermore, fluorescence was recorded from a virtual point source (about 1 fl volume) within the oocyte by use of a confocal microfluorimeter. This minimized problems of spatial inhomogeneities of Ca^{2+} distribution, since diffusional equilibration would occur within this tiny volume in less than 1 ms (Parker & Ivorra, 1993). The main result was that elevations of cytosolic Ca^{2+} resulting from InsP_3-evoked Ca^{2+} liberation and from intracellular injections of exogenous Ca^{2+} were not comparable in their ability to inhibit InsP_3-evoked Ca^{2+} release, suggesting that inhibition is not a simple function of the cytosolic free Ca^{2+} level.

METHODS

Experiments were done on immature ovarian oocytes of albino *Xenopus laevis*, obtained by surgical removal from frogs anaesthetized 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 removing the oocytes. Oocytes were treated with collagenase to remove enveloping cells and placed in a recording chamber superfused with Ringer solution (composition in mM: NaCl, 120; KCl, 2; $CaCl_2$, 1·8; Hepes, 5; at pH about 7·0) at room temperature. The animal hemisphere was viewed through a coverslip forming the base of the chamber by an Olympus IMT2 inverted microscope equipped with a ×40 objective (numerical aperture 1·3). Procedures for preparation of oocytes, intracellular microinjection, photolysis of intracellularly loaded caged InsP_3, and confocal microfluorimetry of intracellular free Ca^{2+} were similar to those described previously (Parker, 1992; Parker & Ivorra, 1993; Yao & Parker, 1993, 1994). In brief, oocytes were loaded with calcium green-5N and with caged InsP_3 (*myo*-inositol 1,4,5-trisphosphate, $P^{4(5)}$-1-(2-nitrophenyl)ethyl ester) to respective final intracellular concentrations of about 40 and 2 μM. Photolysis of caged InsP_3 was induced by applying flashes of UV light (340–400 nm), the durations of which were set by an electronic shutter and which were focused on the oocyte as a spot of about 20 μm diameter, at the same focal depth and concentric with the confocal recording spot. Recordings of Ca^{2+}-dependent fluorescence were made by a Noran Odyssey confocal microscope (Noran Instruments, Middleton, WI, USA) operated in the stationary spot (i.e. non-scanning) mode and interfaced to the inverted microscope through the phototube. Fluorescence excited by the laser spot (488 nm) focused about 5 μm into the oocyte was monitored at wavelengths > 510 nm by a photomultiplier behind a confocal aperture, and signals were recorded on floppy disk by a digital oscilloscope after low-pass filtering at 100–200 Hz. Calcium green-5N was used as the Ca^{2+} indicator since its low affinity (12 μM; Yao & Parker, 1994) minimizes interference with normal Ca^{2+} homeostasis. Fluorescence signals are expressed as fractional changes above the resting baseline ($\Delta F/F$) and a calibration in terms of free Ca^{2+} concentration was obtained by measuring the maximal fluorescence (F_{max}) obtained after microinjecting saturating amounts of Ca^{2+}. F_{max} was about 5 times greater than the largest InsP_3-evoked signals, so that $\Delta F/F$ would be almost linearly proportional to free Ca^{2+} concentration for the responses described here. As a rough guide, a $\Delta F/F$ value of 0·5 is equivalent to about 1·7 μM free Ca^{2+} and the peak Ca^{2+} level following a just-suprathreshold flash was about 2 μM.

In experiments where the basal cytosolic free Ca^{2+} concentration was changed, a micropipette filled with 100 mM $CaCl_2$ was inserted through the oocyte so that its tip lay 20–40 μm inward from the bottom surface of the oocyte viewed by the inverted microscope. Ionophoretic current was applied to either retain Ca^{2+} ions within the pipette, or to inject Ca^{2+}. The pipette tip was located 20–30 μm to the side of the confocal recording spot, to avoid mechanical damage near the recording area and so that the concentration gradient of Ca^{2+} away from the pipette would be shallow near the measuring spot.

Calcium green-5N was obtained from Molecular Probes Inc. (Eugene, OR, USA) and caged InsP_3 from Calbiochem (La Jolla, CA, USA). All other reagents were from Sigma Chemical Co, St Louis, MO, USA.

RESULTS

Inhibition of InsP_3-mediated Ca^{2+} signals with paired flashes

The basic phenomenon is illustrated in Fig. 1*A*, which shows confocal Ca^{2+} signals evoked by paired photorelease of InsP_3. A certain threshold level of InsP_3 (about 60 nM; Parker & Ivorra, 1992) is required to evoke Ca^{2+} liberation, and the test flashes were set to a duration (10 ms) slightly greater than that (8 ms) required to just evoke a response (Fig. 1*B*). These test flashes were preceded 4 s earlier by conditioning flashes of progressively increasing duration. Conditioning flashes shorter than threshold had little effect on the amplitude of the test response, or caused a slight potentiation (Fig. 1*C*). However, once the conditioning flash duration was lengthened sufficiently to just evoke Ca^{2+} release, the test response was dramatically altered. A just-threshold conditioning flash (8 ms) caused the rising phase of the test response to be greatly slowed, although its peak amplitude was little diminished, and a further small increase to 9 ms caused the virtual abolition of the test response.

Time course of onset and recovery from inhibition

The kinetics of the inhibitory process were investigated by experiments like that in Fig. 2*A*, where the interval between two identical, just-suprathreshold flashes was varied. To allow responses evoked by the test flash to be visualized in isolation, we recorded control responses to the conditioning flash alone and subtracted them from paired responses (right-hand traces in Fig. 2*A*). The decay of the

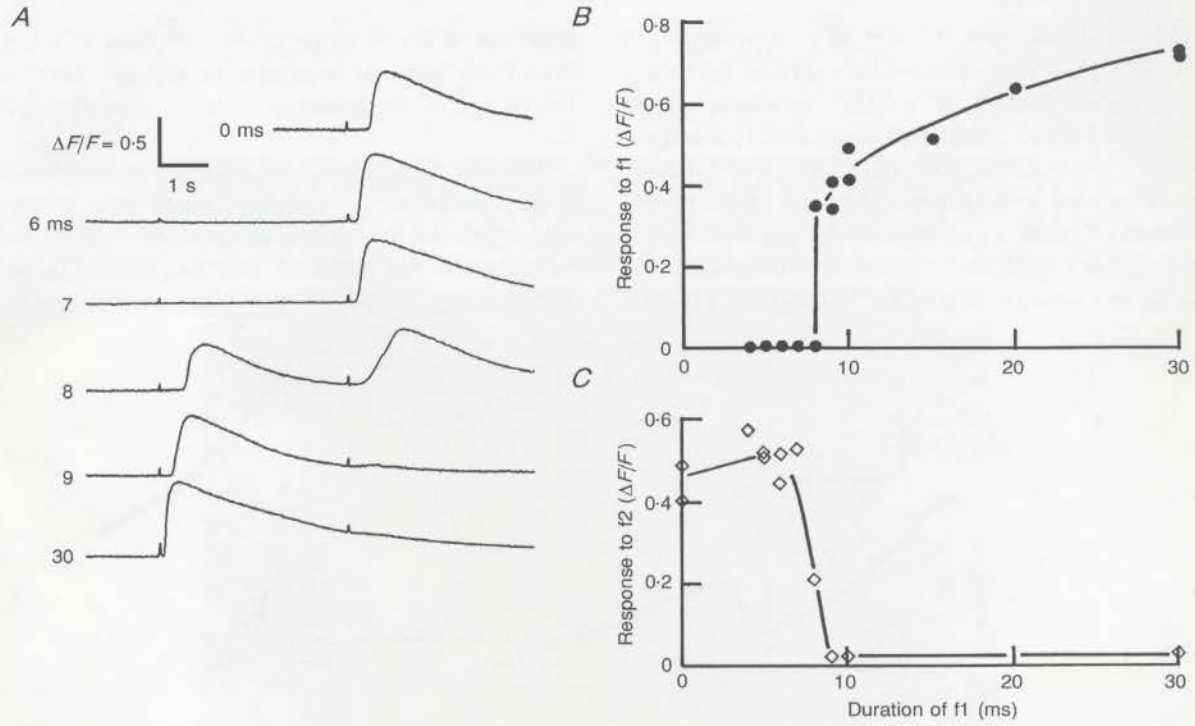

Figure 1. Ca^{2+} release evoked by a conditioning flash causes nearly all-or-none inhibition of release to a subsequent, just-suprathreshold test flash
A, traces show confocal Ca^{2+} transients evoked by paired light flashes delivered to oocytes loaded with caged $InsP_3$ and calcium green-5N. Timing of the flashes can be seen from stimulus artifacts, and the duration of the test (second) flash was always 10 ms. The duration of the conditioning flash (f1) was varied, and is indicated next to each trace. The top record shows a control response to a 10 ms test flash alone. *B*, size of responses to the conditioning flash as a function of its duration. *C*, size of responses to the test flash (f2) as a function of duration of the conditioning flash.

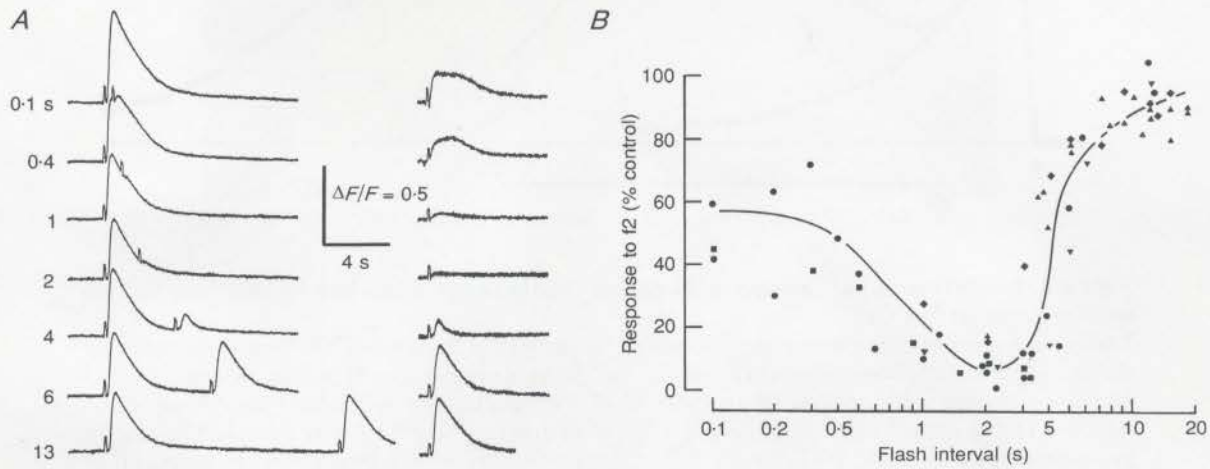

Figure 2. Time course of onset and recovery from inhibition of $InsP_3$-evoked Ca^{2+} liberation
A, traces on left show point confocal measurements of intracellular free Ca^{2+} signals evoked by paired light flashes. The timing of the flashes can be seen from the stimulus artifacts and inter-flash intervals are given next to the traces, in seconds. All light flashes were of identical intensity and duration (50 ms). Traces on the right show the additional signal resulting from the second (test) flash in each pair, and were derived by subtracting the response evoked by a conditioning flash alone from that evoked by a pair of flashes. *B*, peak sizes of Ca^{2+} signals evoked by test flashes (f2) as a function of inter-flash interval. Each point is a single measurement from traces like those in *A*, and data are included from 5 oocytes (different symbols). Responses are expressed as a percentage of a control response evoked by an identical flash following a 2 min rest period.

fluorescence signals occurred at a similar rate to responses evoked by flash photolysis of caged Ca^{2+} (Ivorra & Parker, 1990) and by transient entry of Ca^{2+} through voltage-gated channels (Yao & Parker, 1992), indicating that it is largely determined by reuptake of Ca^{2+} ions and their diffusion from the stimulated area. Since the rising phase of the $InsP_3$-evoked Ca^{2+} signals was rapid in comparison to their decay, their peak height then reflects the integral of the Ca^{2+} flux, i.e. the total amount of Ca^{2+} liberated. A plot of peak size of the response to the test flash as a function of inter-flash interval is shown in Fig. 2*B*, expressed as a percentage of the response to the (identical) conditioning flash.

The onset of inhibition was not instantaneous. Test flashes at intervals < 500 ms evoked increments of Ca^{2+} about 50 % of the control size, which then declined to a minimum as the interval was lengthened to about 2 s. Subsequently, responses recovered over several seconds,

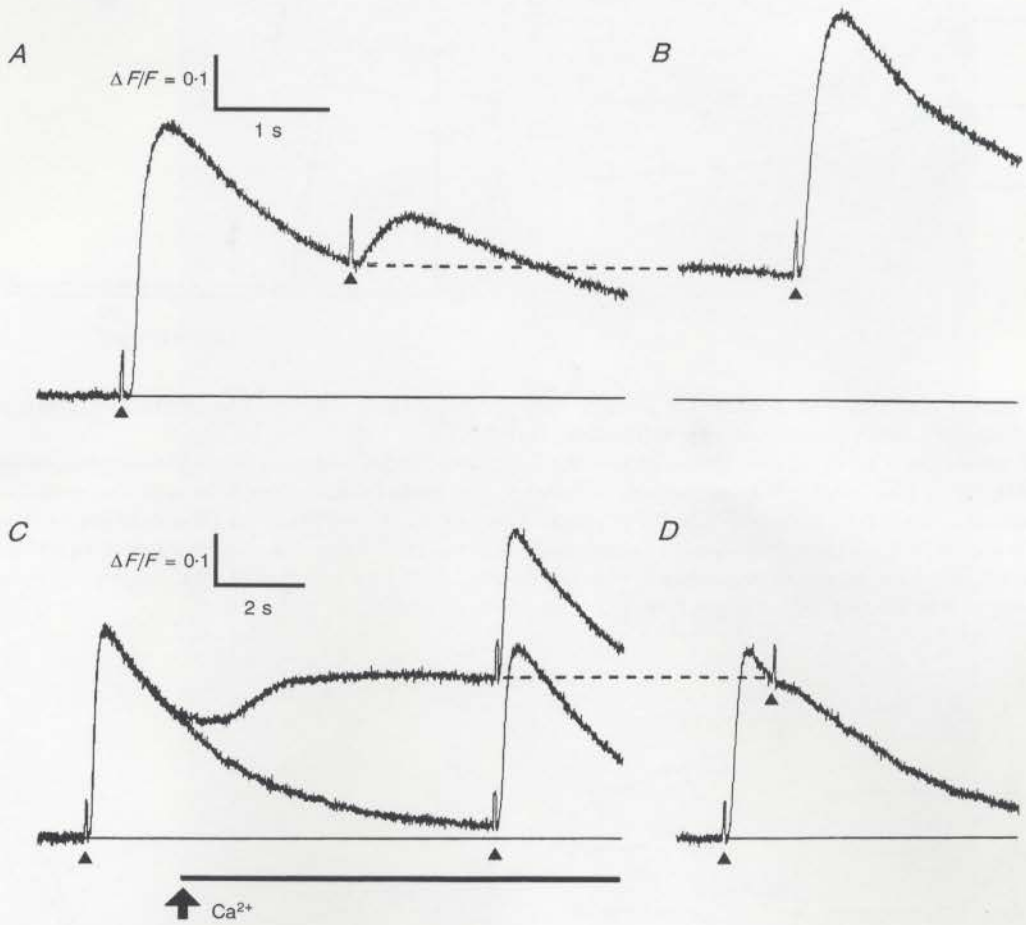

Figure 3. Inhibition of Ca^{2+} release with paired flashes is not mimicked by injecting Ca^{2+} to elevate cytosolic free Ca^{2+}

Traces show confocal fluorescence signals evoked by photolysis flashes delivered when indicated by the arrowheads, and fluorescence at the resting Ca^{2+} level is indicated by thin lines. *A*, two identical photolysis flashes were applied at an interval of 2 s, resulting in substantial inhibition of the response to the second flash. *B*, following a 2 min recovery period, Ca^{2+} was injected through an ionophoretic pipette to elevate cytosolic Ca^{2+} at the measuring spot to a level about the same as that at the time of the second flash in *A* (dashed line). An identical test flash given after holding this Ca^{2+} level for about 5 s still evoked a response almost as large as the control. *C* and *D*, elevation of cytosolic Ca^{2+} between paired photolysis flashes does not appreciably slow recovery from inhibition. Superimposed traces in *C* show responses to two identical pairs of photolysis flashes. The lower trace shows control responses. In the upper trace, Ca^{2+} was injected through a micropipette for the time indicated by the bar, to elevate the cytosolic free Ca^{2+} level during the inter-flash interval. *D*, responses to paired photolysis flashes (identical to those in *C*) at an interval of 1 s. The response to the second flash was greatly depressed, even though the Ca^{2+} level at the time of the flash was no greater than that produced by the Ca^{2+} injection (dashed line).

and were half-maximal after intervals of about 4–5 s. Appreciable recovery was seen only at times when the tail of Ca^{2+} signal evoked by the conditioning flash had fallen by 75 % or more from the peak, and responses then recovered rapidly, even though the further decline of Ca^{2+} was slight (e.g. 4 and 6 s traces in Fig. 2*A*). A final point is that the incremental Ca^{2+} signals evoked by test flashes at short intervals showed a plateau, different to the usual roughly exponential decay. It is not clear whether this arose because the mechanisms removing Ca^{2+} from the cytosol became saturated, or because the test flash evoked a more persistent liberation of Ca^{2+}.

Injections of Ca^{2+} do not mimic paired-flash depression

The results in Fig. 2 already suggested that inhibition of $InsP_3$-mediated Ca^{2+} release could not be an instantaneous function of cytosolic free Ca^{2+} level, since inhibition developed over about 2 s following a conditioning flash, even though peak Ca^{2+} levels were attained within a few hundred milliseconds. To test this point further, and to examine whether recovery from inhibition was contingent upon removal of Ca^{2+} from the cytosol, we compared the effects of elevating cytosolic free Ca^{2+} either by conditioning light flashes or by injecting Ca^{2+} through a micropipette.

In Fig. 3*A* and *B* a pair of identical light flashes were first applied at an interval (2 s) giving pronounced inhibition (Fig. 3*A*). Following a 2 min rest period, Ca^{2+} was injected into the oocyte to raise the free Ca^{2+} at the confocal spot to a level comparable to that at the time the test flash was delivered in *A* (dashed line). An identical test flash then evoked a Ca^{2+} transient much greater than the second of the paired responses and only slightly smaller (5 %) than the control response evoked at resting Ca^{2+} level (Fig. 3*B*). Similar results were obtained in seventeen trials in six oocytes. In all cases, Ca^{2+} injections resulted in inhibitions of $InsP_3$-evoked Ca^{2+} signals that were slight in comparison to the paired-flash inhibition seen at comparable Ca^{2+} levels.

These results are summarized in Fig. 4, which plots the inhibition resulting from conditioning flashes (filled symbols) and Ca^{2+} injections (open symbols) as a function of the elevation of basal Ca^{2+} level at the time of the test flash. Data are shown for both the peak size of the fluorescence signal (Fig. 4*A*), which gives a measure of the amount of calcium released by the test flash, and the rate of rise of fluorescence (Fig. 4*B*), which is proportional to the rate of Ca^{2+} efflux. Although Ca^{2+} injections did inhibit both the amount (cf. Parker & Ivorra, 1990) and rate of $InsP_3$-induced Ca^{2+} liberation, the extent of the suppression was small in comparison to that following conditioning flashes when Ca^{2+} levels were elevated to comparable extents.

A final experiment demonstrated that recovery from inhibition was not slowed when injections were applied to elevate the Ca^{2+} level between paired flashes. This is illustrated in Fig. 3*C* and *D*, which is representative of results in thirteen trials in five oocytes. Figure 3*C* shows superimposed responses to two pairs of identical flashes. During the first pair, Ca^{2+} was allowed to decline normally following the conditioning response, and the interval was chosen to allow substantial recovery of the test response

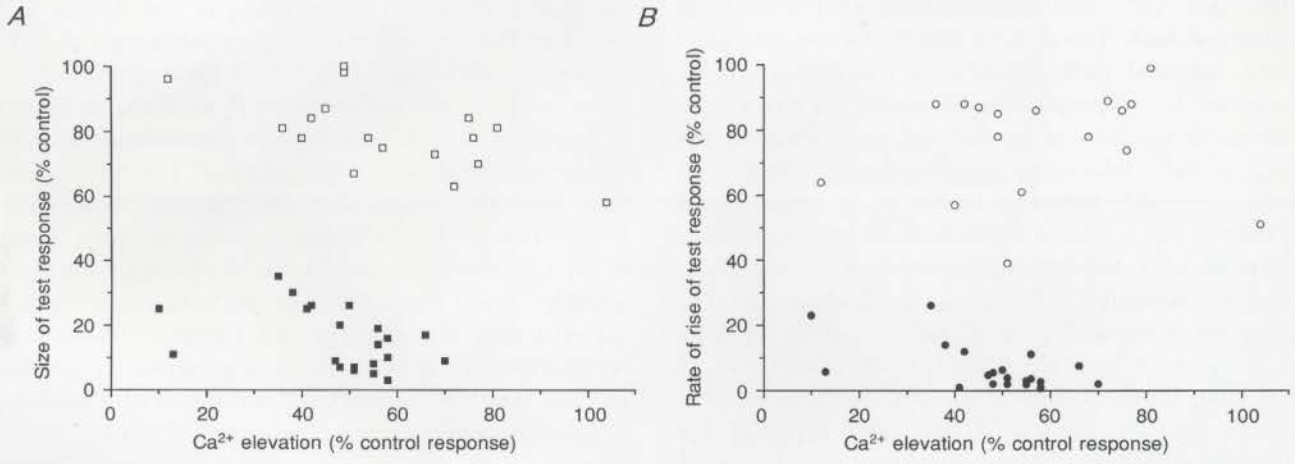

Figure 4. Comparison of inhibition of $InsP_3$-evoked Ca^{2+} release produced by injection of exogenous Ca^{2+} and by Ca^{2+} liberation evoked by a conditioning flash

Graphs show sizes (*A*) and rates of rise (*B*) of fluorescence responses to test flashes, expressed as a percentage of the control response evoked by the same flash after a 2 min period at the resting Ca^{2+} level. Horizontal axes indicate the basal Ca^{2+} level at the time of the test flash, expressed as a percentage of the peak Ca^{2+} level (roughly 2 μM) during control responses to light flashes. Open symbols are measurements following injections of Ca^{2+}. Filled symbols are measurements from the same five oocytes following conditioning photolysis flashes.

(72 % control). After a 2 min rest period the paired flashes were again repeated, but a sustained ionophoretic current was now applied when indicated by the bar, so as to maintain the Ca^{2+} signal at a high level during most of the inter-flash interval. Despite this, the response to the test flash was only slightly reduced (by 20 %) compared to the test response in the control pair. In particular, the extent of inhibition was negligible compared to that observed when the inter-flash interval was shortened so that the Ca^{2+} elevation resulting from the conditioning flash was similar to that produced by the Ca^{2+} injection (Fig. 3*D*).

DISCUSSION

Experiments using light flashes to photolyse caged $InsP_3$ showed that $InsP_3$-evoked Ca^{2+} liberation in *Xenopus* oocytes was strongly inhibited by prior Ca^{2+} liberation evoked by a conditioning flash. However, conditioning flashes just below the threshold to evoke all-or-none Ca^{2+} liberation (Parker & Ivorra, 1993) failed to cause depression, suggesting that inhibition does not arise simply from the presence of $InsP_3$. Also, the inhibition cannot be attributed to consumption of caged $InsP_3$ during the conditioning flash, since only a negligible part of the total would be consumed (Parker & Ivorra, 1992), and because just-subthreshold flashes failed to induce inhibition. Instead, inhibition appears to be concomitant upon the opening of the $InsP_3$-gated Ca^{2+} release channels or on the resulting redistribution of Ca^{2+} ions from intracellular stores to cytosol.

One explanation for the inhibition could be that the stores become depleted during the conditioning response, so that little Ca^{2+} remains available for release by a following test flash. This seems unlikely, because inhibition was not maximal until 2 s after a conditioning flash, whereas the Ca^{2+} stores presumably ceased emptying after the 200 ms rising phase of the Ca^{2+} transient. Further, the slowing of Ca^{2+} release by a just-suprathreshold flash without appreciable reduction in size of the test response (8 ms trace in Fig. 1*A*) is consistent with an inhibition of the release machinery, but not with depletion of stored Ca^{2+}.

Another possibility arises from the finding that the $InsP_3$ receptor–channel is inhibited by relatively high levels of cytosolic free Ca^{2+} (Payne *et al.* 1990; Parker & Ivorra, 1990; Bezprozvanny *et al.* 1991; Finch *et al.* 1991; Levitan *et al.* 1993; Levy & Payne, 1993). Although our results confirm this effect, the inhibition of a flash-evoked response by exogenous elevation of cytosolic $[Ca^{2+}]$ was too small to account for the paired-flash inhibition. Also, inhibition developed over about 2 s following a conditioning flash, even though the Ca^{2+} level declined for much of this time. Thus, it is unlikely that the time course of recovery from inhibition in *Xenopus* oocytes is determined simply by the decline in cytosolic Ca^{2+} lingering from the conditioning response, as proposed for *Limulus* photoreceptors (Levy & Payne, 1993). A concern is whether the fluorescence signal accurately reflects Ca^{2+} levels in the immediate vicinity of the $InsP_3$ receptors and whether fluorescence signals evoked by Ca^{2+} injections and $InsP_3$-evoked release are directly comparable. For example, if release occurred from discrete 'hot spots' that did not happen to lie directly under the confocal point, the inhibition seen with $InsP_3$ could involve a higher level of Ca^{2+} than sensed in our recordings and the 'matching' level of Ca^{2+} produced by injection would be too small. This was not the case, since recordings at many random locations gave similar results, and confocal video imaging of flash-evoked Ca^{2+} signals showed that peak Ca^{2+} levels were almost uniform throughout the area exposed to the photolysis spot. However, the free Ca^{2+} concentration near the mouth of an open Ca^{2+} channel may well have been much higher than the bulk measure reported by even a tiny confocal spot, so that inhibition could have been initially triggered by a transient, locally high Ca^{2+} level close to the activated $InsP_3$ receptor.

What then might be the mechanism underlying inhibition and responsible for its time course? A key observation is that over-expression of a Ca^{2+}-ATPase in oocytes led both to a more rapid decay of Ca^{2+} spikes evoked by a persistent elevation of $InsP_3$ and to an increase in spike frequency (Camacho & Lechleiter, 1993), indicating that the duration of the refractory period following a spike is influenced by Ca^{2+} movements. However, in our paired-flash experiments, elevated cytosolic Ca^{2+} had little influence on recovery from inactivation. We can consider two explanations for this apparent discrepancy. One is that binding of Ca^{2+} to sites mediating inhibition of the $InsP_3$ receptor is enhanced when $InsP_3$ is also bound to the receptor. The recovery of sensitivity following a photolysis flash would then depend upon both the decline of cytosolic free $[Ca^{2+}]$ and the decline of $InsP_3$ as it was metabolized and diffused away from the site of photorelease. Failure of exogenous elevations of cytosolic $[Ca^{2+}]$ to mimic paired-flash inhibition would then be explained as a result of lower affinity of the Ca^{2+} binding site in the initial absence of $InsP_3$. Another possibility is that refilling of $InsP_3$-sensitive stores regulates recovery from inactivation. As argued above, it is unlikely that inhibition results simply because less Ca^{2+} is available for release, but the possibility remains that intralumenal Ca^{2+} levels may modulate $InsP_3$ receptor–channel function (Missiaen *et al.* 1992).

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