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POTENTIATION OF INOSITOL TRISPHOSPHATE-INDUCED Ca^{2+} MOBILIZATION IN *XENOPUS* OOCYTES BY CYTOSOLIC Ca^{2+}

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SUMMARY

1. The ability of cytosolic Ca^{2+} ions to modulate inositol 1,4,5-trisphosphate ($InsP_3$)-induced Ca^{2+} liberation from intracellular stores was studied in *Xenopus* oocytes using light flash photolysis of caged $InsP_3$. Changes in cytosolic free Ca^{2+} level were effected by inducing Ca^{2+} entry through ionophore and voltage-gated plasma membrane channels and by injection of Ca^{2+} through a micropipette. Their effects on Ca^{2+} liberation were monitored by video imaging of Fluo-3 fluorescence and by voltage clamp recording of Ca^{2+}-activated membrane Cl^- currents.

2. Treatment of oocytes with the Ca^{2+} ionophores A23187 and ionomycin caused a transient elevation of cytosolic Ca^{2+} level when cells were bathed in Ca^{2+}-free solution, which probably arose because of release of Ca^{2+} from intracellular stores.

3. Membrane current and Fluo-3 Ca^{2+} signals evoked by photoreleased $InsP_3$ in ionophore-treated oocytes were potentiated when the intracellular Ca^{2+} level was elevated by raising the Ca^{2+} level in the bathing solution.

4. Responses to photoreleased $InsP_3$ were similarly potentiated following activation of Ca^{2+} entry through voltage-gated Ca^{2+} channels expressed in the plasma membrane.

5. Ca^{2+}-activated membrane currents evoked by depolarization developed a delayed 'hump' component during sustained photorelease of $InsP_3$, probably because Ca^{2+} ions entering through the membrane channels triggered liberation of Ca^{2+} from intracellular stores.

6. Ba^{2+} and Sr^{2+} ions were able to substitute for Ca^{2+} in potentiating $InsP_3$-mediated Ca^{2+} liberation.

7. Gradual photorelease of $InsP_3$ by weak photolysis light evoked Ca^{2+} liberation that began at particular foci and then propagated throughout, but not beyond that area of the oocyte exposed to the light. Local elevations of intracellular Ca^{2+} produced by microinjection of Ca^{2+} acted as new foci for the initiation of Ca^{2+} liberation by $InsP_3$.

8. In resting oocytes, intracellular injections of Ca^{2+} resulted only in localized elevation of intracellular Ca^{2+}, and did not evoke propagating waves.

9. The results show that cytosolic Ca^{2+} ions potentiate the ability of $InsP_3$ to liberate Ca^{2+} from intracellular stores. This process may be important for the positive feedback mechanism underlying the generation of Ca^{2+} spikes and waves, and for

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interactions between the InsP_3 pathway and Ca^{2+} ions entering cells through voltage- and ligand-gated channels.

INTRODUCTION

Inositol 1,4,5-trisphosphate (InsP_3) is a ubiquitous second messenger that functions by mobilizing Ca^{2+} ions sequestered within intracellular stores (Berridge & Irvine, 1989). Several observations indicate that the release mechanism is a regenerative process. Specifically, activation of InsP_3 signalling has been found in many different cell types to lead to repetitive spikes in cytosolic free Ca^{2+} (Woods, Cuthbertson & Cobbold, 1986; Meyer & Stryer, 1991), and to the active propagation of Ca^{2+} waves throughout the cell (Meyer, 1991). Furthermore, recordings of free Ca^{2+} from minute subcellular regions show that InsP_3-evoked Ca^{2+} release occurs in a nearly all-or-none manner (Parker & Ivorra, 1990*a*; Parker & Yao, 1991). An essential ingredient to account for all these phenomena is positive feedback; but how does this arise? Three main classes of models have been proposed, all of which involve mobilized Ca^{2+} ions acting at various stages of the messenger pathway. (1) The InsP_3–Ca^{2+} cross-coupling model involves mutual reinforcement of InsP_3-induced Ca^{2+} release and Ca^{2+}-stimulated InsP_3 formation (Swann & Whitaker, 1986; Harootunian, Kao, Paranjape & Tsien, 1991; Meyer & Stryer, 1991). This scheme implicitly requires that spikes of Ca^{2+} be accompanied by spikes in InsP_3 level, whereas the remaining two schemes will show Ca^{2+} spikes at constant InsP_3 levels. (2) Ca^{2+}-induced Ca^{2+} release models postulate the existence of two kinds of non-mitochondrial Ca^{2+} pools; InsP_3-sensitive stores and Ca^{2+}-sensitive (but InsP_3-insensitive) stores (Rooney, Sass & Thomas, 1989; Wakui, Potter & Petersen, 1989; Berridge & Irvine, 1989; Berridge, 1990; Goldbeter, Dupont & Berridge, 1990; Missiaen, Taylor & Berridge, 1991). Activation of the messenger pathway causes a steady rate of release of Ca^{2+} from the InsP_3-sensitive stores, leading to a gradual rise in cytosolic free Ca^{2+}. At a certain level, release of Ca^{2+} from the Ca^{2+}-sensitive store is triggered and, because of the autocatalytic nature of the process, release continues until the Ca^{2+}-sensitive pool is depleted. (3) A novel model is based on the recent findings that cytosolic Ca^{2+} acts as a co-agonist at the InsP_3 receptor (Iino, 1990; Finch, Turner & Goldin, 1991; Bezprozvanny, Watras & Erlich, 1991). Thus, once release begins, Ca^{2+} exerts a positive feedback to promote the Ca^{2+}-mobilizing action of InsP_3.

Xenopus oocytes show Ca^{2+} spikes and waves like those in other cell types (Parker & Ivorra, 1990*b*; Brooker, Seki, Croll & Wahlesteadt, 1990; Lechleiter, Girard, Peralta & Clapham, 1991*a*), and their large size makes them a valuable model for studying Ca^{2+} signalling. In a previous study (Parker & Ivorra, 1990*a*) we found that Ca^{2+} inhibited the ability of InsP_3 to mobilize Ca^{2+} in the oocyte, but did not detect any potentiation. However, measurements in permeabilized cells and microsomal preparations subsequently revealed that Ca^{2+} has a biphasic action on InsP_3-stimulated Ca^{2+} release; low concentrations of Ca^{2+} potentiate release, whereas higher concentrations depress release (Iino, 1990; Finch *et al.* 1991; Bezprozvanny *et al.* 1991). This prompted us to re-examine the effects of small elevations of intracellular free Ca^{2+} on InsP_3-mediated Ca^{2+} release in intact oocytes. For this purpose, we used flash photolysis of caged InsP_3 to evoke reproducible transients in

intracellular $InsP_3$ level, and monitored the resulting mobilization of cytosolic Ca^{2+} both by voltage clamp recording of Ca^{2+}-activated membrane current and by video imaging of fluorescent dye signals. We find that under certain conditions Ca^{2+} potentiates the release process, and present evidence favours the idea that regenerative release occurs because Ca^{2+} acts as a co-agonist at the $InsP_3$ receptor.

METHODS

Experiments were done on oocytes of *Xenopus laevis*, obtained from albino frogs so as to avoid the problems during optical recording encountered with normally pigmented oocytes. Procedures for preparation of oocytes, voltage clamp recording, photolysis of caged $InsP_3$ and video imaging of intracellular free Ca^{2+} were as previously described (Sumikawa, Parker & Miledi, 1989; Parker & Yao, 1991; Parker, 1992). Briefly, oocytes were loaded with roughly 50 pmol Fluo-3 (Minta, Kao & Tsien, 1989) and 5 pmol caged $InsP_3$ D-*myo*-inositol 1,4,5-trisphosphate, $P^{4,5}$-1-(2-nitrophenyl) ethyl ester) (McCray & Trentham, 1989) by pneumatic pressure injection. Flashes of near ultraviolet light were used to photorelease $InsP_3$ in the oocyte, and video images of Ca^{2+}-dependent fluorescence of Fluo-3 were captured by an intensified charge-coupled device camera and stored on videotape for subsequent image processing and analysis. Traces showing changes in fluorescence with time (e.g. Figs 2 and 3) were obtained using a photodiode to monitor the intensity of small regions of a monochrome display screen. Over most of the intensity range used in the present experiments, the photodiode signal was linearly proportional to fluorescence intensity within better than $\pm 5\%$, and at higher intensities the signal underestimated the true intensity by up to 18%. Autofluorescence in the absence of dye loading was less than 10% of the resting fluorescence of Fluo-3-loaded oocytes. Because Fluo-3 does not permit the use of ratio measurements to determine absolute free Ca^{2+} levels, fluorescence data are presented in arbitrary units. Fura-2 measurements indicated that the resting free Ca^{2+} level in the oocyte is about 30 nM, and rises to a maximum of about 200 nM following agonist activation (I. Parker & Y. Yao, unpublished data). In some experiments, voltage clamp recording of Ca^{2+}-activated Cl^- membrane currents (Miledi & Parker, 1984) were used as an alternative, or an additional monitor of intracellular free Ca^{2+} level.

Oocytes were continuously superfused with Ringer solution at room temperature while recording. Normal Ringer solution had the composition (mM): NaCl, 120; KCl, 2; $CaCl_2$, 1·8; HEPES, 5; at pH about 7·0. High-Ca^{2+} solution contained a total of 12 mM $CaCl_2$. Ca^{2+}-free solution was made by omitting $CaCl_2$, adding 5 mM $MgCl_2$ and in some experiments, adding 1 mM EGTA. A strontium Ringer solution was made by substituting 12 mM $SrCl_2$ for $CaCl_2$. Isotonic Ba^{2+} solution contained only 80 mM $BaCl_2$ and 5 mM HEPES, pH about 7·0. Ionomycin and A23187 were prepared as 1 mM stock solutions in dimethyl sulphoxide (DMSO), and diluted in Ringer solution as needed.

Some experiments used oocytes injected a few days before recording with mRNA from rat cerebral cortex, so as to induce the expression of exogenous voltage-gated Ca^{2+} membrane channels. Procedures for mRNA extraction and injection into oocytes were as described previously (Sumikawa, Parker & Miledi, 1989).

RESULTS

Permeabilization of oocytes with Ca^{2+} ionophores

In previous experiments (Parker & Ivorra, 1990*a*) we studied the effects of Ca^{2+} on responses to photoreleased $InsP_3$ by microinjecting Ca^{2+} through an intracellular pipette. However, this method results in steep spatial gradients in Ca^{2+} concentration away from the pipette tip. To obtain a more even elevation of cytosolic free Ca^{2+} we therefore sought ways to induce entry of extracellular Ca^{2+} across the plasma membrane. The first approach tried was to permeabilize the membrane using the Ca^{2+} ionophores, ionomycin and A23187.

The procedure followed was that described by Boton, Dascal, Gillo & Lass (1989),

in which oocytes are transiently exposed to ionophore while bathed in a Ca^{2+}-free solution. Figure 1*A* shows records of Ca^{2+}-dependent fluorescence from an oocyte loaded with Fluo-3 during application of 1 μM ionomycin in a Ringer solution containing 5 mM Mg^{2+}, 1 mM EGTA and no added Ca^{2+}. Although the free Ca^{2+}

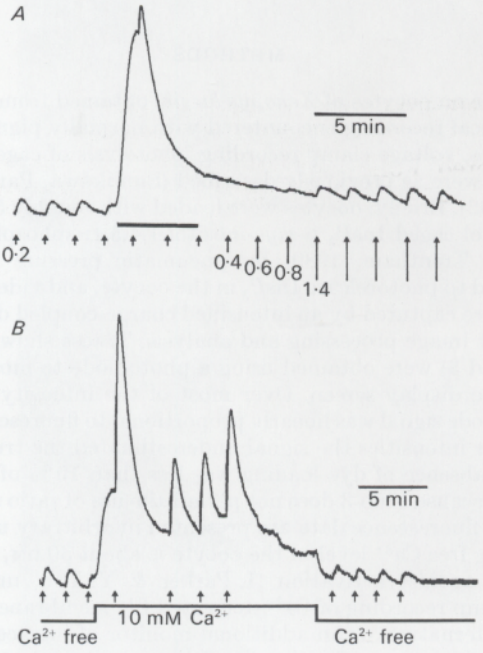

Fig. 1. *A*, ionomycin evokes liberation of Ca^{2+} from intracellular stores and reduces responses evoked by photorelease of $InsP_3$. Traces show Fluo-3 fluorescence; upward deflections correspond to increasing free Ca^{2+} level but the magnitude is uncalibrated. The peak increase in fluorescence corresponds to an increase of 26% above the resting fluorescence. The oocyte was continually superfused with Ca^{2+}-free solution including 1 mM EGTA, and ionomycin (1 μM) was added to this solution for the time indicated by the bar. Repetitive flashes of photolysis light were applied when indicated by the arrows. The length of the arrows denotes the durations of the flashes, and flash durations are also indicated numerically (in seconds). *B*, potentiation of $InsP_3$-evoked Ca^{2+} release by extracellular Ca^{2+} in an ionomycin-treated oocyte. The trace is a continuation of the record in (*A*), obtained about 50 min after treatment with ionomycin. Photolysis light flashes of constant intensity and duration (200 ms) were delivered when marked by the arrows. The oocyte was bathed in Ca^{2+}-free solution (without EGTA) at the beginning and end of the record, but this was exchanged for 10 mM Ca^{2+} solution when indicated by the bar.

concentration in this solution is expected to be very low (< 4 nM, assuming 10 μM total contaminating Ca^{2+}), ionomycin caused a transient increase in Fluo-3 fluorescence. Results like those illustrated were seen in all eight oocytes treated with ionomycin (1 μM), and in three other oocytes treated with A23187 (1 or 2 μM). Voltage clamp recordings of Ca^{2+}-activated Cl^- current were also obtained in several oocytes, and the activation of an inward current (at -60 mV) confirmed that ionomycin caused an elevation of intracellular free Ca^{2+}. Furthermore, intracellular

Ca^{2+} transients were still evoked in five oocytes that had been continuously incubated for 12 h or more, before ionomycin treatment in Ca^{2+}-free solution including 1 or 10 mM EGTA. Thus, it appears that the ionophores cause release of Ca^{2+} from intracellular stores, as well as making the plasma membrane permeable to Ca^{2+}.

Throughout the record in Fig. 1*A*, the oocyte was stimulated by repetitive flashes of photolysis light, to evoke photorelease of $InsP_3$. Before applying ionomycin, flashes of 0·2 s duration evoked small Ca^{2+} transients of roughly constant size. During application, the signal evoked by a flash given at the peak of the Ca^{2+} rise induced by the ionomycin was potentiated, but responses to subsequent flashes of the same duration were completely suppressed, probably because Ca^{2+} in the $InsP_3$-sensitive stores was by then depleted. Following removal of ionomycin, these flashes still failed to evoke signals, but a small response was evident when the flash duration was lengthened to 0·8 s, and a flash of 1·4 s gave responses of similar size to those originally evoked by 0·2 s flashes. In the oocyte illustrated, this reduction in sensitivity to the light flashes was slowly reversible and, about 50 min following ionomycin treatment (during which time the oocyte was transiently exposed to Ca^{2+}-containing solutions), flashes of 0·2 s duration again evoked Ca^{2+} signals of similar size to the controls. However, in three out of six oocytes examined, the caged $InsP_3$ response was abolished following ionomycin treatment and failed to recover.

$InsP_3$-evoked Ca^{2+} signals are potentiated by ionophore-mediated Ca^{2+} entry

Figure 1*B* shows traces of Fluo-3 fluorescence recorded in the same oocyte as described above, beginning about 50 min after ionomycin treatment. In Ca^{2+}-free solution (without EGTA), photolysis light flashes (0·2 s duration) evoked small $InsP_3$-mediated rises in intracellular Ca^{2+}. However, changing to 10 mM Ca^{2+} solution caused the basal intracellular free Ca^{2+} to rise, and responses to the light flashes were greatly potentiated. These effects were reversible; on returning to Ca^{2+}-free solution the basal Ca^{2+} level declined, and responses to light flashes were a little smaller than in the control. Potentiation of the $InsP_3$ response was most prominent shortly after changing to 10 mM Ca^{2+} solution and, in the example shown, the Fluo-3 signal was about ten times greater than the preceding responses in Ca^{2+}-free solution. A similar, though smaller potentiation was seen in 1 mM Ca^{2+} solution (data not shown). Potentiation of $InsP_3$ responses by raised extracellular Ca^{2+} was seen in three oocytes treated with A23187 and in three oocytes treated with ionomycin.

Potentiation by Ca^{2+} entry through voltage-gated channels

Because of the effect of Ca^{2+} ionophores on intracellular stores, we also explored the possibility of elevating intracellular Ca^{2+} in a less invasive way, by triggering influx through voltage-gated Ca^{2+} channels. Native *Xenopus* oocytes show a Ca^{2+}-dependent transient outward (T_{out}) current on depolarization (Miledi, 1982), which arises because of the presence of endogenous Ca^{2+} channels in the oocyte membrane. However, most oocytes possess relatively few Ca^{2+} channels and, to augment the Ca^{2+} influx, we recorded instead from oocytes that had been injected with mRNA from rat brain to induce expression of exogenous Ca^{2+} channels (Miledi, Parker & Sumikawa, 1986). In these oocytes, depolarizing pulses evoked intracellular Fluo-3

signals that were maximal at a potential of about +10 mV but, even in oocytes bathed in 12 mM Ca^{2+}, these were much smaller than the increases that could be evoked by photorelease of InsP_3 (Fig. 2).

Ca^{2+} signals evoked by photorelease of InsP_3 were facilitated when the light flash was preceded shortly beforehand by a depolarizing pulse. In Fig. 2 the response to

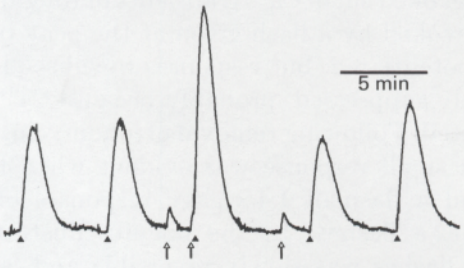

Fig. 2. Potentiation of caged InsP_3 response by Ca^{2+} entry through voltage-gated channels. Trace shows Fluo-3 fluorescence in an oocyte that had been injected with rat brain mRNA to induce the expression of voltage-gated Ca^{2+} channels. InsP_3 was photoreleased by flashes of constant intensity and duration when marked by the arrowheads. At the arrows the membrane potential was depolarized from −80 mV to +10 mV for 10 s, to induce the entry of extracellular Ca^{2+}. The bathing solution included 12 mM Ca^{2+}. The largest fluorescence signal corresponds to an increase of about 8% above the resting level.

a flash delivered 2 s after the end of a depolarizing pulse was about twice as large as expected from a linear summation of the responses to each stimulus alone. However, when the interval between the depolarization and the flash was lengthened to 100 s, no facilitation was apparent.

Ca^{2+}-induced Ca^{2+} release in the presence of InsP_3

Figure 3 shows an experiment in which repetitive depolarizing pulses were used to activate Ca^{2+} entry in a mRNA-injected oocyte during sustained photorelease of intracellular InsP_3 by continued exposure to photolysis light of low intensity. Simultaneous recordings of Fluo-3 fluorescence and Ca^{2+}-activated membrane current provided independent monitors of intracellular free Ca^{2+}.

Before the photolysis light was turned on, depolarizing pulses (to +10 mV) evoked large transient outward (T_{out}) currents, that arose because Ca^{2+} ions entering through voltage-gated channels caused Cl^- channels in the plasma membrane to open (Miledi, 1982; Barish, 1983). The T_{out} currents declined over a few seconds during the pulse, whereas the corresponding Fluo-3 signals continued to rise throughout the depolarization, and subsequently returned to the baseline over several seconds after the membrane potential was stepped back to the holding level.

Photolysis of caged InsP_3 evoked a gradual rise in the Fluo-3 signal that began following a latency of several seconds and, after about 5 min, reached a plateau level over ten times greater than that evoked by the control depolarizations. In contrast to the large increase in intracellular Ca^{2+} monitored by Fluo-3, the membrane current showed virtually no change (< 5 nA inward current), even though Cl^-

currents activated by a given intracellular Ca^{2+} level are expected to be of similar size (though opposite direction) at the holding potential (−60 mV) and at +10 mV (Miledi & Parker, 1984). Depolarizing pulses applied during photorelease of $InsP_3$ evoked transient Fluo-3 signals that grew progressively as the basal Ca^{2+} increased

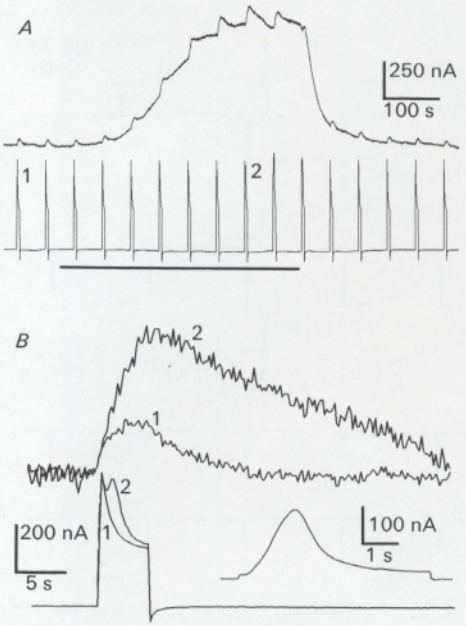

Fig. 3. Intracellular Ca^{2+} transients and Cl^- currents evoked by entry of extracellular Ca^{2+} through voltage-gated channels are enhanced during continued photorelease of $InsP_3$. *A*, upper trace shows intracellular free Ca^{2+} monitored by Fluo-3, and lower trace shows membrane current. The membrane potential was clamped at −80 mV and stepped to +10 mV during 5 s duration pulses at intervals of 50 s. During the time marked by the bar the oocyte was continually illuminated by photolysis light attenuated to 0·5 % of the normal intensity. The maximum rise in fluorescence corresponds to an increase of 33 % above the resting level. *B*, superimposed traces showing, on an expanded time scale, the Fluo-3 and current responses to the depolarizing pulses marked in *A*. For clarity, the Fluo-3 traces were aligned so that the baseline levels preceding the pulses superimpose. The inset trace shows the difference between the two current traces.

and, at the peak, were about three times greater than the control response. Although the peak size of the associated membrane current responses was not much altered, a striking finding was that instead of decaying monotonically, the T_{out} current showed an additional 'hump' component (Fig. 3*B*). The time course of this component is shown in the inset to Fig. 3*B*, obtained after subtracting the control response to depolarization from that during $InsP_3$ release. The additional current began after a latency of about 250 ms, and reached a maximum after 3 s. After extinguishing the photolysis light the basal Fluo-3 signal returned to the resting level, the transient Fluo-3 signals evoked by depolarization reduced to the control size, and the 'hump' in the T_{out} current disappeared.

The facilitation of the depolarization-induced Fluo-3 signals in Fig. 3*A* is representative of findings in eleven out of thirteen oocytes studied. One oocyte showed only a small (< twofold) facilitation, and no facilitation was evident in the remaining oocyte. Recordings of T_{out} current were obtained in eight oocytes that

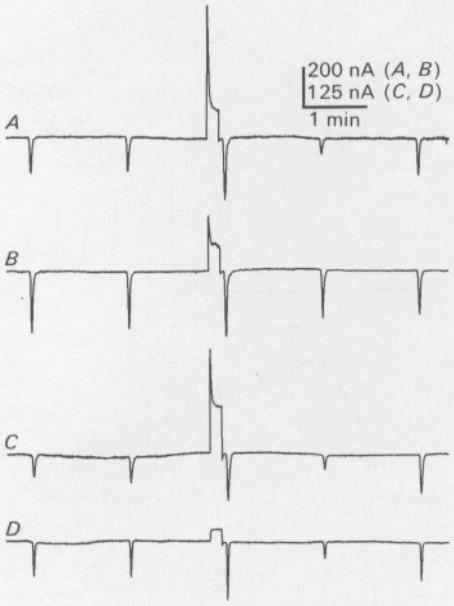

Fig. 4. Potentiation of InsP_3-evoked membrane currents by entry of Ca^{2+} and other divalent ions through voltage-gated membrane channels. Traces show recordings of membrane current at a clamp potential of −80 mV in response to repetitive photolysis light flashes delivered at 90 s intervals. The potential was stepped to +20 mV for 10 s, and returned to −80 mV, 3 s before the third flash in each trace. *A*, records obtained in Ringer solution containing 10 mM Ca^{2+}. *B*, records from the same oocyte after adding 100 μM Cd^{2+} to solution including 10 mM Ca^{2+}. *c*, bathing solution contained 10 mM Sr^{2+} and no added Ca^{2+}. *D*, solution contained 80 mM Ba^{2+} and no added Ca^{2+}.

showed facilitation of the depolarization-induced Fluo-3 signal during photorelease of InsP_3. Of these, six showed responses like that in Fig. 3; that is to say, no hump component was apparent during control depolarizations, but a hump developed during InsP_3 release and subsequently disappeared after extinguishing the photolysis light. In one oocyte the hump current persisted after the photolysis light was extinguished, whereas in the remaining cell this current was small even during photorelease of InsP_3.

Several control experiments indicated that facilitation of the Ca^{2+} signals during InsP_3 action arose because of entry of Ca^{2+} ions through voltage-gated Ca^{2+} channels, and not because of the depolarization *per se*. Firstly, the Fluo-3 signals evoked by depolarization were strongly reduced by adding 0·1 mM Cd^{2+} to the bathing solution to block Ca^{2+} channels (two oocytes examined). Secondly, the size of the Fluo-3 signal showed a voltage dependence like that expected for Ca^{2+} entry through voltage-gated channels (Hille, 1984). The fluorescence signals were maximal during

polarization to about +10 mV, but were not detectable at −10 mV, and reduced to about half the peak size at +50 mV. Finally, depolarizations to +10 mV produced no detectable increase in the $InsP_3$-evoked Ca^{2+} signal in two control (non-mRNA-injected) oocytes that showed only small (50 nA) T_{out} currents.

In fact, depolarization of control oocytes produced a transient decrease in the $InsP_3$-evoked Fluo-3 signal. This probably arose because second messenger-operated Ca^{2+} channels in the plasma membrane became activated during sustained elevation of intracellular $InsP_3$ level (Parker & Miledi, 1987), thus allowing a steady influx of Ca^{2+} at the holding potential (−80 mV) which diminished during depolarization because of the reduced electrical driving force for Ca^{2+} entry. In agreement with this interpretation, the decrease in Ca^{2+} became progressively greater with polarization to increasingly positive potentials, whereas hyperpolarization evoked an increase in the Ca^{2+} signal. Oocytes injected with mRNA also showed a reduction in Ca^{2+} signal with depolarization but, because the effect developed over several minutes, it was apparent only after an initial period when responses to depolarization were facilitated. Also, the depression was most obvious when relatively intense UV illumination was used to evoke strong photorelease of $InsP_3$. In Fig. 3*A* the duration and intensity of the photolysis light exposure were such that depression was not evident.

$InsP_3$-evoked membrane currents are potentiated by divalent cations

Figure 4 shows experiments in which recordings of Ca^{2+}-activated membrane current were used to monitor the potentiation of responses to $InsP_3$ by Ca^{2+} and other divalent cations. Oocytes were stimulated by repetitive light flashes, which evoked transient Cl^- currents (Parker & Miledi, 1989). After recording control responses, the membrane potential was stepped to +20 mV for 10 s, to open voltage-gated Ca^{2+} channels expressed in rat brain mRNA-injected oocytes. In oocytes bathed in 12 mM Ca^{2+} solution a large Ca^{2+}-activated Cl^- current was evoked during the depolarization, and responses to a light flash given 2 s after the end of a depolarizing pulse were potentiated (Fig. 4*A*). The responses to the subsequent flash were reduced, probably because of inhibition of $InsP_3$-mediated Ca^{2+} release by the larger amount of Ca^{2+} liberated by the test flash (Parker & Ivorra, 1990*a*). Addition of 0·1 mM Cd^{2+} to the bathing solution greatly reduced both the T_{out} current and the extent of facilitation of the light flash response (Fig 4*B*), indicating that the facilitation arose through entry of Ca^{2+} into the cell.

To see if other divalent cations could substitute for Ca^{2+} in facilitating the $InsP_3$ response, we replaced Ca^{2+} in the bathing solution by Sr^{2+} (Fig. 4*C*) or Ba^{2+} (Fig. 4*D*), ions that are both expected to be permeant through Ca^{2+} channels (Hille, 1984). Depolarizing pulses applied while bathing the oocyte in solution including 10 mM Sr^{2+} evoked a T_{out} current, since this ion is able to substitute (albeit with lower potency) for Ca^{2+} in activating the Cl^- membrane channels (Miledi & Parker, 1984). Furthermore, the response to a light flash delivered shortly after the depolarization was facilitated. Ba^{2+}, on the other hand, is much less effective in activating the Cl^- channels (Miledi & Parker, 1984), and depolarization in isotonic $BaCl_2$ solution evoked only a passive 'leakage' current. Nevertheless, the light flash response was potentiated following depolarization in Ba^{2+} solution.

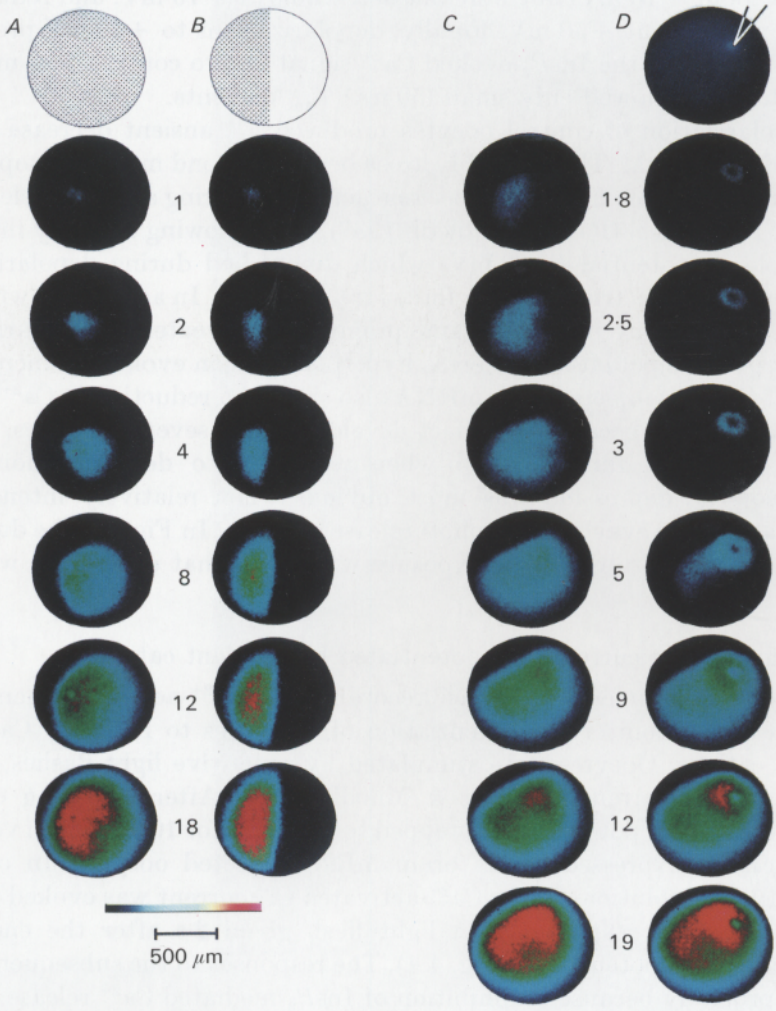

Fig. 5. Ca^{2+} waves evoked by photorelease of $InsP_3$. Figures show pseudocoloured images of Fluo-3 fluorescence after subtraction of resting fluorescence, and thus indicate $InsP_3$-evoked Ca^{2+} release. Increasing levels of Ca^{2+} are depicted on a colour scale (bar at lower left) ranging from black (no increase above basal) to red; absolute concentrations are not quantified. The entire oocyte is visible in each frame but, because of the spherical shape of the cell, fluorescence falls off as a sine function towards the perimeter. *A* and *B* show the time sequence of Ca^{2+} images in a single oocyte that was continually exposed to UV light of fixed, low intensity covering either the entire cell (*A*) or only the left half (*B*), as illustrated by the stippled regions in the diagrams at the top. Numbers next to each pair of images give the time (in seconds) after the first detectable rise in Ca^{2+}. In both cases, this occurred about 20 s after beginning illumination. *C* and *D* show the time sequence of Ca^{2+} images during continued illumination with UV light covering the entire oocyte. Numbers give the time after the first detectable response (as in *A* and *B*), which occurred in both cases about 6 s after beginning illumination. Images in *C* are control records. Those in *D* were recorded in the same oocyte about 4 min after injecting Ca^{2+} into the oocyte. The location of the pipette is indicated in the uppermost image, which shows the resting fluorescence just before turning on the photolysis light. Note that Ca^{2+} remained elevated locally around the pipette tip 4 min after injecting Ca^{2+}. This image was used as the reference background that was subtracted from frames captured during photolysis to derive the remaining images in this column.

Ca^{2+} wave evoked by photorelease of $InsP_3$

Agonist activation of $InsP_3$ signalling in the oocyte leads to the initiation of Ca^{2+} release at one or more foci, from which Ca^{2+} waves then propagate to engulf the entire cell (Lechleiter *et al.* 1991*a*; Lechleiter, Girard, Peralta & Clapham, 1991*b*; Yao & Parker, 1991). Active propagation of Ca^{2+} waves requires the existence of a regenerative positive feedback mechanism (Meyer, 1991). To explore the role of Ca^{2+}-facilitated Ca^{2+} liberation in this process, we used video imaging to monitor Ca^{2+} release induced by diffuse photoliberation of $InsP_3$.

Figure 5 illustrates a typical response. The entire visible area of the oocyte was exposed continually to UV light of low intensity (about 0·5% maximum), so as to evoke a gradual, diffuse photorelease of $InsP_3$. No Ca^{2+} signals were detected until about 20 s after beginning illumination, at which time Ca^{2+} began to rise in a discrete region. Over several seconds a wave of Ca^{2+} then spread from the initial focus, and the intensity of the Ca^{2+} signal rose, until a high Ca^{2+} level was attained throughout the cell.

Among the schemes that have been proposed for positive feedback of Ca^{2+} (see Introduction), that involving Ca^{2+} ions acting as a co-agonist together with $InsP_3$ requires that $InsP_3$ be present in a region of the cell in order for it to sustain a propagating Ca^{2+} wave. On the other hand, if positive feedback arises because Ca^{2+} promotes the formation of $InsP_3$, one would expect that any localized elevation in Ca^{2+} should trigger a Ca^{2+} wave that propagates throughout the remaining, unstimulated regions of the cell. We sought to discriminate between these possibilities in two ways. The first was to arrange the photolysis light so that $InsP_3$ was liberated across only half of the oocyte. As shown in Fig. 5*B*, Ca^{2+} release was then essentially confined to the illuminated area, and the Ca^{2+} wave failed to propagate into that half of the cell where $InsP_3$ was not formed, even though the same oocyte showed a global response when uniformly illuminated (Fig. 5*A*). Similar experiments in more than fifteen other oocytes confirmed that Ca^{2+} liberation remained restricted to that region where $InsP_3$ was photoreleased.

A second approach was to elevate locally intracellular free Ca^{2+} by continuous injection through an intracellular micropipette filled with 5 mM $CaCl_2$. Figure 6*A* shows the resulting distribution of Ca^{2+} at different times after beginning injection, obtained by measuring the increase in Fluo-3 fluorescence along a line section passing through the injection site. Ca^{2+} remained localized close to the pipette tip and, even 25 s after beginning infusion, the Ca^{2+} signal declined to half at a distance of about 50 μm from the injection site. In contrast to the local elevation in Ca^{2+} produced by microinjection of Ca^{2+}, photorelease of $InsP_3$ across the oocyte (before inserting the Ca^{2+} pipette) evoked a Ca^{2+} rise that began near the centre of the cell, and then propagated outwards (Fig. 6*B*). Similar results were obtained in ten oocytes. In all cases, Ca^{2+} injections failed to evoke a Ca^{2+} wave, and the Ca^{2+} level showed a decremental distribution with distance from the injection site.

Phospholipase C inhibitors

In order to test the role of Ca^{2+}-dependent stimulation of phospholipase C in facilitation of $InsP_3$-evoked responses more directly, we attempted to block this enzyme using neomycin (Swann & Whitaker, 1986; Vassbotn, Langeland & Holmsen,

1990) and U-731222 (Smith, Sam, Justen, Bundy, Bala & Bleasdale, 1990). When bath applied at respective concentrations of 5 mM and 10 μM, both agents caused a reversible block of InsP_3-mediated current responses evoked in the oocyte by serum (Tigyi, Dyer, Matute & Miledi, 1990) (five oocytes tested with neomycin and three

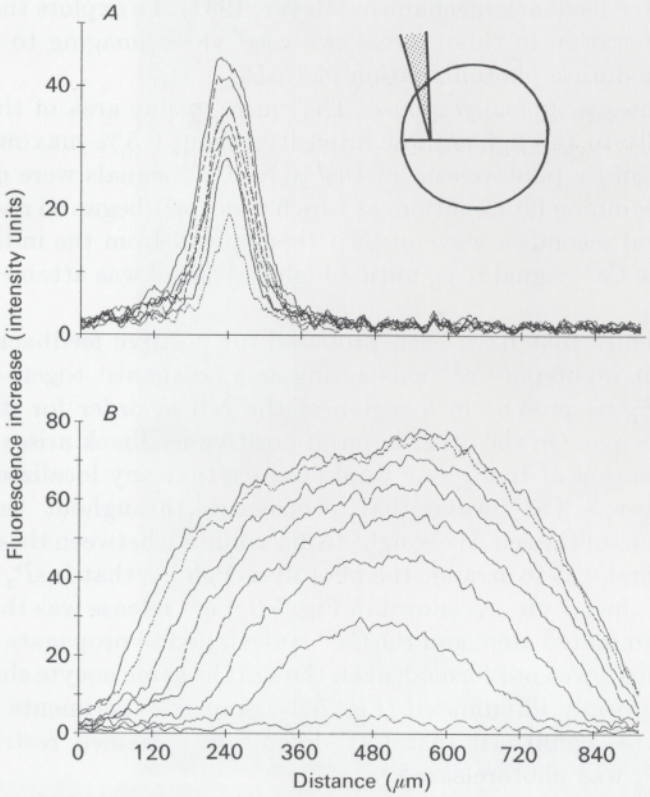

Fig. 6. Distribution of intracellular Ca^{2+} resulting from continuous infusion of Ca^{2+} from a micropipette (*A*) and from photorelease of InsP_3 across the whole oocyte (*B*). Inset shows the experimental design. The circle represents the outline of the oocyte. A micropipette filled with 5 mM $CaCl_2$ was inserted as indicated, and measurements of Fluo-3 fluorescence were taken along a horizontal section extending nearly the full width of the cell as shown by the line. Graphs show fluorescence intensities (on an arbitrary scale from 0 to 80) after subtraction of the resting fluorescence distributions measured just before stimulation. Tick marks on the horizontal axes are in increments of 120 μm. *A*, superimposed traces show fluorescence distributions at 3 s intervals after beginning a steady infusion of Ca^{2+} through the micropipette. The peak fluorescence level rose progressively with time. *B*, similar traces obtained in the same oocyte as *A* at 3 s intervals during continued exposure to photolysis light. The light covered the entire oocyte, and traces were obtained starting when the Ca^{2+} level first began to rise.

with U-73122). However, under the same conditions neither neomycin (three oocytes tested) nor U-73122 (seven oocytes) appreciably reduced the oscillatory currents generated when oocytes were injected with guanosine 5′-*O*-3-thiotriphosphate (GTPγS) to stimulate more directly phospholipase C via G protein activation.

Thus, it seems that neither agent provides a good tool for blocking phospholipase C activity, but that they may interfere with the signalling pathway at the level of the receptor or its interaction with G proteins.

Local elevations of Ca^{2+} act as foci for $InsP_3$ action

Whilst recording $InsP_3$-evoked Ca^{2+} waves we noticed that Ca^{2+} responses frequently began at sites where microelectrodes or micropipettes were inserted into the cell. To confirm this observation, we positioned oocytes so that the site where the injection pipette had been inserted lay outside the recording area, and noted the location where Ca^{2+} first began to rise following uniform illumination by the photolysis light. A micropipette was then impaled a few hundred micrometres away from this focus and, after waiting a few minutes, the photolysis exposure was repeated. In four out of six trials the Ca^{2+} response then began around the impalement site and, although in the remaining two cases the Ca^{2+} rise still began at the original foci, a second, independent release of Ca^{2+} occurred shortly afterwards around the impalement site.

The resting Ca^{2+} level was usually elevated close to where micropipettes were inserted, probably because extracellular Ca^{2+} leaked into the cell around the sides of the pipette. It seemed likely that this local elevation in free intracellular Ca^{2+} might be the factor which initiated $InsP_3$-mediated Ca^{2+} release at the impalement sites, but the effect could also have arisen from damage caused by the pipette. To distinguish between these possibilities, we inserted Ca^{2+} pipettes into oocytes while they were bathed in Ca^{2+}-free Ringer solution. After impalement, but before injecting any Ca^{2+}, photorelease of $InsP_3$ across the oocyte evoked Ca^{2+} release that began at the same sites as before the pipettes were inserted (three trials). However, after injecting Ca^{2+}, subsequent exposures to photolysis light evoked responses that began around the injection site. Typical records are illustrated in Fig. 5*C* and *D*. After insertion of a Ca^{2+} pipette, but before injection of Ca^{2+}, photoliberation of $InsP_3$ evoked Ca^{2+} release that began diffusely at the left centre of the recording field and then propagated across the oocyte (Fig. 5*C*). Injection of a brief pulse of Ca^{2+} resulted in a localized elevation of intracellular Ca^{2+}, that was still evident in a resting Fluo-3 image captured 4 min later (upper frame, Fig. 5*D*). Photorelease of $InsP_3$ across the oocyte then evoked Ca^{2+} liberation that began as a ring around the injection site (Fig. 5*D*). Results like those illustrated were obtained in eight out of ten oocytes, and in the remaining oocytes a secondary focus of Ca^{2+} release occurred around the site of Ca^{2+} injection, although the initial release began at its original locations. Facilitation of $InsP_3$-evoked Ca^{2+} release was most prominent a few minutes after injecting a pulse of Ca^{2+}. In six oocytes that were exposed to photolysis light within about one minute of injection, none showed Ca^{2+} release beginning at the injection site, although in all cases focal release was seen at this site beginning shortly after an initial Ca^{2+} release elsewhere.

In many oocytes, Ca^{2+} release began as an annulus surrounding the injection site, whereas the Ca^{2+} close to the pipette tip was inhibited (e.g. Fig. 5*D*). This did not occur because the Ca^{2+} level resulting from the injection was already sufficient to saturate the dye, since a large increase in fluorescence was seen at later stages of the response, even though the local signal was suppressed for several seconds after Ca^{2+}

first began to rise in surrounding regions (Fig. 5*D*). The mechanism of this suppression remains, however, unclear. One possibility is that InsP_3-mediated Ca^{2+} release was inhibited by the prolonged elevation for resting Ca^{2+} level but, if so, this process must be time dependent, since Ca^{2+} in the immediate vicinity rose much higher during the response to InsP_3.

DISCUSSION

The main finding is that elevations of cytosolic free Ca^{2+} level in *Xenopus* oocytes can potentiate the ability of InsP_3 to mobilize further Ca^{2+} from intracellular stores. This result is opposite to earlier results that intracellular injections of Ca^{2+} into oocytes (Parker & Ivorra, 1990*a*) and photoreceptors (Payne, Flores & Fein, 1990) inhibit the Ca^{2+}-mobilizing action of InsP_3.The explanation for this discrepancy probably lies in differences in the concentration and spatial distribution of intracellular Ca^{2+} between the two sets of experiments. Injection through a micropipette leads to a high Ca^{2+} level sharply localized around the pipette tip, whereas the present experiments were done by inducing a more widespread influx of Ca^{2+} across the plasma membrane. Thus, it seems that cytosolic Ca^{2+} ions may have a biphasic effect on InsP_3-mediated Ca^{2+} liberation, facilitating at low levels and inhibiting at higher levels. In the present experiments we were not able to quantify absolute free Ca^{2+} concentrations, because Fluo-3 (and all other currently available long wavelength indicators) do not permit the use of ratio imaging for calibration and, as discussed later, because there may be large spatial inhomogeneities in the distribution of Ca^{2+} in the cell. In particular, the Fluo-3 signal probably reflects a weighted mean of the free Ca^{2+} concentration over a depth of a few tens of micrometres into the cell, but may seriously underestimate the localized increase in Ca^{2+} close to the plasma membrane that results from Ca^{2+} influx. Nevertheless, although our results are somewhat qualitative, they demonstrate that Ca^{2+} facilitation of InsP_3 action is likely to be important under physiological conditions.

Mechanism of Ca^{2+} facilitation

Several explanations for the facilitation of InsP_3 action by Ca^{2+} can be considered. First, it is unlikely that this arose through any non-linearity of the Fluo-3 signal. The photodiode monitor provided a close-to-linear measure of fluorescence, and changes in fluorescence of the indicator evoked by fixed increments in free Ca^{2+} were expected to decrease, rather than increase, as the Ca^{2+} level rose toward saturation of the dye. Furthermore, facilitation was apparent also in recordings of the Ca^{2+}-activated Cl^- current, which provides a linear measure of Ca^{2+} under conditions similar to those in the present experiments (Parker & Ivorra, 1992). A second possibility is that facilitation arose because the Ca^{2+} buffering systems in the cell approached saturation at high Ca^{2+} levels, so that a constant amount of Ca^{2+} released from InsP_3-sensitive stores resulted in a greater increase in free cytosolic Ca^{2+}. Several observations argue against this, including the linear dependence of Cl^- current activation on amount of Ca^{2+} injected into the oocyte or photoreleased from a caged precursor (Parker & Ivorra, 1992), and the fact that facilitation of the InsP_3-evoked signal was already apparent with small elevations of Ca^{2+} that were well below the

maximum level (e.g. Fig. 2, and responses during the rising phase in Fig. 3*A*). Another possibility is that increases in cytosolic Ca^{2+} level lead to greater filling of intracellular stores, so that they are then able to release more Ca^{2+} when subsequently challenged by $InsP_3$. However, this is unlikely, as signals resulting from Ca^{2+} influx were potentiated during continued photorelease of $InsP_3$, when the intraluminal Ca^{2+} level was expected to be reduced (e.g. Fig. 3*A*).

Another way in which Ca^{2+} may facilitate the $InsP_3$-evoked Ca^{2+} signal is by exerting positive feedback on the release process. Three specific mechanisms have been proposed, and are briefly summarized in the Introduction. Considering each in turn, the proposal that Ca^{2+} stimulates phospholipase C to cause increased production of $InsP_3$ (Swann & Whitaker, 1986; Harootunian *et al.* 1991) implies that a local elevation of cytosolic Ca^{2+} should evoke a self-amplifying release of further Ca^{2+}, which will propagate as a wave to engulf the whole cell. Differing with this, we found that injections of Ca^{2+} into the oocyte produced only a localized elevation of intracellular Ca^{2+}, spreading decrementally from the injection site, and that Ca^{2+} liberation induced by photorelease of $InsP_3$ remained restricted to the area of the cell exposed to photolysis light. A further argument against this model is that if Ca^{2+} stimulates $InsP_3$ production, injections of Ca^{2+} should evoke oscillatory Ca^{2+}-dependent membrane current responses like those seen following injection of $InsP_3$ (Parker & Miledi, 1986). Instead, smoothly graded currents are usually observed (Miledi & Parker, 1984) which vary linearly in size with the amount of Ca^{2+} injected (Parker & Ivorra, 1992). Facilitation of responses to exogenous $InsP_3$ by Ca^{2+}-dependent stimulation of endogenous $InsP_3$ production is, therefore, unlikely to account for our results, although it could still be important during receptor and G protein-linked activation of phospholipase C.

The failure of Ca^{2+} injections to evoke regenerative release of stored Ca^{2+} argues also against Ca^{2+}-induced Ca^{2+} release from $InsP_3$-insensitive stores being the mechanism underlying the facilitation of $InsP_3$ action by Ca^{2+} (Berridge & Irvine, 1989; Rooney *et al.* 1989; Goldbeter *et al.* 1990). Instead, our results seem most consistent with the finding that $InsP_3$ and Ca^{2+} act as co-agonists to cause opening of the Ca^{2+} release channel (Iino, 1990; Bezprozvanny *et al.* 1991; Finch *et al.* 1991). In particular, this mechanism explains why Ca^{2+}-induced Ca^{2+} release and propagating Ca^{2+} waves were seen only when $InsP_3$ levels were elevated, and the bell-shaped dose dependence of Ca^{2+} accounts for the observations that small elevations of cytosolic Ca^{2+} potentiate Ca^{2+} release whereas higher levels inhibit. Nevertheless, it is difficult to exclude completely a two-pool model, in which Ca^{2+} induces regenerative Ca^{2+} release from $InsP_3$-insensitive stores that have been primed by accumulation of Ca^{2+} liberated from $InsP_3$-sensitive stores (Berridge & Irvine, 1989; Rooney *et al.* 1989; Goldbeter *et al.* 1990). Thus, although the experiment of Fig. 5*B* shows that $InsP_3$ is needed for wave propagation, the requirement may not be direct, and the failure of the Ca^{2+} wave to propagate into the unexposed half of the cell could have arisen because there was no priming of Ca^{2+}-sensitive stores.

Although the notion that cytosolic Ca^{2+} exerts positive feedback on $InsP_3$-mediated Ca^{2+} release provides an attractive explanation for many of our results, some observations remain puzzling. One is that the regenerative nature of the Ca^{2+} feedback might be expected to lead to an explosive, all-or-none release characteristic,

whereas the Ca^{2+} signal grew in a smoothly graded manner with increasing levels of $InsP_3$ (Fig. 3*A*; and see Parker & Ivorra, 1990*b*), and the facilitation of the $InsP_3$-evoked response by exogenous Ca^{2+} was also graded. Two observations may help to explain this discrepancy. Firstly, the delayed negative feedback of cytosolic Ca^{2+} on Ca^{2+} liberation (Parker & Ivorra, 1990*a*) could reduce the effect of the positive feedback so that it was no longer regenerative, at least for slowly rising Ca^{2+} levels such as in Fig. 3. Secondly, recent findings have shown that $InsP_3$-sensitive Ca^{2+} stores in the oocyte are arranged as multiple, functionally independent units, that release their contents in a train of repetitive all-or-none Ca^{2+} spikes (Parker & Ivorra, 1990*b*; Parker & Yao, 1991). Different units show varying threshold sensitivities to $InsP_3$ (Parker & Yao, 1991), and their frequency of spiking increases with increasing concentration of $InsP_3$ (Parker & Ivorra, 1993). Thus, the apparently smoothly graded responses recorded in the present experiments from wide areas of the cell probably represent the summated activity of many release units, that each function independently in an all-or-none manner. A related question is why the apparently small rise in cytosolic Ca^{2+} induced by activating voltage-operated Ca^{2+} channels was able to dramatically potentiate $InsP_3$-induced Ca^{2+} release (Fig. 2), whereas the Ca^{2+} rise resulting from $InsP_3$-induced Ca^{2+} mobilization did not, itself, produce a similar potentiation. The answer may lie in the different spatial localization of Ca^{2+} from the two sources. The transient localized Ca^{2+} increase near the membrane resulting from Ca^{2+} influx was probably underestimated by the Fluo-3 signal, but could have strongly potentiated a more prolonged release of Ca^{2+} from adjacent $InsP_3$-sensitive stores.

The finding that Ba^{2+} and Sr^{2+} are able to substitute for Ca^{2+} in facilitating the action of $InsP_3$ may provide a useful tool for further investigation of the mechanism of the facilitation. Differing with our results, Baquero-Leonis & Pintado (1989) reported that these ions inhibited $InsP_3$-mediated Ca^{2+} release in thymocyte microsomes. However, this discrepancy might have arisen through differences in experimental procedures if the actions of Ba^{2+} and Sr^{2+} are like those of Ca^{2+}, and show both time and dose dependence.

$InsP_3$-dependent Ca^{2+}-induced Ca^{2+} release

Depolarization of *Xenopus* oocytes that have been injected with mRNA from rat brain evokes large Ca^{2+}-activated Cl^- currents comprised of two components; a monotonically decaying current T_{out1}, on which a delayed 'hump' (T_{out2}) is sometimes superimposed (Miledi *et al.* 1986; Gillo, Landau, Moriarty, Roberts & Sealfon, 1989). In native (non-mRNA-injected) oocytes T_{out1} is usually present, though small and T_{out2} is rarely seen (Miledi, 1982; Barish, 1983). Gillo *et al.* (1989) proposed that the T_{out2} arose because injection of brain mRNA induced expression in the oocyte of a Ca^{2+} release mechanism. Our results suggest a different interpretation, namely that Ca^{2+} influx through exogenous voltage-gated Ca^{2+} channels is sufficient to trigger a secondary release of Ca^{2+} from $InsP_3$-sensitive stores. Many oocytes have elevated resting levels of intracellular $InsP_3$, as demonstrated by the presence of spontaneous oscillations in membrane current (Kusano, Miledi & Stinnakre, 1982) which can be blocked by caffeine, an inhibitor of $InsP_3$ action (Parker & Ivorra, 1991). Thus, variability in size of T_{out2} between oocytes may reflect differences in resting level of $InsP_3$, and the small Ca^{2+} influx

through native Ca^{2+} channels in non-mRNA-injected oocytes may be insufficient to trigger release of sequestered Ca^{2+}. In agreement with this interpretation, Gillo *et al.* (1989) found that T_{out2} was potentiated by serotonin (which activates the $InsP_3$ signalling pathway) and, although injections of $InsP_3$ gave only slight potentiation, this relative lack of effect may be explained if microinjected $InsP_3$ failed to spread throughout the entire cell. An alternative, though unlikely possibility, is that $InsP_3$ may modulate membrane Ca^{2+} channels to cause a delayed influx of Ca^{2+}. Against this, Gillo *et al.* (1989) found that Ba^{2+} currents through the Ca^{2+} channels did not show any delayed 'hump' component, and our finding that Ca^{2+} influx potentiates responses to subsequent photorelease of $InsP_3$ is consistent with potentiation of intracellular Ca^{2+} liberation.

As well as accounting for the delayed Ca^{2+}-activated current on depolarization (T_{out2}), Ca^{2+}-induced release of Ca^{2+} from $InsP_3$-sensitive stores is also likely to be involved in the slowly developing, Ca^{2+}-dependent chloride current (I_{Tin}) evoked when oocytes are hyperpolarized during activation of $InsP_3$ signalling (Parker, Gundersen & Miledi, 1985). In this case, the increased electrical driving force enhances entry of Ca^{2+} ions into the cell through $InsP_3$-operated channels in the surface membrane (Parker & Miledi, 1987), but the slow time course of the Cl^- current suggests that it may also arise through triggered release of intracellular Ca^{2+}, as well as resulting directly from the entry of extracellular Ca^{2+}.

Discrepancies between fluorescence and membrane current Ca^{2+} signals

Voltage clamp recordings of Ca^{2+}-dependent Cl^- membrane current (Miledi & Parker, 1984) provide a convenient monitor of intracellular free Ca^{2+}, but several discrepancies have already been noted between the time course of the current and that of intracellular Ca^{2+} signals monitored by various optical probes (Parker & Miledi, 1986; Parker & Ivorra, 1990*a*, *b*; Lechleiter *et al.* 1991*b*). Figure 3 illustrates a further, dramatic discrepancy. Gradual photorelease of $InsP_3$ evoked a rise in Fluo-3 fluorescence that grew over a few minutes to become more than ten times greater than the transient signals evoked by control depolarizing pulses. Very different to this, the Ca^{2+}-activated Cl^- currents during the pulses were about 100 times larger than that evoked by $InsP_3$. Reasons for this difference are not entirely clear, but may involve at least two factors. The first is the spatial distribution of Ca^{2+} in the cell. The Ca^{2+}-activated current presumably reflects Ca^{2+} levels next to the inner membrane surface, whereas the Fluo-3 signal is a weighted average of Ca^{2+} over depths of a few tens of millimetres into the cell (limited by the turbidity of the cytoplasm). Thus, transient Ca^{2+} entry through membrane channels is expected to result in a steeply decaying gradient of Ca^{2+} inward from the plasma membrane, in contrast to a more homogeneous distribution as Ca^{2+} ions diffuse over several tens of seconds from $InsP_3$-activated stores located within the cell. Active extrusion of Ca^{2+} ions across the plasma membrane may be sufficient to maintain a low Ca^{2+} level near the Cl^- channels in the face of restricted diffusion of Ca^{2+} from $InsP_3$-mediated release sites, but could be transiently overwhelmed by Ca^{2+} influx through membrane channels. A second factor may be inactivation of the membrane Cl^- channels during prolonged elevation of intracellular free Ca^{2+}, since brief large (several microamps) currents can be evoked by brief flashes that give Fluo-3 signals comparable to that in Fig. 3. However, if inactivation of the Cl^- channels is responsible for the failure of the

gradually rising Ca^{2+} level in Fig. 3 to evoke appreciable current, this is difficult to reconcile with the observation that the currents evoked by depolarizations were not also inactivated.

Physiological role of Ca^{2+} facilitation of $InsP_3$-mediated Ca^{2+} liberation

Ca^{2+} potentiation of $InsP_3$-mediated Ca^{2+} release is likely to be of physiological importance in at least two respects. Firstly, positive feedback by cytosolic Ca^{2+} ions provides a mechanism for the regenerative Ca^{2+} release process that underlies the production of Ca^{2+} spikes and waves (Meyer, 1991; Meyer & Stryer, 1991). Although we had originally proposed that a delayed negative feedback of Ca^{2+} on $InsP_3$ action might be sufficient to account for oscillatory liberation of Ca^{2+} (Parker & Ivorra, 1990*a*), recent observations, including the nearly all-or-none dose dependence of Ca^{2+} liberation (Parker & Ivorra, 1990*b*; Parker & Yao, 1991) and the active propagation of Ca^{2+} waves (Lechleiter *et al.* 1991*a*), clearly indicates that an additional process of positive feedback must exist. A second role for Ca^{2+} potentiation of $InsP_3$ action, which is likely to be of particular importance in neurones, is that it provides a mechanism for cross-modulation between the $InsP_3$ signalling pathway and responses evoked by Ca^{2+} entry through voltage- and ligand-gated membrane channels. For example, slow synaptic responses mediated by receptors that couple to the phosphoinositide pathway may be potentiated by a preceding action potential which opens membrane Ca^{2+} channels, or by activation of NMDA receptor/channels. Conversely, responses evoked by Ca^{2+} entry across the cell membrane may be amplified by a process of $InsP_3$-dependent Ca^{2+}-induced Ca^{2+} release, even though little or no response is directly evoked by the $InsP_3$ itself. Furthermore, the finding that localized elevations of Ca^{2+} act as a focus for initiation of Ca^{2+} liberation by $InsP_3$ raises the possibility that the spatial pattern of Ca^{2+} waves in a neuron may be determined by the distribution of active synapses.

Note added in proof. After this paper went to press, DeLisle & Welsh (1992) and Lachleiter & Clapham (1992) also reported that propagation of Ca^{2+} waves in the oocyte requires the presence of $InsP_3$.

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