Characteristics of membrane currents evoked by photoreleased inositol trisphosphate in *Xenopus* oocytes

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Parker, Ian, and Isabel Ivorra. Characteristics of membrane currents evoked by photoreleased inositol trisphosphate in *Xenopus* oocytes. *Am. J. Physiol.* 263 (*Cell Physiol.* 32): C154-C165, 1992.—Photorelease of inositol 1,4,5-trisphosphate ($InsP_3$) from a caged precursor was used to study characteristics of Ca^{2+}-activated Cl^- currents activated in *Xenopus* oocytes by the $InsP_3$-Ca^{2+} signaling pathway. Photolysis flashes shorter than a threshold duration evoked no response, but the current amplitude then grew about linearly as the flash duration was further lengthened. Currents directly evoked by photorelease of Ca^{2+} from a caged precursor grew linearly with increasing flash duration and showed a small threshold before they were activated. However, the major part of the threshold of $InsP_3$-evoked responses appears to arise because a certain concentration of $InsP_3$ (estimated to be ~60 nM) is required to evoke Ca^{2+} liberation. Subthreshold conditioning flashes potentiated responses to subsequent flashes, and the potentiation increased linearly with increasing conditioning flash duration before abruptly declining. The potentiation decayed exponentially with a time constant of ~17 s with increasing interflash interval. Currents evoked by photoreleased $InsP_3$ began after a latency that shortened from 10 s or longer to 100 ms as the photolysis intensity was increased. This dose dependence of the latency could be quantitatively explained by the time required for the $InsP_3$ concentration to rise above threshold. Intracellular injection of heparin (a competitive antagonist at the $InsP_3$ receptor) increased the threshold for $InsP_3$ action, as did increased temperature. We conclude that several characteristics of $InsP_3$-evoked responses, including their dose dependence, latency, and facilitation with paired stimuli, arise because a distinct threshold level of $InsP_3$ is required to evoke release of Ca^{2+} from intracellular stores.

calcium; caged inositol trisphosphate

INOSITOL 1,4,5-trisphosphate ($InsP_3$) serves as an intracellular second messenger molecule in almost all cell types, where it functions in part by causing the liberation of calcium ions sequestered in intracellular stores (2). The mechanisms by which $InsP_3$ liberates Ca^{2+} show considerable complexities regarding dose dependence and spatial and temporal characteristics, many of which are poorly understood (2, 11, 19, 20, 26). Because $InsP_3$ does not cross the cell membrane, it has been difficult to study such phenomena in intact single cells. However, the recent availability of photolabile "caged" $InsP_3$ (17) now provides a way to rapidly and precisely elevate intracellular levels of $InsP_3$ in defined regions of a cell (28, 31, 35, 36).

We previously described the use of caged $InsP_3$ to study $InsP_3$ signaling in *Xenopus* oocytes (31-33, 35). Photolytic release of $InsP_3$ from its caged precursor evoked transient Cl^- currents, which arose because $InsP_3$ liberated intracellular Ca^{2+} and that, in turn, activated Ca^{2+}-dependent Cl^- channels in the plasma membrane (21). An important finding was that the Cl^- currents increased in a strongly nonlinear manner with increasing photorelease of $InsP_3$ (35). Subsequent experiments using fluorescent indicators to monitor intracellular Ca^{2+} have shown that the nonlinearity arises primarily through the process of $InsP_3$-mediated Ca^{2+} liberation rather than activation of the Cl^- channels (15, 33) and are beginning to provide clues as to the underlying mechanism (35). The present paper is concerned not with the origin of the nonlinearity but instead with exploring how it determines various aspects of $InsP_3$-mediated cellular responses, including their dose dependence, latency to onset, and facilitation with paired stimuli.

METHODS

Experiments were done on oocytes of *Xenopus laevis*, obtained after killing donor frogs by decerebration and pithing. Details of preparation of oocytes and electrophysiological recording were as previously described (37). Briefly, membrane currents were recorded in defolliculated oocytes using a two-electrode voltage clamp to hold the membrane potential at −60 mV. During recording, the oocyte was continually superfused with Ringer solution with the following composition (in mM): 115 NaCl, 2 KCl, 1.8 $CaCl_2$, and 5 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) at pH ~7.0. The solution temperature was monitored by an electrically isolated thermocouple placed just downstream of the oocyte and was normally at room temperature (21-24°C). A heat exchanger mounted before the inflow to the recording chamber allowed the temperature to be varied when desired. Intraoocyte injections were made by pneumatic pressure pulses from micropipettes. Volumes of fluid injected were estimated by measuring the diameters of fluid droplets expelled with the pipette tip in the air. All injection solutions included 5 mM HEPES (pH 7.0) and were passed through a 0.22-μm Millipore filter. Except for solutions containing Ca^{2+} or caged Ca^{2+}, 50 μM EDTA was further added to chelate contaminating Ca^{2+}. Caged $InsP_3$ [*myo*-inositol 1,4,5-trisphosphate, $P^{4(5)}$-1-(2-nitrophenyl)ethyl ester] was obtained from Calbiochem (La Jolla, CA) and injected at a concentration of 1 mM. Low-molecular-weight heparin (4,000-6,000) was obtained from Sigma and injected as a 50 mg/ml aqueous solution. Approximate molar amounts of heparin given in the text were calculated assuming a mean molecular mass of 5,000 Da. Dimethoxynitrophenamine (DM-nitrophen; caged Ca^{2+}) was obtained from Calbiochem and injected as a 50 mM solution after a roughly equimolar amount of $CaCl_2$ was added.

Oocytes were normally loaded with between 1 and 5 pmol caged $InsP_3$ (corresponding to final intracellular concentrations of a few μM) and were allowed to rest for 30 min or more before beginning recordings to allow distribution of the caged $InsP_3$ throughout the cell. Near-ultraviolet (UV) light (between ~340 and 400 nm) from a xenon arc lamp was used for photolysis. The flash duration was set by an electronic shutter (Newport, Fountain Valley, CA), and the light was focused onto the oocyte through a microscope objective lens as a square or rectangle of variable size. For most experiments a ×6.3 Neofluar objective (Zeiss) was used and, except where otherwise noted, the light was arranged as a square of ~50 μm/side. Measurements of the

near-UV light output from this objective using a Gentec ED100A joulemeter gave an energy of 1.2 μJ for a 3-ms duration flash illuminating an area of 0.1 mm^2, which corresponds to an irradiance of ~4 mW/mm^2. Oocytes were usually mounted with the equator facing the lens, and the light square was positioned close to the equator on the vegetal hemisphere. This position was chosen to maximize the responses. Currents evoked by injections of $InsP_3$ are larger at the animal compared with the vegetal pole (1), but the pigmentation of the animal hemisphere strongly attenuates the photolysis light. By comparison with the photolysis system used in earlier experiments (30, 35) the present system gave a more stable and reproducible light output by virtue of an improved shutter mechanism and by mounting the lamp vertically to avoid arc "wander." Further details of the optical system, and procedures for loading caged $InsP_3$, are described by Parker (31). Experiments with caged Ca^{2+} were done using the same procedures as described for caged $InsP_3$, except that the amount loaded into the oocyte was greater (usually ~500 pmol) and recordings began after allowing ~10 min for equilibration.

To measure the extent of photolysis resulting from a given light flash, a bioluminescent luciferin-luciferase assay was used to determine the amount of ATP released from a caged precursor. Because caged $InsP_3$ and caged ATP use the same caging group, this procedure provides a calibration for photorelease of $InsP_3$ but is not applicable to caged Ca^{2+}, since DM-nitrophen uses a very different photolytic reaction. A droplet of 1 μl volume containing undiluted ATP assay mix (FL-AAM, Sigma) together with 100 μM caged ATP [adenosine-5′-triphosphate, P^3-1-(2-nitrophenyl)ethyl ester; Calbiochem] was placed in a small humidified chamber at the focus of the objective lens. The field aperture on the photolysis unit was enlarged to cover the complete droplet, but otherwise the settings of the photolysis system were as used during oocyte experiments. After UV light flashes of various durations, the luminescence of the droplet was measured by a photomultiplier mounted on the microscope phototube to give a relative measure of the amount of free ATP that had been formed. The intensity of luminescence increases linearly with the amount of ATP (Sigma Technical Bulletin BAAB-1). A shutter protected the photomultiplier from overload during the light flashes, and control experiments with droplets from which caged ATP was omitted showed that UV light flashes caused no increase in light emission from the ATP assay mix alone.

RESULTS

Currents evoked by elevating intracellular free Ca^{2+}. Many of the experiments described here concern the relationship between $InsP_3$ and Ca^{2+} liberation from intracellular stores. Because measurements of the resulting Ca^{2+}-activated Cl^- membrane current (21) were used as an indicator of intracellular Ca^{2+}, we first determined the relationship between Ca^{2+} and the Cl^- current. This was done in two ways, by recording currents evoked by microinjection of different amounts of Ca^{2+} into the oocyte and by flash photolysis of caged Ca^{2+} (DM-nitrophen) loaded into the oocyte (17). Ca^{2+} injections allow the total amount of free Ca^{2+} to be estimated, but it is technically difficult to achieve reproducible injections, and the resulting distribution of free Ca^{2+} in the oocyte is spatially inhomogeneous. Flash photolysis of caged Ca^{2+}, on the other hand, is expected to produce a uniform release of Ca^{2+} near the plasma membrane. The extent of photolysis varies linearly with flash duration, but it is difficult to quantify the absolute amounts of Ca^{2+}.

Figure 1*A* illustrates membrane currents evoked by injections of different amounts of Ca^{2+} into the animal hemisphere of a voltage-clamped oocyte. Ca^{2+} injections evoked transient responses, which usually began within ≤100 ms of the pressure pulse, reached a peak after ~1 s, and then declined to the baseline over a few seconds. These currents were inwardly directed at a potential of −60 mV but inverted direction at about −25 mV, which corresponds to the Cl^- equilibrium potential in the oocyte (16). In addition to these transient responses, we found that some oocytes also showed long-lasting (several minutes) oscillatory currents. However, in agreement with previous reports (7, 8, 21), these oscillatory responses developed only after many repeated injections of Ca^{2+} or after injections that evoked large (several μA) currents, and oocytes were discarded if they began to show oscillations.

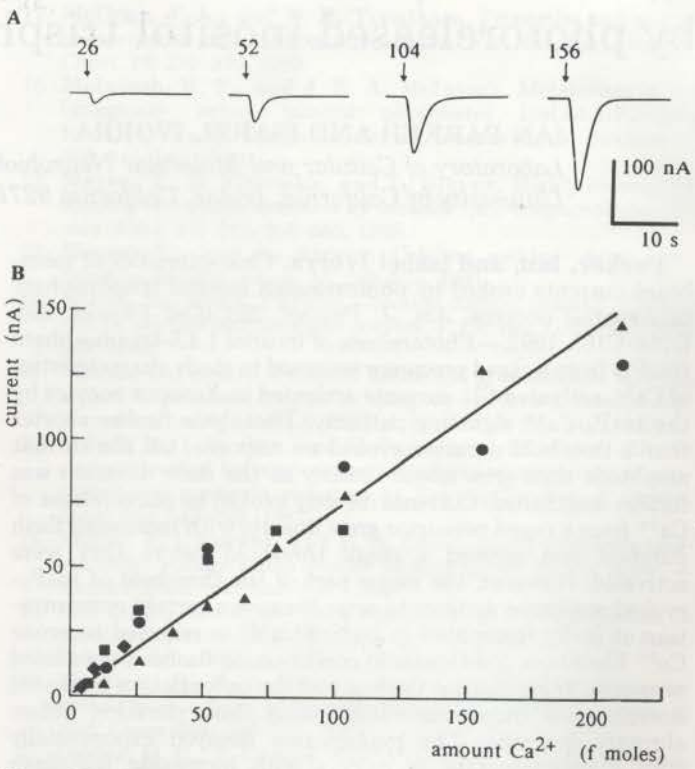

Fig. 1. Membrane currents evoked by injections of different amounts of Ca^{2+} into animal hemisphere of a single oocyte. Injections were made from a micropipette filled with 0.5 mM $CaCl_2$, using pneumatic pressure pulses of 40–200 kPa and durations of 80 ms. Amount of Ca^{2+} injected was varied in each run by applying different numbers of pulses (1, 2, 4, etc.) in rapid succession. *A*: sample records of currents recorded at a clamp potential of −60 mV. Arrows, times of Ca^{2+} injections; amounts injected are indicated in femtomoles next to each trace. In this and other figures, downward deflections correspond to inward membrane currents. *B*: variation in peak size of response with amount of Ca^{2+} injected. Different symbols correspond to different runs using different pneumatic pressures. Line drawn by eye.

Measurements of peak sizes of currents evoked by injection of different amounts of Ca^{2+} into the animal hemisphere of one oocyte are shown in Fig. 1*B*. Responses were first detected to injection of as little as 5 fmol Ca^{2+} (10 pl of 0.5 mM $CaCl_2$), and the current then increased about linearly with amounts up to at least 200 fmol. A straight line drawn through the data passed close to the origin, indicating that there was no detectable

threshold in the amount of Ca^{2+} required to evoke a response. Furthermore, there was no evidence of saturation of the current with larger amounts of Ca^{2+}, probably because the largest currents in this experiment (~200 nA) were still much smaller than those (several μA) that can be evoked by injections of large amounts of Ca^{2+} (21).

Similar results were obtained in a total of five oocytes (obtained from two frogs). When plotted on double-logarithmic coordinates (data not shown), regression lines fitted to the measurements from each oocyte had a mean slope of 1.09 ± 0.09 (SE). The limiting slope of the dose-response relationship on double-logarithmic axes (Hill coefficient) gives a measure of the cooperativity of the response, and a slope close to unity therefore indicates that the peak current varied linearly with the amount of Ca^{2+} injected.

Figure 2*A* shows currents evoked by photolysis light flashes of various durations applied to an oocyte loaded with Ca^{2+}-saturated DM-nitrophen at a final intracellular concentration of ~0.5 mM. The flashes gave inward currents that began almost instantaneously (latency <10 ms) and decayed within ~2 s. This time course was more

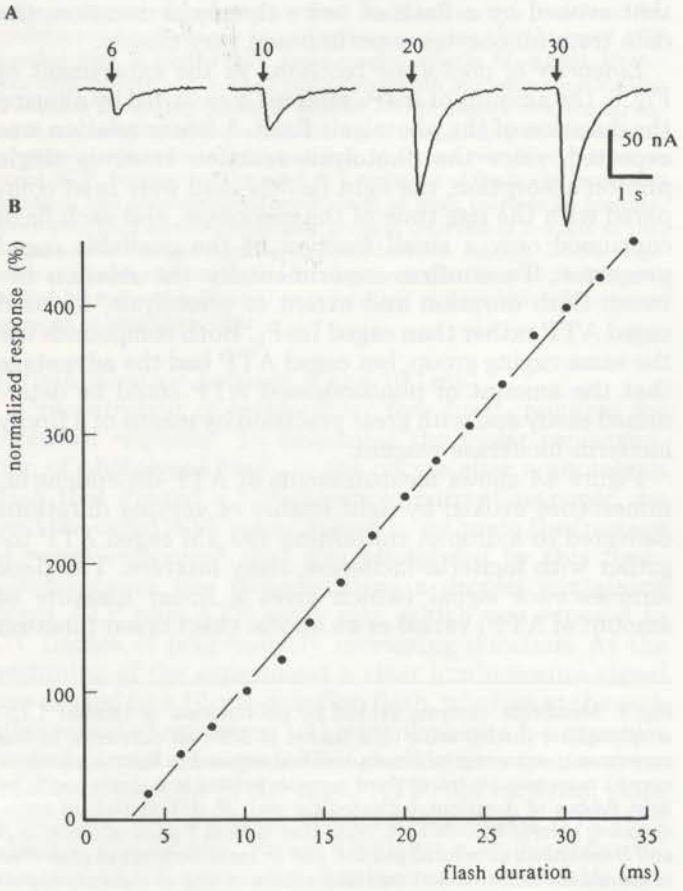

Fig. 2. Membrane currents evoked by photolysis of caged Ca^{2+} [dimethoxynitrophenamine (DM-nitrophen)]. Data are from an oocyte that was loaded with ~10 nl of 50 mM Ca^{2+}-saturated DM-nitrophen. *A*: sample records of currents evoked by photolysis flashes of various durations (indicated in ms next to each trace). Arrows mark times of flashes. *B*: peak sizes of currents measured from traces like those in *A*, plotted as a function of flash duration. Currents are expressed as a percentage of that evoked by 10-ms test flashes delivered at intervals throughout experiment. Currents evoked by 10-ms flashes declined from 54 nA at start of experiment to 36 nA at end.

rapid than that seen with Ca^{2+} injections, probably because Ca^{2+} was photoreleased homogeneously in the cytoplasm close to the plasma membrane rather than diffusing from a point source at some distance into the cell. Measurements of current amplitudes were complicated because responses to constant test flashes gradually declined over several minutes, possibly because of consumption of caged Ca^{2+} or slow diffusional equilibration of caged Ca^{2+} throughout the oocyte. To correct for this, 10-ms-duration test flashes were interspersed at intervals throughout an experiment, and responses evoked by flashes of various durations were scaled as a percentage of the interpolated 10-ms control responses. As illustrated in Fig. 2*B*, the normalized current response varied as an almost exactly linear function of flash duration over a 20-fold range of currents, but a small threshold was apparent and the current extrapolated to zero at a flash duration of 2.5 ms. Similar results were obtained in two further oocytes.

Dose-response relationship of $InsP_3$-evoked currents. Oocytes loaded with caged $InsP_3$ show transient currents on stimulation by brief light flashes (e.g., Fig. 3*A*). These arise through the liberation of intracellular Ca^{2+}, since they are abolished by intracellular injection of the Ca^{2+}-chelating agent ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), but are unaffected by removal of extracellular Ca^{2+} (35). Furthermore, the currents reverse direction at about the Cl^- equilibrium potential in the oocyte, indicating that they are carried by a flux of chloride ions. Although activation of muscarinic and other receptors leads to the generation of both Cl^- and K^+ currents in follicle-enclosed oocytes, the K^+ currents arise in follicular cells (which are electrically coupled to the oocyte proper) and are activated by adenosine 3',5'-cyclic monophosphate (cAMP), not $InsP_3$ (25). The present experiments were made in oocytes from which the follicular cells were removed by collagenase treatment, and the records are uncontaminated by K^+ currents.

The $InsP_3$-evoked Cl^- currents show an apparent threshold, in that the light flash must exceed a certain duration or intensity before any current is detected. We had previously proposed (35) that this effect may arise if Ca^{2+} liberation increases as a steep power function of the level of $InsP_3$ (19) so that low levels of $InsP_3$ evoke responses that are too small to detect. To test this hypothesis, we repeated experiments to measure currents evoked by flashes of different durations, under conditions that enhanced the stability and resolution of the recordings.

Figure 3*A* shows currents evoked in an oocyte loaded with caged $InsP_3$. In this, and all other experiments, the cell was voltage clamped at a potential of −60 mV. A flash of 25 ms duration gave no (<0.25 nA) current, whereas a small increase in flash duration to 27 ms evoked a clear (8 nA) response. Further prolongation of the flash evoked progressively larger currents, which began after shorter latencies and showed a faster rate of rise (Fig. 3*B*). Measurements of the peak size of membrane currents are plotted in Fig. 3*C* as a function of flash duration. The responses began abruptly as the flash duration was lengthened beyond ~25 ms, and for durations between 30

and 110 ms the current increased about linearly with flash duration. These results cannot be satisfactorily described by a power relation. To obtain a reasonable fit to data near the "foot" of the dose-response curve, a sixth or higher order power function was required (dotted curve in inset to Fig. 3*C*). However, this predicted that the response would increase extremely steeply as the flash duration was further lengthened, whereas a linear increase was observed.

Figure 3*D* shows a similar analysis of the maximal rate of rise of currents evoked by flashes of different duration. The rate of rise was zero for flash durations shorter than ~25 ms but then increased progressively as the flash was lengthened and varied about linearly with flash durations between ~40 and 110 ms. A sixth-power relationship provided a reasonable fit to the foot of the relationship but increased much more steeply than the experimental data for longer flash durations.

Results like those in Fig. 3*C* were seen in more than 12 oocytes examined. Although the absolute flash duration and amplitude of currents evoked by suprathreshold flashes varied considerably between oocytes (probably because of variations in amount of caged $InsP_3$ injected as well as differing sensitivities between cells), the form of the relationship between flash duration and evoked current was remarkably consistent, and all oocytes showed an abrupt onset of responses with increasing flash duration followed by a linear increase. This is illustrated in Fig. 3*E*, which shows pooled data from five oocytes. The threshold flash durations in these oocytes varied between 10 and 22.5 ms, and the currents evoked by flashes with durations twice the threshold varied between 68 and 200 nA. However, when the measurements were normalized, by expressing flash durations as multiples of the threshold in each oocyte and the currents as multiples of that evoked by a flash of twice threshold duration, the data from all oocytes superimposed very closely.

Linearity of photolysis reaction. In the experiment of Fig. 3, the amount of $InsP_3$ released was varied by altering the duration of the photolysis flash. A linear relation was expected, since the photolysis reaction involves single photon absorption, the light flashes used were brief compared with the rise time of the responses, and each flash consumed only a small fraction of the available caged precursor. To confirm experimentally the relation between flash duration and extent of photolysis, we used caged ATP rather than caged $InsP_3$. Both compounds use the same caging group, but caged ATP had the advantage that the amount of photoreleased ATP could be determined easily and with great precision by means of a firefly luciferin-luciferase reagent.

Figure 4*A* shows measurements of ATP-dependent luminescence evoked by light flashes of varying durations delivered to a droplet containing 100 μM caged ATP together with luciferin-luciferase assay mixture. The peak luminescence signal (which gives a linear measure of amount of ATP) varied as an almost exact linear function

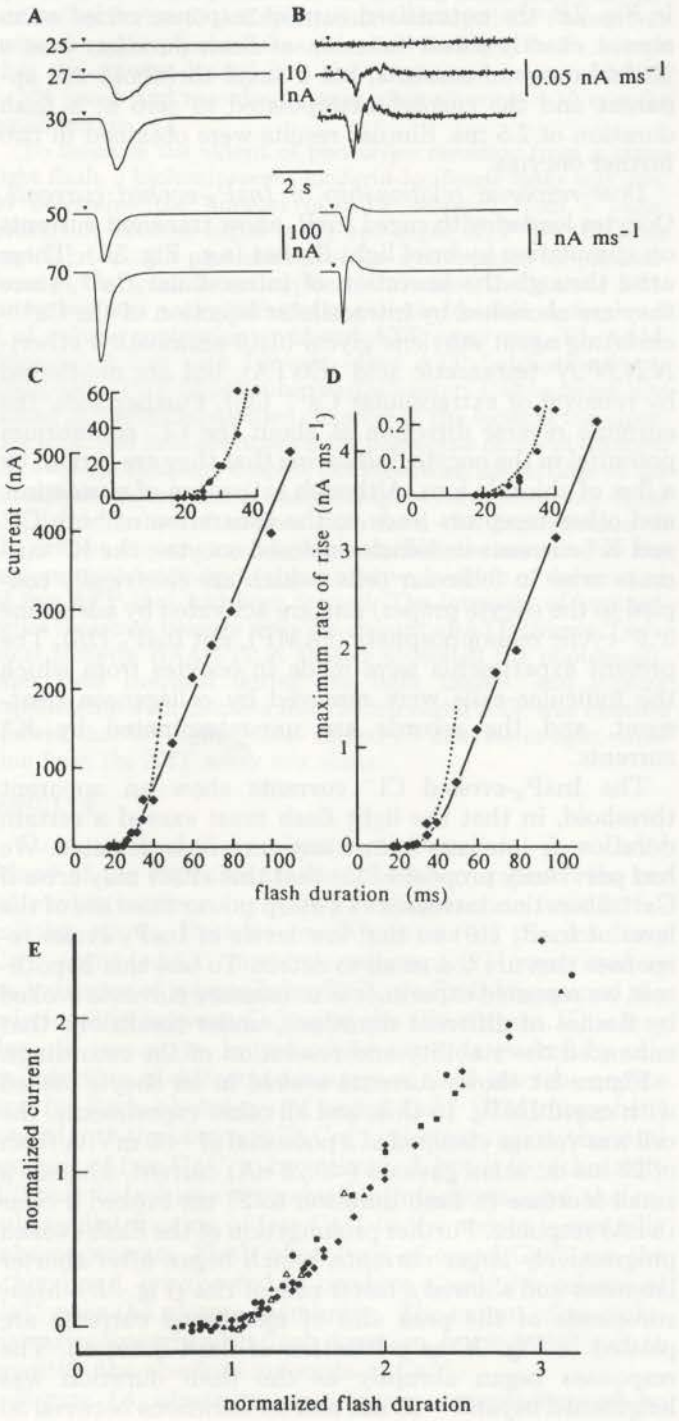

Fig. 3. Membrane currents evoked by photorelease of inositol 1,4,5-trisphosphate ($InsP_3$) using light flashes of different durations. In this experiment, entire vegetal hemisphere was exposed to light to maximize current responses. *A*: traces show currents evoked in a single oocyte by light flashes of durations indicated (in ms). *B*: differentials of corresponding current records in *A*. Note that bottom 2 pairs of traces in *A* and *B* are shown at reduced gains. *C* and *D*: measurements of peak sizes of membrane currents and maximum rates of rise of current, respectively, plotted as functions of flash duration. *Insets*: measurements close to threshold, replotted on expanded scales. Dotted curves in main and inset graphs show 6th power relationships, scaled to obtain a good fit to data points near threshold. Solid curves in main graphs are drawn by eye. *E*: pooled data from 5 oocytes (indicated by different symbols) showing relationship between flash duration and peak size of evoked current. Flash durations are normalized with respect to durations that evoked just-threshold responses in each oocyte, and currents are normalized with respect to currents evoked in each oocyte by a flash of twice threshold duration.

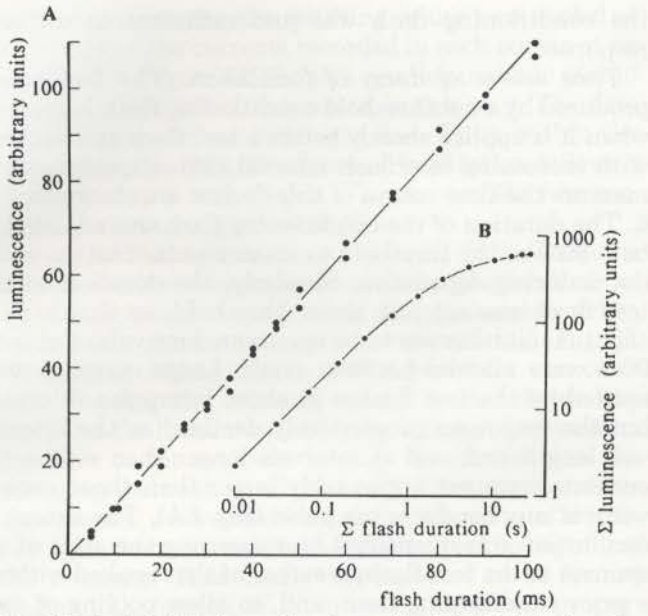

Fig. 4. Extent of photolysis resulting from ultraviolet flashes of varying duration, estimated by using a bioluminescence reaction to assay release of ATP from a caged precursor. *A*: amplitude of ATP-dependent luminescence signals evoked by photolysis flashes of various durations. Recordings were made from a 20-μl droplet containing 100 μM caged ATP together with undiluted ATP assay reagent (Sigma FL-AAM). Photolysis light covered only a small part of droplet to minimize consumption of caged ATP during course of experiment. *B*: results of a similar experiment, except that the whole of a small (0.5 μl) droplet was exposed to ultraviolet light for a sufficient time to almost completely photolyse caged ATP. Droplet was exposed to a sequence of flashes of increasing duration, and measurements were made of peak increase in luminescence after each flash. Flashes were given at intervals of 2–3 min so that luminescence decayed back to baseline between stimuli. Double logarithmic plot shows cumulative luminescence (i.e., sum of peak signals evoked by successive flashes) as a function of cumulative duration of exposure to photolysis light.

of flash duration between 5 and 100 ms.

Concentration of intracellular free $InsP_3$ required for threshold response. To determine the molar concentration of photolysed $InsP_3$ in the oocyte after a photolysis flash that evoked a just-threshold current response, we used the caged ATP assay system to estimate the fraction of caged compound that was photolysed by this flash. Luminescence was recorded from a droplet containing caged ATP plus ATP assay mix, which was exposed to UV flashes of progressively increasing duration. At the beginning of the experiment a clear luminescence signal was evoked by a 10-ms-duration flash, whereas at the end, after repeated exposures to longer flashes, a 5-s exposure evoked almost no signal. This decline in response arose from consumption of the caged ATP, not luciferin, since sensitivity was restored when further caged ATP was added to the droplet. Figure 4*B* shows a double-logarithmic plot of the cumulative luminescence evoked by successive flashes as a function of the cumulative duration of UV exposure. For durations up to a few hundred milliseconds the relationship was linear, but then it curved off to reach a maximum after exposure for a total of ~20 s as the reserve of caged ATP became exhausted. Thus the total cumulative luminescence corresponded to photorelease of all the ATP, and the fractional amount released by a given light flash could be calculated from the ratio of the luminescence resulting from that flash compared with the total luminescence.

In Figure 3, a just-threshold response was evoked by a flash of 25 ms duration applied to an oocyte that was loaded with ~5 pmol caged $InsP_3$. If we assume a cytosolic volume of 1 μl, the resulting final intracellular concentration would have been ~5 μM. From the data of Fig. 4*B*, the maximal luminescence of the calibration drop was 108 times greater than that evoked by a 25-ms flash, so that a flash of this duration would have photolysed ~0.9% of the total amount of caged $InsP_3$ in the region of the cell exposed to light. Thus the concentration of $InsP_3$ required to evoke a threshold Ca^{2+} signal was calculated to be ~45 nM. Other estimates made in this way in 14 oocytes yielded a mean value of 61 ± 14 (SE) nM for the threshold concentration of $InsP_3$. These measurements are subject to several errors and probably overestimate the true threshold concentration of $InsP_3$. First, we made no correction for absorption of photolysis light by the cytoplasm, which is highly turbid. Second, the concentration of free $InsP_3$ in the stimulated region of the oocyte may have declined during the latent period after the flash and before the onset of the current, as a result of diffusion of $InsP_3$ into surrounding unexposed areas of the cell, and into deeper regions where the photolysis light was attenuated.

Facilitation and depression. Responses to a test flash are facilitated when it is preceded by a subthreshold conditioning flash (32, 35). The dependence of facilitation of the current on the duration of the conditioning flash was quantified in experiments like that in Fig. 5*A*. The test flash was set at a duration such that, when applied without any conditioning flash, it evoked small (<5 nA) currents. It was preceded, 3 s earlier, by a conditioning flash of variable duration. As the duration of the conditioning flash was increased from 0 to 30 ms, the size of the test response grew progressively, reaching a maximal size ~16 times that evoked by the test flash alone. However, lengthening the conditioning flash to 35 ms then caused the test response to decline slightly, and a further increase to 40 ms produced a large decrease. In the oocyte illustrated, the threshold flash duration was just less than 40 ms, so that conditioning flashes with durations of ≤35 ms failed to evoke detectable currents, whereas the 40-ms flash gave a small response.

Measurements of responses to test and conditioning flashes obtained in three oocytes are shown in Fig. 5*B*. Because the sizes of the responses and the threshold flash durations differed between the oocytes (maximal sizes 25–77 nA, threshold durations 7–35 ms), the data were normalized by expressing the flash durations as a percentage of that giving maximal facilitation in each oocyte, and the response sizes were expressed as a percentage of the maximally facilitated currents. As the conditioning flash duration was increased, the size of the test response at first grew about linearly but then decreased abruptly. For example, increasing the flash duration by 20% above that giving maximal facilitation caused the average test response to reduce to about one-half.

The inhibition seen with longer conditioning flashes

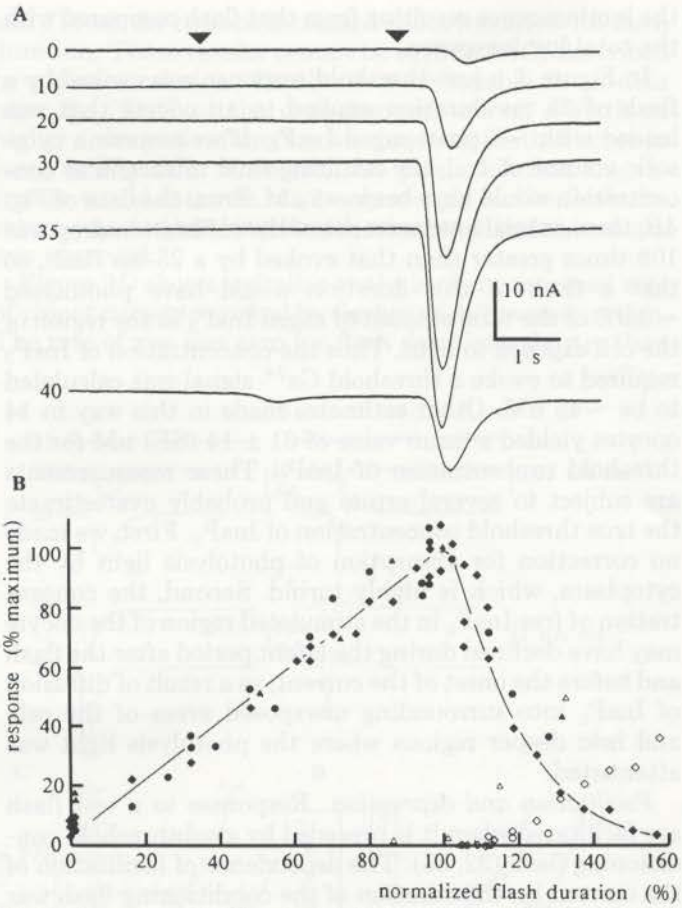

Fig. 5. Currents evoked by a test light flash are both potentiated and depressed by a preceding conditioning flash. *A*: membrane currents evoked by paired flashes, applied when indicated by arrowheads. Duration of second (test) light flash remained fixed at 42 ms, and durations of first (conditioning) flash are indicated (in ms) next to each trace. Interval between conditioning and test flashes was 3 s. *B*: sizes of membrane currents evoked by test (solid symbols) and conditioning flashes (open symbols), for conditioning flashes of various durations. Data are shown from 3 oocytes (different symbols) and were normalized as a percentage of maximally facilitated test response in each oocyte. Similarly, conditioning flash durations were normalized as a percentage of that which gave maximal facilitation in each oocyte. Conditioning flashes with normalized durations shorter than 100% failed themselves to evoke any currents, and these points have been omitted for clarity.

may have arisen because the conditioning flashes themselves caused liberation of Ca^{2+}, and the resulting rise in intracellular free Ca^{2+} inhibited the ability of $InsP_3$ released by the test flash to liberate further Ca^{2+} (32). Measurements of the currents evoked by the conditioning flashes themselves are therefore included in Fig. 5*B* (open symbols) to provide an indication of Ca^{2+} liberation. In two oocytes (open circles and diamonds in Fig. 5*B*) responses to the conditioning flash were not seen until the flash duration was lengthened to ~110% of that giving maximal facilitation. Thus inhibition was already apparent with flashes that themselves failed to evoke any detectable current (e.g., 35-ms trace in Fig. 5*A*), and just-threshold conditioning flashes depressed subsequent test responses to ~75% of the maximally facilitated value. In the third oocyte (open triangle in Fig. 5*B*) responses to the second flash began to decrease when the duration of the conditioning flash was just sufficient to evoke a response.

Time course of decay of facilitation. The facilitation produced by a subthreshold conditioning flash is greatest when it is applied shortly before a test flash and declines with increasing interflash interval (35). Experiments to measure the time course of this decline are shown in Fig. 6. The duration of the conditioning flash was adjusted to be ~90% of the threshold to maximize facilitation without inducing depression. Similarly, the duration of the test flash was set just above threshold, so that even a slight facilitation would be apparent. Intervals of at least 90 s were allowed between trials. Large currents were evoked by the test flashes at short interpulse intervals, but the responses progressively declined as the interval was lengthened, and at intervals longer than ~60 s the currents were not appreciably larger than those evoked without any conditioning pulse (Fig. 6*A*). The extent of facilitation was quantified by measuring the sizes of responses to the test flash in excess of that evoked without a prior conditioning flash, and, to allow pooling of data

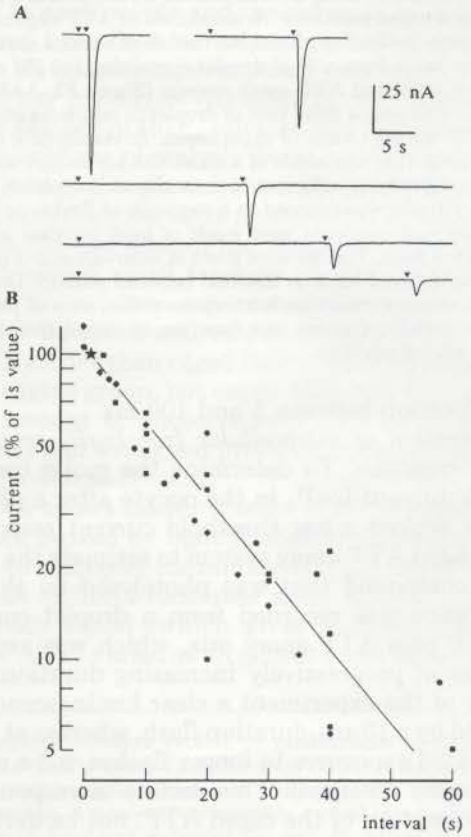

Fig. 6. Time course of decay of facilitation. *A*: membrane currents evoked by paired flashes delivered at different intervals. Arrowheads mark times of each flash. Duration of first flash in each pair was 18 ms and of second was 22 ms. No responses were evoked by first flash in each pair, whereas second evoked small (3–5 nA) currents when applied after long (90 s) intervals. Photolysis light was focused on oocyte as a square of ~150 μm/side. *B*: semilogarithmic plot of peak currents evoked by second light flash as a function of interval between flashes in each pair. Data are shown from 3 oocytes (different symbols) and have been normalized as a percentage of responses obtained in each oocyte at an interval of 1 s. Currents evoked by second flash alone (i.e., after an interval of 90 s) have been subtracted from measurements. Line was drawn by eye and corresponds to a time constant of 17 s.

from different oocytes, the resulting values were scaled as a percentage of the currents recorded in each oocyte at an interval of 1 s. Figure 6*B* shows a semilogarithmic plot of the decay of facilitation estimated in this way. The data points lie fairly well on a straight line. Thus, within the errors of measurement, the decay of facilitation follows a single exponential time course. The slope of the line corresponds to a time constant of 17 s.

Latency to onset of responses. After a light flash, the membrane current responses evoked by photorelease of $InsP_3$ did not begin until after an appreciable delay, which became shorter with stronger stimuli (e.g., Fig. 3). Interpretation of latencies in that experiment was complicated because the stimulus strength was adjusted by varying the flash duration, and the longest flashes were comparable to the response latency. To study better the relationship between level of $InsP_3$ formation and response latency we instead varied the intensity of a photolysis light that was maintained until after the beginning of the response. Thus $InsP_3$ was expected to be formed throughout the period of illumination at a rate proportional to the light intensity.

Figure 7*A* shows currents evoked in one oocyte by different intensities of photolysis light, set by neutral density filters in the light path. The onset of the currents after an initial latency was extremely abrupt and could be determined with little (<10 ms) error. At the full intensity of the light source, the latency was ~100 ms and large currents were evoked. As the intensity was reduced, the latency lengthened to ~10 s and the peak currents became smaller.

A possible explanation for the origin of the latency is that it arises because of the time required for the intracellular level of $InsP_3$ to rise above a threshold required for Ca^{2+} liberation (2, 22, 30). The simplest case is that $InsP_3$ was formed at a steady rate during illumination, whereas its subsequent removal proceeded at a rate proportional to the intracellular concentration. Thus, after the onset of illumination, the intracellular level of $InsP_3$ would rise toward a steady-state value along an exponential time course, giving rise to a latency (t) between the onset of illumination and the time at which the $InsP_3$ level just exceeded the threshold. This scheme is illustrated in the inset to Fig. 7*B*. A light intensity just sufficient to evoke any current is expected to produce a very long latency response because the steady-state level of $InsP_3$ will rise only slightly above threshold (dotted curve in inset). On the other hand, the latency will be short with strong stimulation, since $InsP_3$ is formed rapidly (solid curve). The duration of the latency is given by the following equation (24), assuming that the rate of $InsP_3$ formation is linearly proportional to light intensity and that its degradation follows first-order kinetics

$$t = \Upsilon \times \ln[I/(I - I_{th})] \qquad (1)$$

where Υ is time constant of rise in $InsP_3$ level, I is intensity of photolysis light, and I_{th} is threshold intensity of light required to evoke a just-detectable response.

Figure 7*B* shows measurements in two oocytes of the relationship between response latency and intensity of the photolysis light. The solid curves drawn through the

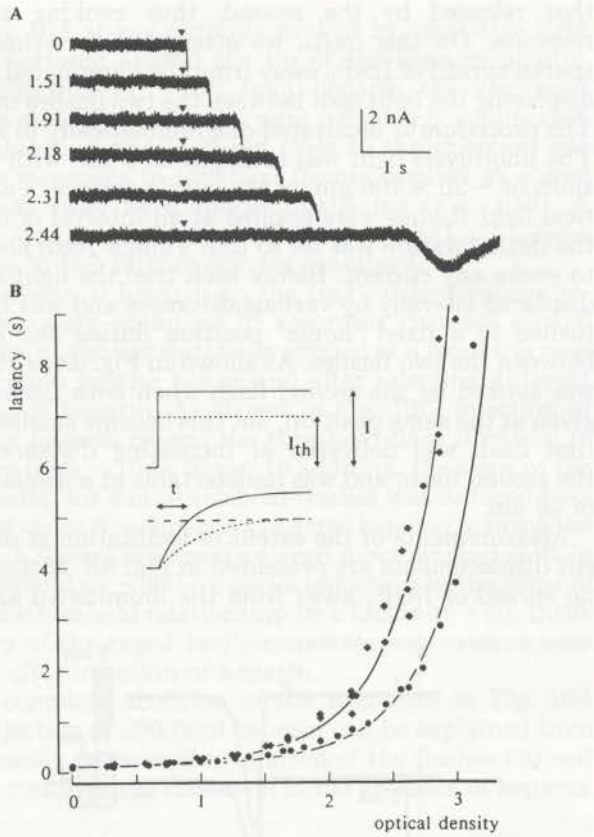

Fig. 7. Latencies to onset of membrane currents in response to photolysis of caged inositol 1,4,5-trisphosphate ($InsP_3$) by lights of different intensities. *A*: records of membrane currents at high gain, to better illustrate onset of responses. With exception of bottom trace, peak currents are off scale. Numbers indicate optical density of neutral filters placed in light path. In each trace, light was turned on at arrowhead and was extinguished shortly after beginning of response. Photolysis light spot was a square of ~70 μm/side. *B*: variation in response latency with optical density of filters. Data are from 2 oocytes (different symbols; points marked by diamonds correspond to oocyte in *A*). *Inset*: model scheme used to derive curves fitted to data points. Top part indicates stimulation by light of 2 different intensities (I), the lesser of which is just sufficient to evoke a threshold response (I_{th}). Bottom part shows predicted changes in level of $InsP_3$ resulting from these stimuli. Dashed line, threshold level of $InsP_3$ required for Ca^{2+} liberation. Latency t to onset of current response is marked for more intense stimulus. See text for further details.

data were calculated from *Eq. 1*. For each oocyte, I_{th} was estimated as the minimum light intensity required to evoke a current during prolonged (30 s) illumination. The time constant Υ for the rise in $InsP_3$ level corresponds to the time constant for its degradation, and a value of 17 s was used on the basis of the results in Fig. 6. A good fit was obtained between the observations and the predicted curves, suggesting that the majority of the latency can be accounted for by the scheme represented by *Eq. 1*. However, an exception was that the model predicts that the latency should approach zero with intense stimulation, whereas the observed latencies reduced to a limiting value of ~100 ms. The curves in Fig. 7*B* were therefore drawn with a constant latency of 100 ms added to the values predicted from *Eq. 1*.

Spatial spread of facilitation. As described above, the facilitation seen with paired flashes may arise because residual $InsP_3$ liberated by the first flash summates with

that released by the second, thus evoking a larger response. On this basis, we attempted to estimate the spatial spread of $InsP_3$ away from the illuminated area by displacing the light spot between the two flashes in a pair. The procedure is illustrated diagrammatically in Fig. 8*A*. The photolysis light was arranged as a slit, with dimensions of $\sim 20 \times 100$ μm on the oocyte surface. Two identical light flashes were applied at an interval of 3 s, and the flash duration was set so that a single flash just failed to evoke any current. Before each trial the light slit was displaced laterally by various distances and was then returned to a fixed "home" position during the interval between the two flashes. As shown in Fig. 8*A*, a response was evoked by the second flash when both flashes were given at the same position, but this became smaller as the first flash was delivered at increasing distances from the second flash, and was undetectable at a displacement of 46 μm.

Measurements of the extent of facilitation at different slit displacements are presented in Fig. 8*B*. If there were no spread of $InsP_3$ away from the illuminated area, the facilitation is expected to vary linearly with the extent of overlap between the slits used for the first and second flashes, as indicated by the dashed line. The data points lie to the right of this line, by a displacement of ~20 μm. Thus it seems that in the 3 s after the initial flash, $InsP_3$ may diffuse by ~20 μm from its site of photorelease.

Cooling enhances caged $InsP_3$ responses. Changes in temperature greatly affected the sizes of currents evoked by photorelease of $InsP_3$, which, as illustrated in Fig. 9*A*, became larger on cooling. Responses evoked by 30-ms flashes delivered at 40-s intervals evoked large currents while the temperature of the bathing solution was maintained at 19.5°C. However, when the oocyte was warmed to 25°C the response initially declined by ~90% and then increased in size about twofold as the oocyte was kept at this temperature for 2 min. On cooling again to 19.5°C the response size at first overshot the control value but subsequently returned to the original size.

The effect of temperature was most prominent with light flashes close to threshold, suggesting that it might arise if the level of $InsP_3$ required to evoke a response was lower at reduced temperature. This was tested by measuring at different temperatures the relationship between flash duration and size of evoked current (Fig. 9, *B* and *C*). Two changes in the relationship were evident; the threshold flash duration was shorter at lower temperature and the slope of the relationship for suprathreshold flashes was steeper. At temperatures intermediate between the extremes plotted in Fig. 9*C* (13.5 and 23.5°C) the threshold flash duration shifted progressively, but we did not examine the full dose-response relations. Similar

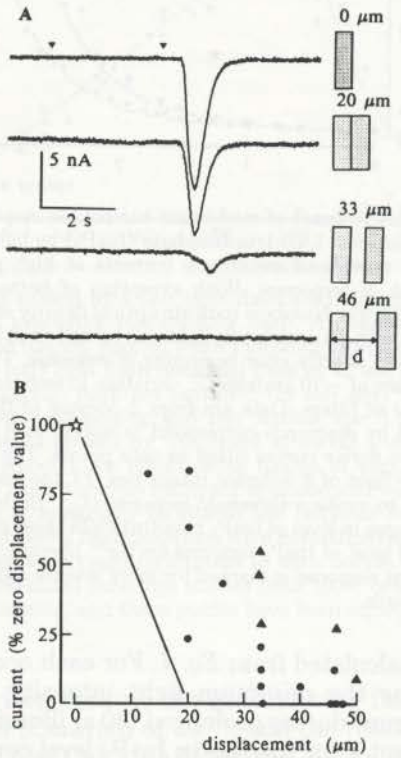

Fig. 8. Spatial spread of facilitation. *A*: photolysis light was focused on oocyte as a slit with dimensions of $\sim 20 \times 100$ μm. An objective lens of ×20 magnification was used for this experiment (rather than normal ×6.3) to provide better localization of illuminated area. Two light flashes of the same intensity and duration were applied at an interval of 3 s, and slit was moved laterally by a distance *d* between the two flashes. Flash duration (90 ms) was such that a single flash was just below threshold. Traces show currents evoked by paired flashes (times marked by arrowheads) with different displacements of light slit between flashes. In top trace, slit remained fixed. *B*: peak sizes of currents evoked by second flash in each pair, plotted against displacement of slit. Control records with no slit displacement were obtained at intervals throughout experiments, and responses are expressed as a percentage of interpolated control values to correct for any changes in sensitivity with time. Solid line, percentage area of overlap between 2 slit positions for different displacements. Data are from 2 oocytes (different symbols). Points marked by circles are from same oocyte as *A*.

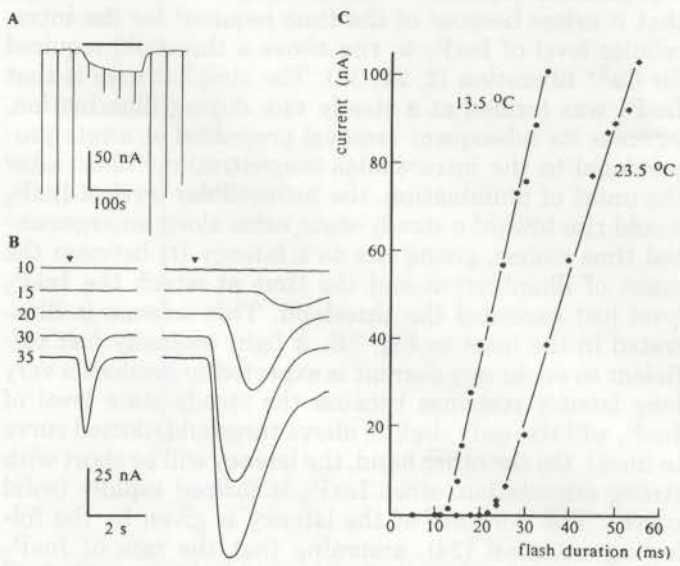

Fig. 9. Temperature dependence of currents evoked by photolysis of caged $InsP_3$. *A*: chart record showing currents evoked by repetitive light flashes (30 ms duration at 40-s intervals). During time marked by bar the oocyte was warmed to 25°C from a holding temperature of 19.5°C. *B*: sample records from a different oocyte showing currents evoked by light flashes of durations indicated (in ms). Traces on left were obtained at a temperature of 23.5°C, and those on right were obtained after cooling to 13.5°C. Arrowheads mark times of flashes. Photolysis light covered entire oocyte, which was positioned with the equator facing light. *C*: measurements of peak sizes of evoked currents plotted as a function of flash duration. Points marked by circles were obtained at a temperature of 13.5°C and points marked by diamonds at 23.5°C.

indicates that Ca^{2+}-induced Ca^{2+} release (9) is not significant in the oocyte. It has been proposed that Ca^{2+} may activate two different Cl^- conductances in the oocyte (5), one of which activates and inactivates rapidly, while the other displays slower kinetics. The fast transient currents evoked by flash photolysis of both caged $InsP_3$ and caged Ca^{2+} presumably arose through activation of the fast conductance, but the more prolonged currents evoked by Ca^{2+} injections could reflect activation of the slow conductance.

We had previously sought to explain the apparent threshold in $InsP_3$-evoked responses (35) on the basis that Ca^{2+} liberation may vary as a steep power function of $InsP_3$ concentration (19). The Cl^- currents would thus decrease steeply as the stimulus was reduced and at some point would fall below the resolution of the recording. However, the measurements presented here, obtained with improved resolution, indicate that this explanation is not applicable because a sixth (or higher) order power function was needed to adequately fit the abrupt onset of the response with increasing flash duration. This is a steeper dependence than the third- or fourth-power relation described by Meyer et al. (19). Moreover, it predicts that the current should rise extremely steeply as the flash is lengthened beyond the threshold, whereas a close to linear relationship was found. The dose-response relationship of the $InsP_3$-evoked Cl^- current may best be described, therefore, as a discrete threshold, followed by a linear increase that ultimately curves off as the response approaches a maximal value.

Recent findings (33, 36) indicate that the threshold may arise because the $InsP_3$-sensitive Ca^{2+} stores in the oocyte are arranged as multiple, autonomous units that each release their contents in a nearly all-or-none manner. The threshold amount of $InsP_3$ required to evoke a membrane current thus corresponds to the activation threshold of the most sensitive units, whereas the graded whole cell response with suprathreshold stimuli reflects the recruitment of additional units with higher thresholds. Furthermore, the abrupt threshold of individual units indicates that Ca^{2+} release probably arises through a regenerative process involving positive feedback (36). An attractive mechanism for this feedback is suggested by experiments on microsomes and lipid bilayers which reveal that, at low concentrations, calcium ions act as a co-agonist at the $InsP_3$ receptor to augment the opening of the Ca^{2+}-release channel (3, 10, 13).

By estimating the proportion of caged $InsP_3$ in the oocyte that was photolysed by a just-threshold light flash, we calculated the threshold increment in $InsP_3$ concentration required to evoke a response to be $\leq$60 nM. This value is consistent with findings that $InsP_3$ concentrations of a few tens of nanomolar are sufficient to evoke Ca^{2+} release in permeabilized cell preparations (4) but is much lower than measurements of the resting levels of $InsP_3$, which are in the low micromolar range in various cell types (see Ref. 4 for references), including the *Xenopus* oocyte (D. Dyer, personal communication). Clearly, our result implies that the $InsP_3$ signaling system in the oocyte should be perpetually activated by such a high resting level of $InsP_3$, and an increment by 60 nM would have little discernible effect. One resolution of this paradox may be that a large fraction of the $InsP_3$ is compartmentalized within the cell. Other possibilities are that the measurements of either the threshold or resting levels of $InsP_3$ are seriously in error. Regarding the threshold, the fact that injection of a 1 μM solution of $InsP_3$ into the oocyte evokes oscillatory responses (34) supports the idea that the threshold concentration of $InsP_3$ must be submicromolar, since the volume of solution injected (50 pl) would have been rapidly diluted in the large ($\sim$1 μl volume) oocyte.

Facilitation. Membrane current and intracellular Ca^{2+} signals evoked by photoreleased $InsP_3$ were facilitated by subthreshold conditioning light flashes. This could arise in various ways, but we favor the simplest explanation, that $InsP_3$ released by the conditioning flash remains present for some time and summates with additional $InsP_3$ released by a subsequent test flash so that the total level of $InsP_3$ then exceeds the threshold and evokes a response (30, 35). Other possibilities are that the binding of subthreshold amounts of $InsP_3$ to receptor sites may facilitate the Ca^{2+} release system or that facilitation arises from a metabolic product of $InsP_3$. An argument against the latter mechanism is that the time course of facilitation showed a monotonic decrease with increasing interval between flashes, whereas a biphasic rise and fall would be expected if it were determined by the formation and subsequent degradation of a metabolic intermediate. However, if the kinetics of production of the metabolite were rapid, it might not be observed in the decay curve.

The degree of facilitation produced by subthreshold conditioning flashes varied linearly over a wide range with duration of the conditioning flash. This suggests that the interaction of $InsP_3$ with the Ca^{2+}-liberating mechanism is linear, even at concentrations that are below threshold and fail directly to cause Ca^{2+} release. However, when the conditioning flashes were lengthened beyond a certain duration, the responses to the test flash began abruptly to decline. This probably resulted because of feedback inhibition by Ca^{2+} released in response to the conditioning flash on the subsequent release of Ca^{2+} by $InsP_3$ formed during the test flash (32). An interesting point was that in some oocytes the onset of the inhibition occurred with conditioning flashes that were themselves too short to evoke a detectable Cl^- current. Thus it seems that sufficient Ca^{2+} was liberated to inhibit $InsP_3$-sensitive Ca^{2+} release but that this was not enough to give a current response. In agreement with this idea, currents evoked by photoreleased Ca^{2+} showed a slight threshold, and Ca^{2+} signals evoked by photorelease of $InsP_3$ were detected by fluorescent indicator dyes at flash durations below the threshold to evoke current responses (33). Furthermore, a higher threshold for the Cl^- current is not surprising, since the feedback inhibition probably arises through calcium ions acting at sites very close to where they are released from intracellular organelles, whereas activation of the Cl^- current will be reduced by diffusion and uptake of calcium ions before they reach the plasma membrane channels. It should be noted, however, that the threshold for the current was only $\sim$10% greater than

for the onset of inhibition or of Ca^{2+} liberation as monitored by fluorescent dyes (33), so that most of the threshold in activation of the $InsP_3$-evoked current arises from the process of Ca^{2+} liberation.

The facilitation resulting from a subthreshold conditioning flash decayed exponentially, with a time constant of ~17 s. As discussed above, a simple interpretation is that this reflects the disappearance of $InsP_3$ from the cytoplasm. However, measurements of the metabolism of radiolabeled $InsP_3$ injected into *Xenopus* oocytes (18) indicated that $InsP_3$ disappeared more slowly, over several minutes. This difference may have arisen if injection of $InsP_3$ resulted in a very high concentration localized around the pipette tip, sufficient to saturate the metabolic enzymes. Also, in our experiments, diffusion of $InsP_3$ may have contributed to its removal, in addition to metabolic degradation. The oocyte cytoplasm is relatively opaque so that the photolysis light would have penetrated only a few tens of microns into the cell. $InsP_3$ would thus be liberated in a thin layer close to the plasma membrane (35), and its subsequent diffusion into the interior of the cell may have contributed to the decline in facilitation. Regardless of mechanism, it seems that the decline in facilitation measured with caged $InsP_3$ should give a good indication of the time course of facilitation induced by physiological stimuli that result in the formation of $InsP_3$ by breakdown of inositol phospholipids in the surface membrane.

Latency of $InsP_3$-evoked currents. The latency to onset of membrane currents evoked by photorelease of $InsP_3$ could be described by the sum of two components: a minimal latency of ~100 ms that remained even with intense stimuli and a latency that varied steeply with light intensity and lengthened to several seconds with weak stimuli. It has previously been proposed (2, 22, 31) that a dose-dependent latency may arise in $InsP_3$ signaling from the time taken before the intracellular concentration of $InsP_3$ rises above a threshold. The results in Fig. 7 support this idea by showing that the observed latency is explained quantitatively by a model in which a steady rate of formation of $InsP_3$ results in an intracellular concentration that rises exponentially toward a steady-state level, with a time constant corresponding to that estimated for the removal of $InsP_3$.

The minimal latency at high light levels is unlikely to arise from the photolysis reaction, since the formation of $InsP_3$ is almost complete within ~10 ms after a light flash (27, 28), and a similar latency was observed after injections of large amounts of free $InsP_3$ into the oocyte (22). Measurements using fluorescent Ca^{2+} indicators showed a quiescent period of ~50 ms before the Ca^{2+} level began to rise after photolysis of caged $InsP_3$ (33), so that about one-half of the total latency may be attributed to the $InsP_3$-mediated Ca^{2+} release mechanism. Regarding the remaining part, this probably did not arise from a delay in activation of the Cl^- channels, since currents evoked by photolysis of caged Ca^{2+} in the oocyte began within 5 ms of the light flash (14). Instead, the additional latency may arise through the diffusion of calcium ions from their sites of release to the inner surface of the plasma membrane.

Some factors affecting threshold for $InsP_3$ action. The threshold flash duration required to evoke a current response became shorter at lower temperatures, leading to a pronounced enhancement of responses evoked by near-threshold stimuli. Because photolysis of caged $InsP_3$ is expected to be slowed by cooling, this effect probably arose because of an inverse temperature sensitivity of some stage in the $InsP_3$ signaling pathway. Consistent with this interpretation, we had previously found that oocytes injected with $InsP_3$ showed large currents when they were rapidly cooled, suggesting that the sensitivity to $InsP_3$ was enhanced at lower temperature (23). Because the threshold arises primarily from the process of Ca^{2+} liberation, temperature dependence of the Cl^- channels cannot be important in determining the threshold, but the mechanism is presently unclear. Possibilities include a change in affinity of the $InsP_3$ receptor (6), a reduction or slowing of feedback inhibition by Ca^{2+} on $InsP_3$-mediated Ca^{2+} release (32), a slowing of $InsP_3$ metabolism, or an elevation of the resting level of $InsP_3$ in the cell.

Intracellular injections of heparin, a competitive antagonist at the $InsP_3$ receptor (12, 39), increased the threshold for $InsP_3$ action and reduced currents evoked by suprathreshold stimuli. These results are consistent with the idea that threshold activation of Ca^{2+} release requires the binding of a certain amount of $InsP_3$ to heparin-sensitive receptor sites, though we cannot exclude the possibility that heparin also affected various enzymes in the inositol polyphosphate pathway or bound to $InsP_4$ receptors. A complication was that the action of heparin was localized near the injection site, probably because of restricted diffusion of heparin in the cytoplasm. Injections of heparin thus failed to reduce appreciably responses evoked by bath application of agonist to the whole oocyte surface, a consideration that may be important for the use of heparin as an $InsP_3$ antagonist in other large cells.

Summary. We find that intracellular levels of $InsP_3$ must exceed a definite threshold before Ca^{2+} liberation can begin and that this threshold may account for several properties of the $InsP_3$ signaling pathway, including facilitation and latency. These are likely to be important in signal transduction in many cell types and especially in neurons in which slow synaptic responses are mediated by the $InsP_3$ pathway. As we have discussed (30, 35), the finding of a threshold for $InsP_3$ action suggests the basis for a novel form of synaptic integration, based on a summation of $InsP_3$ levels rather than a summation of postsynaptic potentials. In contrast to electrical signaling, this biochemical integration will be spatially more restricted, since it is limited by diffusion of $InsP_3$, but temporally it will be longer lasting and determined by metabolism of $InsP_3$ rather than the electrical time constant of the cell. Furthermore, the $InsP_3$ pathway offers many possibilities for modulation of its integrating functions, since several stages including metabolic enzymes, $InsP_3$ receptors, and Ca^{2+}-activated membrane channels are potential sites for modulation by other second messenger systems (2, 39). Finally, because activation of the

inositol phospholipid signaling system produces two second messengers, $InsP_3$ and diacylglycerol (2), the finding of a threshold in the $InsP_3$ arm of the pathway raises the possibility that qualitatively different responses may be evoked by a given agonist, depending on its concentration.

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