

Inhibition by Ca^{2+} of inositol trisphosphate-mediated Ca^{2+} liberation: A possible mechanism for oscillatory release of Ca^{2+}

(caged inositol 1,4,5-trisphosphate/fluo-3/*Xenopus* oocyte/chloride current)

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ABSTRACT Light-flash photolysis of caged inositol 1,4,5-trisphosphate (InsP_3) was used to generate reproducible transients of free InsP_3 in *Xenopus* oocytes, and the resulting liberation of Ca^{2+} from intracellular stores was monitored by recording Ca^{2+} -activated membrane currents and by use of the fluorescent Ca^{2+} indicator fluo-3. InsP_3 -mediated Ca^{2+} release was inhibited by elevating the intracellular free Ca^{2+} level, either by microinjecting Ca^{2+} into the cell or by applying conditioning light flashes to liberate Ca^{2+} . This inhibition followed a slow time course, being maximal after about 2 s and subsequently declining over several seconds. Negative feedback of Ca^{2+} ions on InsP_3 -mediated Ca^{2+} liberation may explain the oscillatory release of Ca^{2+} seen during activation of inositol phospholipid signaling in the oocyte, and the time course of the inhibition is consistent with the period of the oscillations.

Inositol 1,4,5-trisphosphate (InsP_3) serves as a second messenger in many cell types, where it acts primarily to cause the liberation of Ca^{2+} ions from intracellular stores (1-3). A characteristic feature of this system is that Ca^{2+} is often liberated in a cyclical manner, resulting in oscillations of intracellular free Ca^{2+} concentration. These have been directly monitored by the use of calcium indicators (4-7) or inferred from oscillations of Ca^{2+} -dependent membrane conductances (8-10). Because oscillations are still observed in voltage-clamped cells in the absence of extracellular Ca^{2+} (8, 11) it is clear that they cannot arise through regenerative electrical activity but, instead, must involve some intracellular biochemical oscillator. The mechanism of this is presently unclear but probably involves feedback at some stage in the messenger pathway (12-14). Ca^{2+} ions have been found to inhibit InsP_3 -mediated Ca^{2+} liberation in various cell types, and this inhibition has been postulated as a basis for oscillatory behavior (15-17). Here, we characterize the inhibitory action of Ca^{2+} in *Xenopus* oocytes, using light-flash photolysis of caged InsP_3 (17-21) to obtain a precisely controlled release of InsP_3 within the oocyte. The resulting liberation of Ca^{2+} from intracellular stores was monitored by recording Ca^{2+} -activated chloride membrane current (22) and by using the fluorescent Ca^{2+} indicator dye fluo-3 (23). An important finding is that the kinetics of Ca^{2+} inhibition of Ca^{2+} liberation are relatively slow and would thus introduce a delay in the feedback loop consistent with the observed period of the oscillations.

MATERIALS AND METHODS

Experiments were made on ovarian oocytes of *Xenopus laevis*, which were treated with collagenase to remove enveloping cells (24). Membrane currents were recorded using a two-electrode voltage clamp, with the membrane potential

held to -60 mV (24). During recording, oocytes were continually perfused with frog Ringer's solution at room temperature ($21-25^\circ\text{C}$). Intracellular injections were made through glass micropipettes, using pneumatic pressure pulses (11, 24). Caged InsP_3 [*myo*-inositol 1,4,5-trisphosphate $P^{4,5}$ -1-(2-nitrophenyl)ethyl ester] was obtained from Calbiochem and injected as a 1 or 5 mM aqueous solution. Each oocyte was loaded with a total of 1-10 pmol of caged InsP_3 . Ca^{2+} was injected as a 5 mM solution of CaCl_2 .

Procedures for light-flash photolysis of caged InsP_3 loaded into oocytes were as described (17). Briefly, UV light was focused on the oocyte as a square of $150 \mu\text{m}$ side, positioned close to the equator on the vegetal hemisphere, and the flash duration was set by an electronic shutter. Because of facilitation between light flashes (17), intervals of 80 s were allowed before each trial. Pipettes for injection of Ca^{2+} were inserted into the oocyte near the center of the light spot.

To monitor intracellular free Ca^{2+} , fluo-3 (Molecular Probes) was injected as a 1.5 mM aqueous solution through a separate micropipette, after loading the oocyte with caged InsP_3 . Fluorescence measurements were made using a second epifluorescence system, fitted with a fluorescein filter set and stacked above that used for photolysis of caged InsP_3 . Fluorescence emission was monitored by a photomultiplier. Increases in fluorescence emission correspond to increases in free Ca^{2+} concentration (23). Because fluo-3 does not show any shift in excitation or emission spectra with Ca^{2+} , it was not possible to calibrate the fluorescence signals, and the vertical scale is arbitrary for these traces. Nevertheless, comparisons of signals obtained under the same conditions give a relative measure of free Ca^{2+} changes. The fluorescence excitation light was focused as a circular spot on the oocyte, coincident with the photolysis light. Fluorescence records are blanked out during the photolysis light flashes.

RESULTS

Inhibition of Caged InsP_3 Responses by Ca^{2+} . Flashes of UV light applied to oocytes loaded with caged InsP_3 evoked transient inward membrane currents (Fig. 1). These do not arise from the light itself (25) or from side products of the photolysis reaction (17). Instead, the liberation of free InsP_3 causes Ca^{2+} to be released from intracellular stores and this, in turn, opens Ca^{2+} -dependent chloride channels in the cell membrane (8, 17, 22). Since each flash photolyzes only a tiny fraction of the caged InsP_3 in the oocyte (17), it was possible to evoke many responses without appreciably depleting the reserve of caged InsP_3 .

This paper concerns the inhibitory effect of intracellular Ca^{2+} on the caged InsP_3 response (17), and Fig. 1 illustrates the basic phenomenon. In Fig. 1A an oocyte was stimulated by repetitive flashes to give a train of current responses of roughly constant size. Injection of about 200 fmol of Ca^{2+}

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Abbreviation: InsP_3 , inositol 1,4,5-trisphosphate.

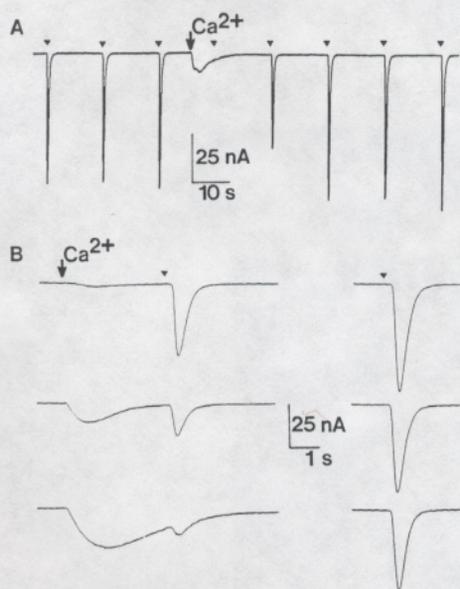


FIG. 1. Inhibition of responses evoked by photolysis of caged InsP_3 following intracellular injection of Ca^{2+} . In this, and other figures, traces show membrane currents at a clamp potential of -60 mV in response to brief (5–100 ms) flashes of light, given at the times indicated by the arrowheads. (A) Repetitive light flashes were given at 15-s intervals, beginning before the record shown, to allow the response size to facilitate to a roughly constant size. Injection of a single dose of about 200 fmol of Ca^{2+} was made when indicated by the arrow. (B) Dose dependence of Ca^{2+} inhibition. Traces on the left show responses evoked by light flashes given 3.5 s after injecting various amounts of Ca^{2+} , and those on the right show corresponding control records obtained 80 s later in response to the light flash alone. Approximate amounts of Ca^{2+} injected were, from top to bottom, 50, 200, and 400 fmol.

almost completely suppressed the response to a light flash 6.5 s later, and the response to a subsequent flash 15 s later was still depressed. This inhibitory action varied with the amount of Ca^{2+} , as illustrated in Fig. 1B. Injection of a small amount of Ca^{2+} , which itself evoked only a barely detectable chloride current, already produced an appreciable (35%) depression of the response to a subsequent light flash, and the extent of the depression grew progressively with increasing amount of Ca^{2+} . The inhibitory action of Ca^{2+} was localized to the site of injection, and no inhibition was observed when the photolysis light spot was displaced about 200 μm away from the tip of the Ca^{2+} pipette.

Time Course of Ca^{2+} Inhibition. Fig. 2A illustrates an experiment to study the time course of the inhibition of the caged InsP_3 response by Ca^{2+} . A light flash given 1 s after injection of Ca^{2+} evoked a response only slightly (23%) smaller than the control, even though the current directly evoked by Ca^{2+} injection was maximal at this time. However, as the interval between the Ca^{2+} injection and the light flash was progressively lengthened, the caged InsP_3 response at first became more depressed and then, at intervals longer than about 8 s, gradually recovered back to the control value.

The maximal inhibition varied between >90% and <50% in different oocytes and with different amounts of Ca^{2+} . To combine results from several oocytes we normalized the data by expressing them as a percentage of the maximal inhibition seen in each oocyte. Mean measurements from three oocytes are shown in this way in Fig. 2B. The maximal inhibition was attained between about 8 and 10 s following Ca^{2+} injection.

Ca^{2+} Does Not Alter the Threshold for InsP_3 Action. A threshold level of InsP_3 is required to trigger release of Ca^{2+} from intracellular stores in the oocyte (17, 26). We were therefore interested to see if the inhibitory action of Ca^{2+}

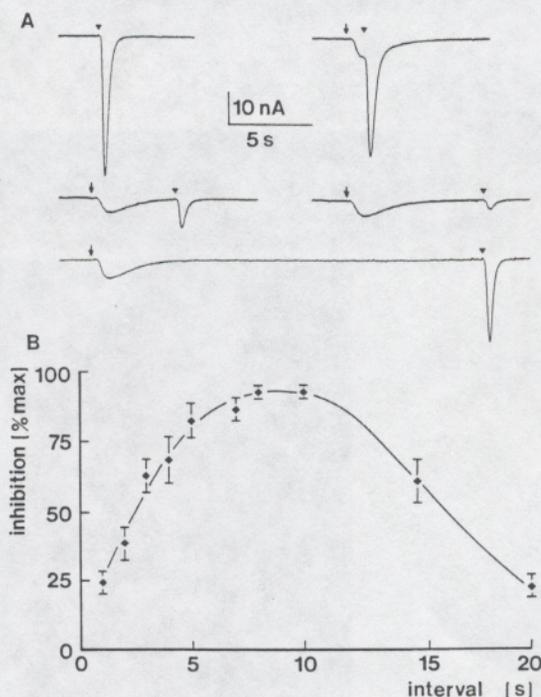


FIG. 2. Time course of inhibition by Ca^{2+} of the caged InsP_3 response. (A) Responses to light flashes of fixed intensity and duration delivered at various times (arrowheads) following injection of about 200 fmol of Ca^{2+} (marked by arrows). The first trace shows a control response to the light flash alone. (B) Measurements from three oocytes with inhibition of the caged InsP_3 response plotted as a function of the interval between the Ca^{2+} injection and the light flash. Data from each oocyte are scaled as a percentage of the maximal inhibition, and points indicate mean \pm SEM from five to seven observations.

injections arose because of an elevation of this threshold or because of a reduction in size of currents evoked by supra-threshold levels of InsP_3 . Light flashes of varying duration were given, so as to obtain dose–response relations for the currents evoked by photo release of different amounts of InsP_3 . Alternate records were obtained with flashes presented alone or following injection of nominally constant amounts of Ca^{2+} (Fig. 3A). Dose–response curves from one oocyte are shown in Fig. 3B. These indicate that the threshold duration to evoke a detectable current remained virtually unchanged following Ca^{2+} injection but that the currents evoked by longer flashes were proportionally reduced. Similar results were obtained in two other oocytes.

Inhibition with Paired Responses to Photolysis of Caged InsP_3 . The slow onset of Ca^{2+} inhibition illustrated in Fig. 2 might not accurately reflect the time course of the inhibitory process, because Ca^{2+} ions ejected from the injection pipette would diffuse slowly throughout the volume of the cell encompassed by the photolysis light spot. To circumvent this problem, we looked to see whether Ca^{2+} ions released diffusely by InsP_3 liberated by one light flash would inhibit the response to a subsequent light flash.

Fig. 4A shows a paired-flash experiment, where the duration of the initial (conditioning) flash was varied, and the second (test) flash was constant. When the conditioning flash was shorter than threshold, the response to the test flash was facilitated, because of summation of the levels of InsP_3 liberated by each flash (17). However, as the duration of the conditioning flash was increased above threshold, the size of the test response began abruptly to decrease and reduced progressively as the conditioning flash was increased so as to evoke larger conditioning responses (Fig. 4B).

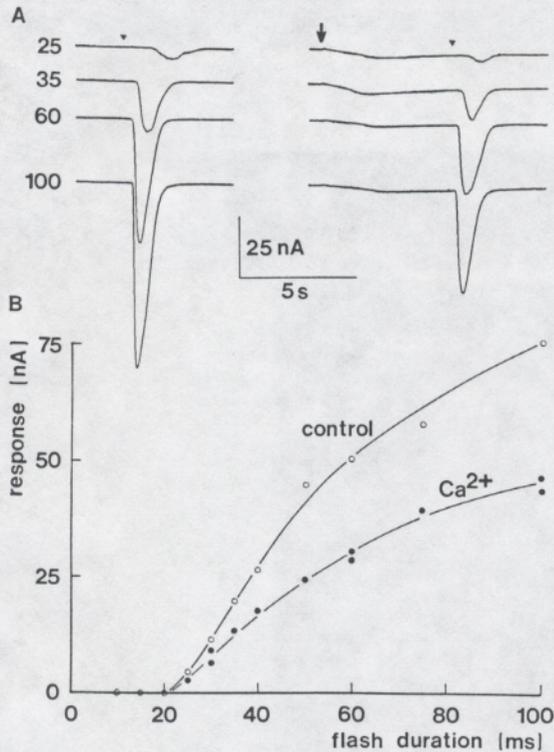


FIG. 3. Ca^{2+} inhibits the caged InsP_3 response by reducing the size of the evoked chloride current, not by increasing the threshold for InsP_3 action. (A) Traces on the left show currents evoked by light flashes of different durations (indicated in ms next to each trace), given when marked by the arrowheads. Traces on the right show responses to the corresponding light flashes when preceded by injection of about 100 fmol of Ca^{2+} (arrow). (B) Plot of peak evoked membrane current versus flash duration for control responses (open symbols) and responses evoked 5.5 s after injection of Ca^{2+} . Data are from the same oocyte as in A. Similar results were obtained in two additional oocytes.

The time course of inhibition with paired flashes was studied by varying the interval between the two flashes (Fig. 5). Each was of the same intensity and duration, so that the response to the first served as a control. At intervals shorter than about 1 s, it was not possible to resolve separate peaks in response to each flash. However, the combined response was more than double that to a single flash, indicating that the contribution from the second flash must have been facilitated. At an interval of 1 s, separate responses were apparent, and the size of the second response (estimated from the falling tail of the first response) was of similar size to the control. Lengthening the interval to 2 s produced a strong inhibition, but the currents subsequently grew as the interval was further increased, and at 12 s responses was again greater than the control. Finally, at increasing intervals between about 15 and 80 s the response gradually returned to the control size (data not shown).

We believe the time course of the paired flash response arises from a superimposition of two processes. One is the facilitation of InsP_3 levels mentioned above. This is maximal immediately following the conditioning flash and declines with a roughly exponential time course over several tens of seconds (17). The second process is an inhibition, resulting from Ca^{2+} liberated during the conditioning response. From Fig. 5B it appears that this inhibition is maximal about 2 s after the conditioning flash and that the subsequent recovery from inhibition is half-complete after about 6 s.

Changes in Intracellular Free Ca^{2+} . The above results clearly show that intracellular Ca^{2+} depresses the membrane currents evoked by photolysis of caged InsP_3 . This depres-

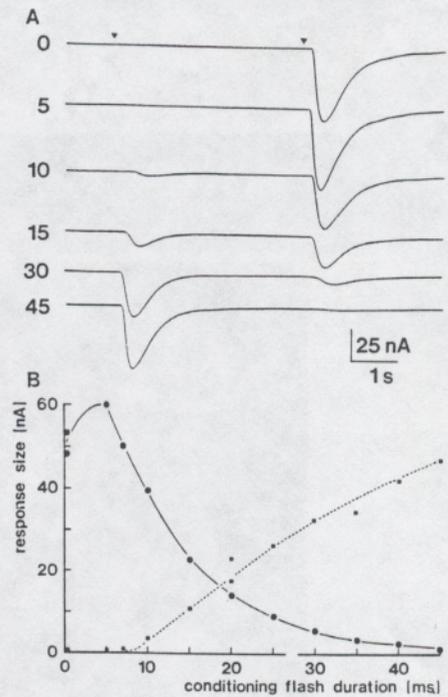


FIG. 4. Facilitation and depression with paired responses to photolysis of caged InsP_3 . (A) Traces show currents evoked by pairs of light flashes applied at an interval of 4 s. Flashes were of identical intensity. The duration of the second was fixed at 45 ms, whereas the duration of the first was varied and is indicated (in ms) next to each trace. (B) Sizes of responses to the first (■) and second (●) light flashes are plotted as a function of the duration of the first light flash. Same oocyte as A.

sion might arise if Ca^{2+} ions inhibit the action of InsP_3 to cause further release of Ca^{2+} from intracellular stores or if the channels mediating the membrane current are inactivated by Ca^{2+} . It already seemed that the latter explanation was unlikely, because currents evoked by paired Ca^{2+} injections show little depression (unpublished data). However, to fur-

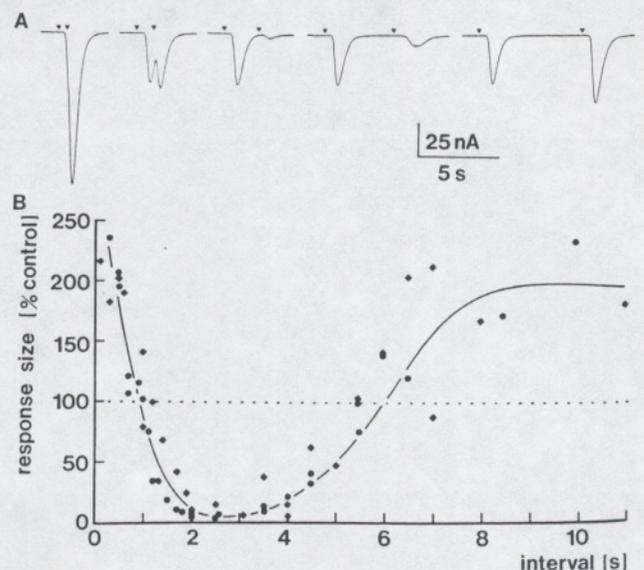


FIG. 5. Time course of facilitation and depression measured with paired light flashes. (A) Records show currents evoked by paired light flashes of identical intensity and duration, given at various intervals as indicated by the arrowheads. (B) Size of the response to the second flash plotted against interval between the flashes. Data are shown from two oocytes (different symbols) and are scaled as percentage of the response evoked by the first flash in each trial.

ther discriminate between these two possibilities, we used the fluorescent calcium probe fluo-3 (23) to monitor elevations in intracellular free calcium evoked by photolysis of caged InsP_3 . Unlike previous fluorescent Ca^{2+} indicators, the peak excitation wavelength for fluo-3 lies in the visible spectrum, so that it was possible to monitor its fluorescence without causing appreciable photolysis of caged InsP_3 .

Simultaneous records of fluo-3 fluorescence and membrane current are shown in Fig. 6 in response to photolysis of caged InsP_3 by identical light flashes delivered alone (left) or following injection of Ca^{2+} (right). The fluo-3 Ca^{2+} signal evoked by the light flash was strongly reduced by a preceding injection of Ca^{2+} . The mean size of the fluo-3 signal measured in six trials, like that illustrated, was reduced to $34.0\% \pm 2.7\%$ (mean \pm SEM) of the control following injection of Ca^{2+} , whereas the corresponding value for the membrane current was $67.8\% \pm 3.7\%$.

Depression of the fluo-3 signal was seen also in experiments using paired light flashes (Fig. 7). A test flash given 2 s following a conditioning flash evoked only a small increment in the Ca^{2+} signal but, similar to the results described above for the membrane current, less depression was evident at shorter and longer intervals.

DISCUSSION

The results demonstrate that an elevation of intracellular free Ca^{2+} level inhibits membrane current responses evoked by photolysis of caged InsP_3 in *Xenopus* oocytes. Because inhibition was seen also using fluo-3 as a monitor of intracellular free Ca^{2+} , it seems that this effect does not arise because of desensitization of the Ca^{2+} -activated chloride current. Furthermore, it seems unlikely that the Ca^{2+} inhibition arose because less Ca^{2+} was released by a given light flash, since elevations in Ca^{2+} did not alter the threshold flash duration required to evoke a response. We thus conclude that cytoplasmic free Ca^{2+} inhibits the action of InsP_3 to liberate Ca^{2+} sequestered in intracellular stores.

The time course of the Ca^{2+} inhibition was measured in two ways: by injecting Ca^{2+} from a micropipette and by using a conditioning light flash to liberate Ca^{2+} . Both methods indicated that inhibition was maximal only after several seconds, but the time course was consistently slower following Ca^{2+} injections. The paired flash experiments probably give a better indication of the actual time course of Ca^{2+} inhibition, because the spread of Ca^{2+} ions from the pipette tip throughout that part of the cell exposed to the photolysis light may be slow. Following a conditioning flash, the maximal inhibition of membrane current and intracellular Ca^{2+} responses

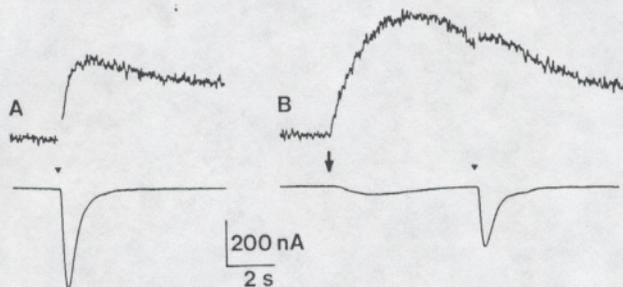


FIG. 6. Inhibition by Ca^{2+} of membrane current and fluo-3 signals evoked by photolysis of caged InsP_3 . Upper traces show fluo-3 fluorescence, and lower traces show membrane current. The vertical scale for the fluorescence is uncalibrated; upward deflections correspond to increasing free Ca^{2+} . (A) Control responses to a light flash applied when marked by the arrowhead. (B) The same light flash was repeated 6 s after injection of about 25 fmol of Ca^{2+} into the oocyte (marked by arrow).

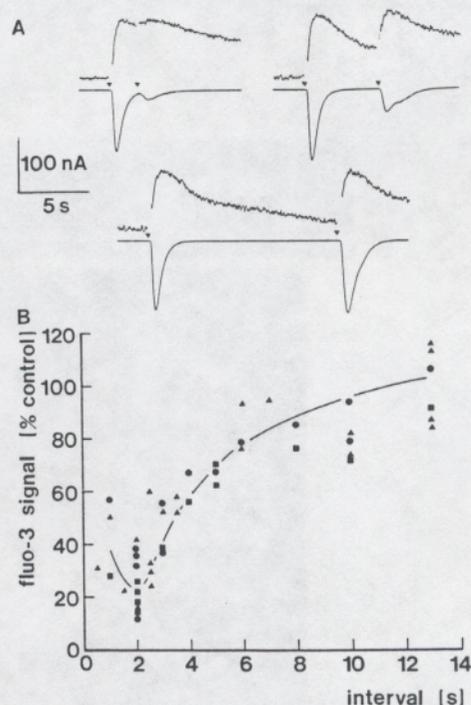


FIG. 7. Fluo-3 and membrane current signals evoked by paired light flashes. (A) The upper trace in each frame shows fluo-3 fluorescence, and the lower trace shows membrane current. Light flashes of fixed intensity and duration were given when marked by the arrowheads. (B) Relative sizes of the fluo-3 signal evoked by the second light flash plotted against the interval between flashes. The size of the second response was measured as the increment in fluorescence above the tail of the first response and is expressed as a percentage of the response to the first flash in each pair. The duration of the light flashes in these experiments was about five times the threshold, so little facilitation was apparent in either the current or the fluo-3 signals. Data were from three oocytes (different symbols).

was seen after about 2 s, and the subsequent recovery from inhibition was half-complete after about 5–6 s.

The binding of InsP_3 to its intracellular receptor is inhibited by low levels of free Ca^{2+} (27), and Ca^{2+} release from microsomes is reduced by Ca^{2+} (28). These effects are thought to arise through a distinct Ca^{2+} -binding protein (calmodin) (27), and this indirect action of Ca^{2+} on the InsP_3 receptor might account for the slow time course of the inhibition.

Several models have been proposed to account for the oscillatory liberation of Ca^{2+} during inositol phospholipid signaling, all of which involve Ca^{2+} feedback on earlier stages in the pathway (for reviews, see refs. 3, 13, and 14). In one class of model, Ca^{2+} modulates the rates of InsP_3 formation or breakdown, thus leading to fluctuating levels of InsP_3 (14, 29). This mechanism is probably not important in the oocyte, because intracellular injection of a single dose of InsP_3 evokes oscillatory responses (8, 10), whereas injection of Ca^{2+} normally evokes only a transient spike of current (22). Furthermore, in pancreatic acinar cells, oscillations are produced by a nonmetabolizable analogue of InsP_3 (30). A second class of model invokes Ca^{2+} -induced Ca^{2+} release to account for the oscillations (14, 31). This explanation also does not seem applicable in the oocyte, because intracellular injections of Ca^{2+} yield transient currents that increase linearly with increasing amount of Ca^{2+} (unpublished data) and are not enhanced when Ca^{2+} is injected during oscillatory activity (11). Finally, a third model involves negative feed-

back of Ca^{2+} on InsP_3 -mediated Ca^{2+} release (15–17, 28). The present results lend strong support to this scheme.

We envision that a rise in intracellular level of InsP_3 leads initially to a rapid liberation of Ca^{2+} , thus evoking a large chloride current. However, within 1 or 2 s Ca^{2+} liberation is inhibited by the elevated level of intracellular free Ca^{2+} , so that the Ca^{2+} level and the associated chloride current decline as Ca^{2+} ions are sequestered or extruded. After several seconds the Ca^{2+} release mechanism recovers from inactivation and, because InsP_3 is still present, Ca^{2+} liberation begins again. Thus, a delayed negative feedback of Ca^{2+} ions on Ca^{2+} liberation will result in cyclical release of Ca^{2+} in the presence of a constant level of InsP_3 . The period of InsP_3 -induced oscillations in the oocyte is about 6 s at room temperature (12) and does not vary appreciably with amount of InsP_3 (unpublished data). This value appears consistent with the time course of recovery of InsP_3 -mediated Ca^{2+} liberation from inhibition by Ca^{2+} (half-recovery, 5–6 s at room temperature). As well as giving rise to the oscillations, the mechanism of Ca^{2+} inhibition may also account for the characteristic time course of responses to agonist activation or injection of InsP_3 , which often show an initial large spike of current followed by gradually increasing oscillations (8, 32).

An important finding yet to be explained is the rapid decline of Ca^{2+} -activated membrane current as compared to the decline of the fluo-3 Ca^{2+} signal. This is probably not due to any artifactual property of the Ca^{2+} monitor, since earlier experiments using aequorin as a Ca^{2+} indicator showed that oscillatory currents are accompanied by an apparently sustained elevation of intracellular free calcium (8). Furthermore, it is unlikely that these differences arise because of modulation of the properties of the Ca^{2+} -activated channels. Instead, there may be spatial differences in the cellular mechanisms of Ca^{2+} release and sequestration close to the plasma membrane.

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