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INOSITOL TRISPHOSPHATE-MEDIATED Ca^{2+} INFLUX INTO *XENOPUS* OOCYTES TRIGGERS Ca^{2+} LIBERATION FROM INTRACELLULAR STORES

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SUMMARY

1. Inositol 1,4,5-trisphosphate ($InsP_3$) functions as a second messenger by liberating Ca^{2+} from intracellular stores and by promoting influx of extracellular Ca^{2+}. We examined whether Ca^{2+} influx modulates intracellular Ca^{2+} liberation in *Xenopus* oocytes by fluorescence monitoring of cytosolic free Ca^{2+} together with voltage clamp recording of Ca^{2+}-activated Cl^- membrane currents. Sustained activation of membrane Ca^{2+} permeability was induced by intracellular injections of a non-metabolizable $InsP_3$ analogue, 3-deoxy-3-fluoro-D-*myo*-inositol 1,4,5-trisphosphate (3-F-$InsP_3$), and Ca^{2+} influx was controlled by applying step changes in membrane potential to alter the driving force for Ca^{2+} entry.

2. Negative-going potential steps evoked intracellular Ca^{2+} signals comprising two components; an initial transient peak followed by a slower rise. The initial transient grew steeply over a narrow (*ca* 40 mV) voltage range but then increased little with further polarization, whereas the second component showed a nearly linear voltage dependence.

3. The transient Ca^{2+} signal continued to rise almost unchanged when Ca^{2+} influx was interrupted by stepping the potential to more positive values after brief hyperpolarization. In contrast, Ca^{2+} levels declined monotonically when positive-going steps were applied after longer intervals during the second component of the Ca^{2+} signal.

4. Large Ca^{2+}-dependent transient inward (T_{in}) membrane currents were evoked during the rising phase of the initial Ca^{2+} signal, but little current was associated with the second component of the Ca^{2+} signal.

5. The T_{in} currents evoked by hyperpolarization were mimicked at fixed clamp potential by re-admitting Ca^{2+} to the bathing solution, and by flash photolysis of caged Ca^{2+} loaded into the oocyte.

6. T_{in} currents were strongly inhibited by prior release of Ca^{2+} from $InsP_3$-sensitive intracellular stores, and vice versa. Experiments with paired hyperpolarizing pulses and paired photorelease of $InsP_3$ showed that responses to both stimuli recovered with similar time courses.

7. We conclude that the transient Ca^{2+} signal and associated T_{in} current evoked by hyperpolarization arise because Ca^{2+} entering the oocyte triggers regenerative release

of Ca^{2+} from InsP_3-sensitive intracellular stores. Since membrane currents evoked by liberated Ca^{2+} were much greater than those evoked by Ca^{2+} entry *per se*, a major function of InsP_3-mediated Ca^{2+} entry may be to modulate the activity of intracellular Ca^{2+} stores.

INTRODUCTION

Inositol 1,4,5-trisphosphate (InsP_3) serves as an intracellular second messenger in a multitude of cell types, in which it functions by elevating cytosolic free Ca^{2+} levels (Berridge & Irvine, 1989). The source of this Ca^{2+} is twofold. InsP_3 acts on receptors in intracellular organelles to liberate sequestered Ca^{2+} ions (Berridge & Irvine, 1989; Taylor & Richardson, 1991; Ferris & Snyder, 1992) and, by mechanisms that are less well understood, increases the permeability of the plasma membrane to Ca^{2+} so as to allow a passive influx of extracellular Ca^{2+} ions (Berridge & Irvine, 1989; Matthews, Neher & Penner, 1989; Jacob, 1990; Putney, 1990; Irvine, 1992). Ca^{2+} ions contributed from both sources have often been assumed to act independently, so that the resulting elevation in free cytosolic Ca^{2+} reflects simply the summation of Ca^{2+} originating from intracellular stores and from the extracellular medium. In particular, the initial Ca^{2+} transient evoked in many cells by Ca^{2+}-mobilizing agonists is thought to arise through intracellular liberation, whereas Ca^{2+} influx provides the subsequent, sustained elevation of free Ca^{2+} (Berridge & Irvine, 1989; Taylor & Richardson, 1991; Irvine, 1992).

We had previously studied InsP_3-mediated Ca^{2+} influx in *Xenopus* oocytes, by recording membrane currents evoked when the driving force for Ca^{2+} entry was enhanced by stepping to more negative potentials (Parker, Gundersen & Miledi, 1985; Parker & Miledi, 1987). In oocytes where InsP_3 levels were elevated by agonist activation, or by intracellular injection of InsP_3, hyperpolarizing pulses evoked transient inward (T_{in}) Cl^- currents, that were abolished by removal of extracellular Ca^{2+} and by intracellular injection of the Ca^{2+}-chelating agent EGTA. Thus, it appeared that InsP_3 increased the permeability of the plasma membrane to Ca^{2+}, so that increases in electrical driving force enhanced the influx of Ca^{2+} ions into the cell, leading to the opening of Ca^{2+}-dependent Cl^- membrane channels (Miledi & Parker, 1984). However, the kinetics of the T_{in} current were difficult to account for on the basis that the current arose simply from the opening of Cl^- channels by Ca^{2+} ions that entered the cell. Specifically, the current began with a lag varying between tens and hundreds of milliseconds following the onset of hyperpolarization, and subsequently decayed within about 1 s, even though measurements with aequorin indicated that Ca^{2+} influx persisted for much longer (Parker & Miledi, 1987).

A different explanation for origin of the T_{in} current arises from recent findings that cytosolic Ca^{2+} ions exert a biphasic action on InsP_3-stimulated liberation of Ca^{2+} from intracellular stores, producing a transient facilitation followed by a more slowly developing inhibition (Iino, 1990; Parker & Ivorra, 1990*a*; Finch, Turner & Goldin, 1991; Bezprozvanny, Watras & Erlich, 1991; Yao & Parker, 1992*a*). Thus, it is possible that Ca^{2+} ions entering across the plasma membrane could trigger a transient liberation of Ca^{2+} from intracellular stores, followed by a more prolonged inhibition of InsP_3-mediated Ca^{2+} liberation.

In the present paper we examine whether InsP_3-mediated entry of extracellular

Ca^{2+} modulates the liberation of Ca^{2+} ions from $InsP_3$-sensitive intracellular stores. Oocytes were injected with a non-metabolizable $InsP_3$ derivative, so as to induce a prolonged and stable increase in membrane permeability to Ca^{2+}, and we then recorded intracellular Ca^{2+} signals and Ca^{2+}-activated membrane currents evoked using voltage steps to vary the electrical driving force for Ca^{2+} influx. The results support the idea that Ca^{2+} entry triggers regenerative liberation of sequestered intracellular Ca^{2+} and, because Ca^{2+}-activated membrane currents associated with this Ca^{2+} release were much larger than those arising from the Ca^{2+} entry *per se*, suggest that a major role for $InsP_3$-mediated Ca^{2+} entry may be to regulate the activity of intracellular stores.

Abstracts describing some of this work have appeared (Yao & Parker, 1992*b*; Parker & Yao, 1993).

METHODS

Experiments were done on isolated oocytes of *Xenopus laevis*, obtained from albino frogs to avoid problems during optical recording and stimulation encountered with normally pigmented oocytes. Procedures for preparation of oocytes, voltage clamp recording, photolysis of caged compounds and fluorescence recordings of intracellular free Ca^{2+} were mostly as described (Sumikawa, Parker & Miledi, 1989; Parker & Yao, 1991; Parker, 1992). However, an important difference was that calcium green-5N (Molecular Probes, Eugene, OR, USA) was used as the Ca^{2+} indicator, instead of fluo-3. This newly available probe has a low affinity for Ca^{2+}, (*ca* 3·3 μM; manufacturers' data), shows a high fluorescence in the presence of Ca^{2+} and is relatively resistant to bleaching. A particular advantage for the present experiments was that good Ca^{2+} signals could be obtained with levels of dye loading that caused little change in $InsP_3$-evoked membrane current responses (final intracellular concentration about 50 μM). In contrast, after loading sufficient fluo-3 to obtain adequate fluorescence signals (> about 20 μM), the currents evoked by hyperpolarization and by flash photolysis of caged $InsP_3$ were reduced in amplitude, or even abolished, suggesting that the dye caused appreciable buffering of intracellular Ca^{2+}.

Fluorescence recordings from the entire visible hemisphere of the oocyte were made through an upright microscope (Zeiss) fitted with a 6·3× objective lens, and fluorescence intensity was measured either by a photomultiplier, or by a photodiode placed behind the intensifier tube of an intensified CCD camera mounted on the microscope phototube. Calcium green-5N shows no shifts in excitation or emission wavelengths on Ca^{2+} bindings, so it was not possible to use ratio measurements to calibrate fluorescence signals in terms of absolute free Ca^{2+} signals. Also, because the fluorescence signal arises throughout a depth of about 20 μm into the cell (limited by turbidity of the cytoplasm), it gives only a weighted average of the Ca^{2+} concentrations throughout this volume, whereas spatial gradients in Ca^{2+} are likely to exist, (Yao & Parker, 1992*a*). For these reasons, fluorescence measurements are presented uncalibrated, or as fractional changes in fluorescence above the resting baseline ($\Delta F/F$). Nevertheless, because of the low affinity of calcium green-5N, the size of fluorescence signals recorded in a given experiment provide a roughly linear indication of relative changes in free Ca^{2+}. Autofluorescence of oocytes not injected with dye was negligible (< 10% of the resting level of cells injected with calcium green-5N).

We attempted to calibrate the average Ca^{2+} concentration changes by ratio measurements with fura-2, but failed because this indicator interfered strongly with the Ca^{2+} signal. The T_{in} current was completely suppressed in oocytes loaded with fura-2 to a final intracellular concentration of about 30 μM, and was reduced to about one-half at a concentration of 6 μM. Although fluorescence Ca^{2+} signals could be detected with as little as 6 μM fura-2, reliable ratio measurements could not be made because autofluorescence of the oocyte was greater than the fura-2 fluorescence and showed marked bleaching.

Oocytes were usually loaded with about 10 nl of a solution containing 5 mM calcium green-5N together with 15 μM 3-deoxy-3-fluoro-D-*myo*-inositol 1,4,5-trisphosphate (3-F-$InsP_3$) about 30 min before beginning recording. Respective final intracellular concentrations would have been about 50 μM and 150 nM, assuming uniform distribution throughout a cytosolic volume of 1 μl. For some

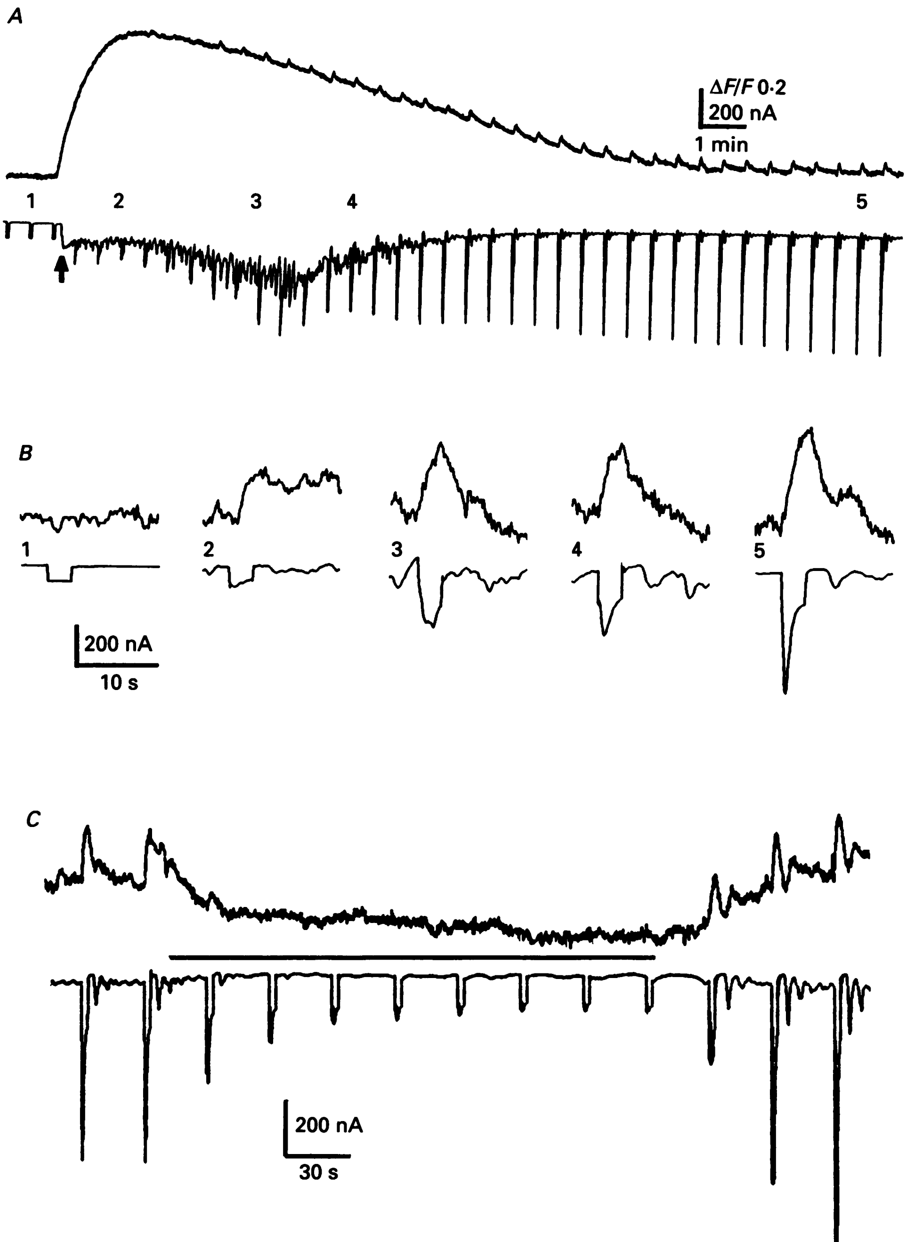

Fig. 1. Mobilization of intracellular Ca^{2+} and prolonged activation of Ca^{2+} entry by intracellular injection of 3-F-InsP_3. In each frame, the upper traces show intracellular Ca^{2+} signals monitored by recording calcium green-5N fluorescence from the entire visible hemisphere of the oocyte and the lower traces show membrane current. *A*, the oocyte was voltage clamped at a holding potential of −50 mV and stepped repeatedly at 30 s intervals to −110 mV during 3 s pulses. An intracellular injection of about 100 fmol 3-F-InsP_3 (1 nl of 100 μM solution) was given at the arrow. The bathing solution was normal (1·8 mM Ca^{2+}) Ringer solution. *B*, selected responses to hyperpolarizing pulses in *A* shown at faster sweep speed; numbers correspond to the marked stimuli in *A*. *C*, Ca^{2+} signals and

experiments, oocytes were injected also with various caged compounds, so that flashes of near UV light could be used to photorelease $InsP_3$ or Ca^{2+} in the oocyte. Caged $InsP_3$ (*myo*-inositol 1,4,5-trisphosphate, $P^{4(5)}$-1-(2-nitrophenyl)ethyl ester) (Walker, Feeney & Trentham, 1989; McCray & Trentham, 1989) was injected as a 0·5 mM solution, and oocytes were normally loaded with about 5 pmol. DM-nitrophen (caged Ca^{2+}) was prepared as a 30 mM solution, together with 1·42 mM Ca^{2+} and 29·44 mM Mg^{2+}. Assuming respective apparent affinities of non-photolysed DM-nitrophen for Ca^{2+} and Mg^{2+} of 5 nM and 500 nM (Kaplan & Ellis-Davies, 1988), the free concentrations of Ca^{2+} and Mg^{2+} in the injection solution would have been, respectively, about 50 nM and 1 mM; thus approximating the resting free concentrations of these ions in the cytosol. Oocytes were injected with about 30 nl of DM-nitrophen solution, resulting in a final intracellular concentration of about 1 mM. Although DM-nitrophen acts as a caged Mg^{2+} as well as caged Ca^{2+}, the amounts of Mg^{2+} released were probably negligible as compared to the resting level, since each light flash photolysed only a small proportion of the total amount of DM-nitrophen present.

Unless otherwise noted, oocytes were continually superfused during recording with Ringer solution at room temperature. The composition of this solution was (mM): NaCl, 120; KCl, 2; $CaCl_2$, 1·8; Hepes, 5; at pH about 7·0. Ca^{2+}-free solution was made by omitting $CaCl_2$ and adding 1 mM EGTA together with 5 mM $MgCl_2$.

Calcium green-5N was obtained from Molecular Probes Inc. (Eugene, OR, USA); caged $InsP_3$, 3-F-$InsP_3$ and DM-nitrophen were from Calbiochem (La Jolla, CA, USA); all other reagents were from Sigma Chemical Co. (St Louis, MO, USA).

RESULTS

Induction of Ca^{2+} influx by a non-metabolizable $InsP_3$ derivative

Intracellular injections of $InsP_3$ into *Xenopus* oocytes increase the membrane permeability to Ca^{2+} for several minutes (Parker & Miledi, 1987) but, for the present experiments, we wanted a sustained, stable increase in permeability so as to allow repeatable measurements during longer recording periods. This was accomplished by injecting oocytes with a non-metabolizable $InsP_3$ analogue, 3-deoxy-3-fluoro-D-*myo*-inositol 1,4,5-trisphosphate (3-F-$InsP_3$), which is about equipotent to $InsP_3$ in liberating intracellular Ca^{2+}, but cannot be converted to inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P_4) by the action of 3-kinases since the 3-position is blocked by fluorine (Kozikowski, Fauq, Aksoy, Seewald & Powis, 1990). When injected into oocytes 3-F-$InsP_3$ evoked transient responses resulting from intracellular Ca^{2+} liberation, and also activated a prolonged increase in Ca^{2+} permeability of the cell membrane (Yao & Parker, 1992*a*, *b*).

Figure 1 shows typical responses evoked by injection of 3-F-$InsP_3$, measured using the fluorescent indicator calcium green-5N to monitor average intracellular free Ca^{2+} levels across the oocyte together with voltage clamp recording of Ca^{2+}-activated Cl^- membrane currents (Miledi & Parker, 1984). Injection of about 100 fmol 3-F-$InsP_3$ produced a rapid increase in fluorescence, together with oscillatory Cl^- currents like those induced by $InsP_3$ (Parker & Miledi, 1986). These responses persisted for several minutes and were independent of extracellular Ca^{2+}, since they were seen in oocytes bathed in Ca^{2+}-free Ringer solution. Ca^{2+} entry was monitored by applying repetitive hyperpolarizing steps, to transiently increase the driving force for Ca^{2+} entry (Parker *et al.* 1985; Parker & Miledi, 1987). In the resting oocyte, hyperpolarization evoked

membrane currents evoked by hyperpolarization are abolished in Ca^{2+}-free solution. The traces are continuations of those in *A*. During the time marked by the bar, the bathing solution was changed to Ca^{2+}-free Ringer solution.

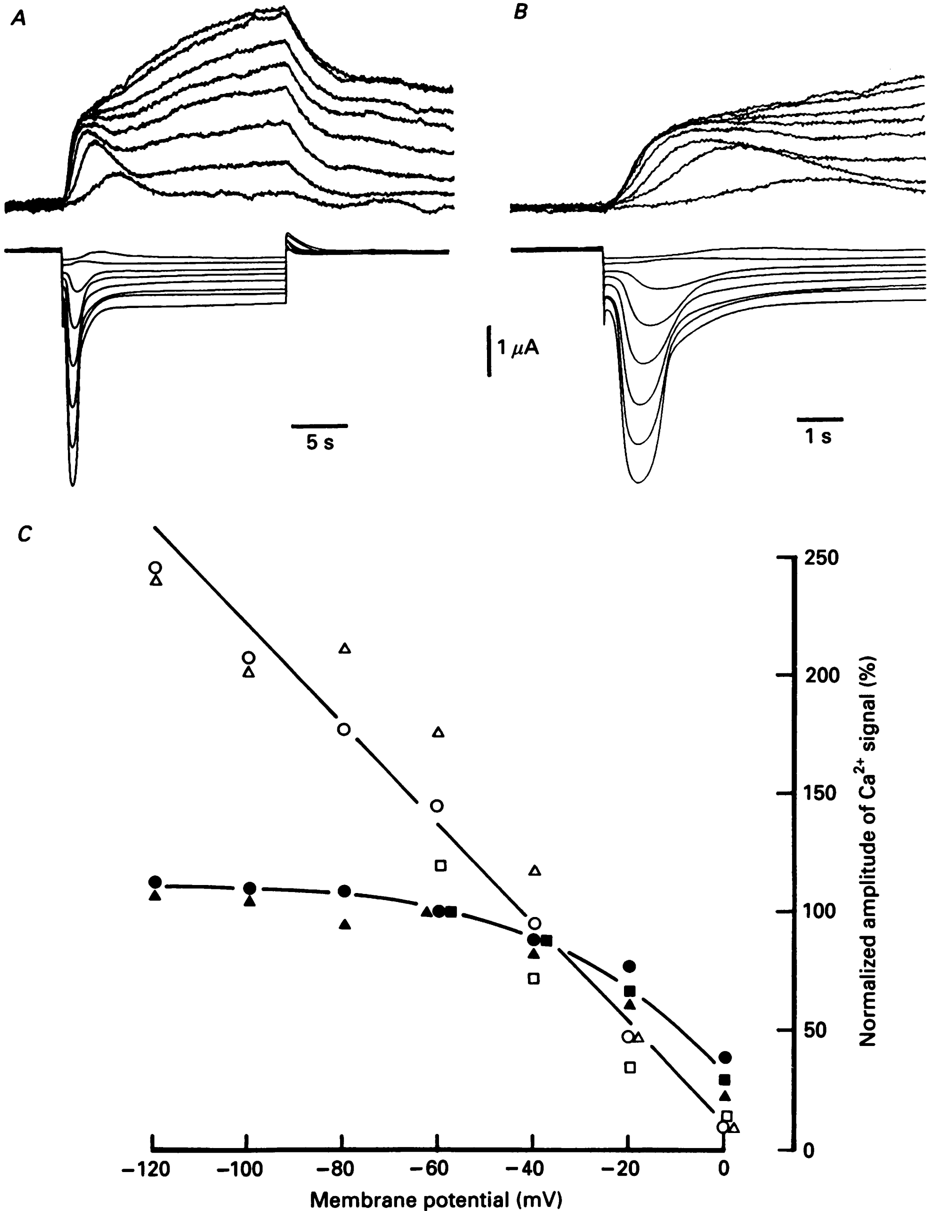

Fig. 2. Transient and sustained Ca^{2+} signals evoked by polarization to increasingly negative potentials. *A*, superimposed records show calcium green-5N fluorescence signals (upper traces) and membrane currents (lower traces) evoked by steps from a holding potential of +20 mV to potentials from 0 to −120 mV in 20 mV increments. The oocyte was loaded with 3-F-InsP_3 about 1 h before recording. *B*, the same records as *A* shown at faster sweep speed to illustrate better the transient responses. *C*, measurements of fluorescence Ca^{2+} signals made at the peak of the initial transient response (filled symbols) and at the end of the 20 s duration polarizing steps (open symbols). Data are from three oocytes, denoted by different symbols, and measurements marked by circles are from the oocyte illustrated in *A*. Curves are drawn by eye. Because the absolute fluorescence signals varied in size between the oocytes, measurements of both transient and sustained Ca^{2+}

only passive 'leakage' currents, and no detectable fluorescence signal. However, identical steps applied after injecting 3-F-InsP_3 evoked T_{in} currents and fluorescence signals, that grew progressively to reach a fairly stable level after about 15 min. Both of these responses depended on the presence of Ca^{2+} in the bathing medium, since they were abolished after removal of extracellular Ca^{2+}, but subsequently recovered when Ca^{2+} was replaced (Fig. 1*C*). A similar activation of both intracellular Ca^{2+} liberation and Ca^{2+} entry by 3-F-InsP_3 was seen in eleven other oocytes in which membrane currents and fluorescence signals were monitored during injection of 3-F-InsP_3. Furthermore, Ca^{2+} influx was consistently activated in more than fifty oocytes preloaded with 3-F-InsP_3 before beginning recording.

The T_{in} currents and associated fluorescence signals induced by 3-F-InsP_3 persisted for an hour or more after injection. For example, in the oocyte of Fig. 1 the T_{in} current evoked by polarization to -110 mV was 350 nA 15 min after injection, and was still 320 nA 1 h later. In contrast, the large Ca^{2+} signal and oscillatory currents arising from intracellular Ca^{2+} liberation usually subsided within about 15 min – possibly as a result of dilution of 3-F-InsP_3 as it diffused throughout the large oocyte cell from the injection point. Thus, after waiting for about 20 min, it was possible to record responses evoked by changes in Ca^{2+} entry, with little interference from on-going intracellular Ca^{2+} release. The persistent activation of Ca^{2+} entry suggests that 3-F-InsP_3 is resistant not only to phosphorylation by the 3-kinase, but also to degradation by phosphatase enzymes.

Ca^{2+} entry evokes biphasic intracellular Ca^{2+} signals

To study how intracellular free Ca^{2+} levels varied under conditions of differing Ca^{2+} influx, we applied voltage steps to 3-F-InsP_3-loaded oocytes bathed in normal Ringer solution (1·8 mM Ca^{2+}), so as to rapidly change the electrical driving force for Ca^{2+} entry. Figure 2*A* and *B* shows representative Ca^{2+} signals and Ca^{2+}-activated membrane currents evoked by a series of pulses to increasingly negative potentials, from a holding potential of $+20$ mV. Because the equilibrium potential for Cl^- ions in the oocyte is about -25 mV (Kusano, Miledi & Stinnakre, 1982), Ca^{2+}-activated Cl^- currents are outwardly directed (upward deflections) at potentials more positive than this, and inwardly directed at more negative potentials. Following the nomenclature introduced by Parker *et al.* (1985) we continue to refer to these responses as T_{in} (transient inward) currents, even though they appear as small outwardly directed currents at voltages positive to the Cl^- equilibrium potential.

The intracellular Ca^{2+} signals monitored by calcium green-5N displayed two distinct components, with different voltage and time dependence (Fig. 2*A* and *B*). Small (20 or 40 mV) negative-going steps from a holding potential of $+20$ mV gave an initial transient increase in Ca^{2+} lasting a few seconds, which became larger and rose more rapidly as the voltage was increased. Following this initial peak, the Ca^{2+} level fell to a trough before rising more slowly for the remainder of the polarizing step, and finally declined over a few seconds when the potential was returned to the

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responses were normalized as a percentage of the transient signal evoked at -60 mV in each oocyte. The abscissa shows membrane potential during the pulse, and the initial holding potential was $+20$ mV in all oocytes.

holding level. With further polarization to potentials more negative than about -20 mV the first component increased little, but the second component continued to grow progressively. At -120 mV there was no longer a trough in Ca^{2+} between the two components, but the Ca^{2+} signal still displayed a distinct shoulder before rising to a level about twice that of the initial transient. The mean fluorescence change ($\Delta F/F$) at the peak of the transient Ca^{2+} signal was $0{\cdot}106 \pm 0{\cdot}027$ (± 1 S.E.M.; 7 oocytes).

Measurements in three oocytes showing the voltage dependence of the transient and sustained Ca^{2+} components are plotted in Fig. 2*C*. The transient signal at first grew steeply as the potential was made more negative, but then showed only slight voltage dependence at potentials more negative than about -40 mV. In marked contrast, the size of the sustained Ca^{2+} component, measured at the end of 20 s hyperpolarizing pulses showed a more graded, and nearly linear variation with potential.

The traces in Fig. 2*A* and *B* show striking differences in time course of the Ca^{2+} signals monitored by calcium green-5N fluorescence and the associated Ca^{2+}-activated membrane currents. At all potentials the peak current was maximal during the rising phase of the initial Ca^{2+} component, but then decayed rapidly so that relatively little current remained at the end of the pulse – even at very negative voltages when the final Ca^{2+} signal was appreciably greater than at the peak of the T_{in} current. Possible reasons for these discrepancies are considered in the Discussion but, in brief, it seems that the current amplitude reflects primarily the rate of rise of the fluorescence Ca^{2+} signal rather than its amplitude. In agreement, the rising phase of the initial Ca^{2+} component continued to steepen at potentials more negative than about -20 mV, even though its amplitude failed to increase appreciably. However, we did not attempt to analyse further the relation between Ca^{2+} and magnitude of the current, since the latter is complicated by the non-linear voltage dependence of Cl^- ion flux (Miledi & Parker, 1984).

The T_{in} current does not require changes in membrane potential

Since the T_{in} current was abolished in Ca^{2+}-free solution it was already clear that it was not activated as a direct consequence of a change in membrane potential. However, the possibility remained that generation of the current required both enhancement of Ca^{2+} influx and a voltage change. To test this, we rapidly increased the Ca^{2+} concentration of the extracellular solution while holding the clamp potential steady at -60 mV. As illustrated in Fig. 3, this evoked a transient membrane current response similar to the T_{in} current evoked in the same oocyte by a hyperpolarizing step. Activation of the current response required a fast exchange of solutions and, in Fig. 3, this was achieved by using a high flow rate. Little response to increasing extracellular Ca^{2+} concentration is evident in Fig. 1*C*, probably because the flow of the superfusate was slow, so that the Ca^{2+} concentration around the oocyte increased gradually. Responses like that in Fig. 3*B* were evoked by rapid rises in extracellular Ca^{2+} in three further oocytes loaded with 3-F-InsP_3, whereas three control (non-injected) oocytes from the same donor frog showed no appreciable inward current responses.

Does the initial Ca^{2+} signal arise through Ca^{2+} liberation or transitory Ca^{2+} influx?

The brief duration of the initial Ca^{2+} signal evoked on hyperpolarization is consistent with the notion that an increased influx of extracellular Ca^{2+} triggers a transient liberation of Ca^{2+} from intracellular stores. On the other hand, the Ca^{2+}

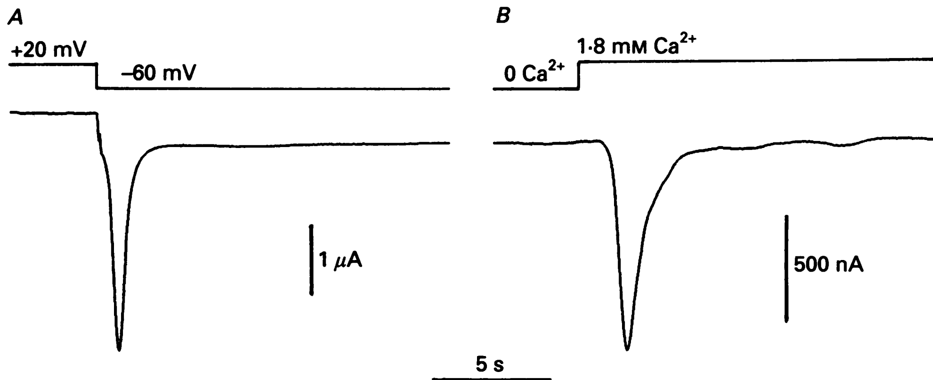

Fig. 3. The T_{in} current activated by hyperpolarization is mimicked at a fixed clamp potential by rapidly increasing the extracellular free Ca^{2+} concentration. *A*, currents evoked in an oocyte loaded with 3-F-$InsP_3$ by stepping the membrane potential from +20 to −60 mV. *B*, currents evoked in the same oocyte by changing the superfusate from Ca^{2+}-free solution (including 1 mM EGTA) to normal Ringer solution including 1·8 mM Ca^{2+}. The clamp potential was −60 mV.

signal instead could arise because of a transient influx of extracellular Ca^{2+}. For example, the $InsP_3$-mediated Ca^{2+} permeability of the plasma membrane might inactivate to a low level within a few seconds of onset of hyperpolarization, in a way analogous to the Ca^{2+}-dependent inactivation of voltage-gated Ca^{2+} channels (Eckert & Chad, 1984). Experiments described in the following sections were done to distinguish between these possibilities. They provide convincing evidence in favour of the former mechanism.

The transient Ca^{2+} signal continues to rise when Ca^{2+} entry is interrupted

Ca^{2+} liberation from $InsP_3$-sensitive stores is regenerative (Parker & Ivorra, 1990*b*; Parker & Yao, 1991). Thus, if the Ca^{2+} transient on hyperpolarization arises through Ca^{2+} release from internal stores, we expected that, once triggered, it should continue almost unchanged even if further Ca^{2+} influx was suppressed by stepping to more positive potentials. On the other hand, if the Ca^{2+} transient arises directly from Ca^{2+} entering the cell, it should be cut short by suppressing Ca^{2+} influx.

Figure 4*A* shows superimposed membrane currents and intracellular Ca^{2+} signals evoked by two hyperpolarizing pulses of differing durations. The longer pulse (2·5 s duration) ended after the T_{in} current had almost completely decayed, whereas the shorter pulse (0·5 s duration) ended before the T_{in} current had peaked. When the membrane potential was returned to 0 mV at the end of the long pulse, the

fluorescence Ca^{2+} signal decayed monotonically, and only a small, brief Ca^{2+}-activated tail current was seen. In contrast, the Ca^{2+} signal continued to rise for about 1 s after the end of the short pulse, reaching a peak amplitude similar to that of the 'shoulder' in response to the longer pulse. Also, the tail current was larger and

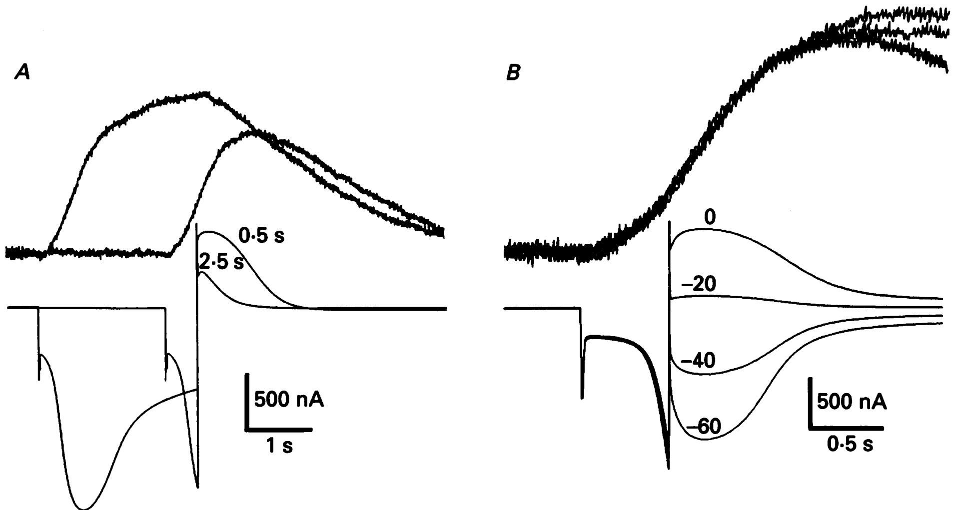

Fig. 4. The transient Ca^{2+} signal shows regenerative characteristics and continues to rise when the driving force for Ca^{2+} entry is reduced by stepping to more positive potentials. *A*, superimposed records show Ca^{2+} signals (upper traces) and membrane currents (lower) evoked by stepping the clamp potential from 0 to −80 mV during pulses lasting 2·5 and 0·5 s. The traces are aligned so that the ends of the two pulses superimpose. Tail currents resulting from each pulse are indicated. *B*, superimposed records showing Ca^{2+} signals and membrane currents evoked by pulses of −0·5 s duration from −20 to −80 mV, following which the potential was returned to the various levels indicated (in mV). Data are from a single oocyte that was loaded with 3-F-InsP_3 and calcium green-5N.

more prolonged than following the longer pulse. Further evidence indicating that the initial Ca^{2+} transient continues to rise after interruption of Ca^{2+} entry is shown in Fig. 4*B*, where the membrane potential was returned to various levels between 0 and −60 mV after a response was initiated by a brief (0·6 s) hyperpolarization. There was virtually no difference in the rising phases of the Ca^{2+} signals, or of their amplitudes at the 'shoulder', despite the widely differing Ca^{2+} influx expected at the different potentials.

One interpretation of this result is that entry of a 'trigger' amount of Ca^{2+} evoked a continuing regenerative liberation of Ca^{2+} from intracellular stores. However, an alternative possibility is that the fluorescence signal continued to rise after Ca^{2+} influx was terminated, because a rapidly inactivating influx during hyperpolarization led to a localized increase in intracellular free Ca^{2+} concentration adjacent to the plasma membrane. If the local concentration was sufficient to saturate the indicator the Ca^{2+} level would initially be underestimated, but the fluorescence would then rise as Ca^{2+} ions diffused further into the cell – even though the total amount of Ca^{2+} did not increase. An argument against this is that the Ca^{2+}-activated Cl^- tail currents,

which presumably reflect the local Ca^{2+} concentration at the inner surface of the cell membrane, continued to rise after interruption of hyperpolarizing pulses (Fig. 4).

Ca^{2+}-dependent inactivation of the T_{in} current and associated Ca^{2+} signal

InsP_3-mediated liberation of sequestered Ca^{2+} is inhibited by raised cytosolic Ca^{2+} levels, becoming suppressed within about 1 s and subsequently recovering over several seconds (Parker & Ivorra, 1990*a*; Iino, 1990; Bezprozvanny *et al.* 1991). If the T_{in} current activated on hyperpolarization arises primarily through triggered release of intracellular Ca^{2+}, it should, therefore, be inhibited by prior elevation of intracellular Ca^{2+} levels, and recover with a time course like that of InsP_3-mediated Ca^{2+} liberation.

To test the first of these predictions we photoreleased InsP_3 from a caged precursor loaded into the oocyte, and examined whether the resulting liberation of intracellular Ca^{2+} inhibited T_{in} currents and Ca^{2+} signals evoked by subsequent hyperpolarizing pulses. A problem in designing this experiment was that the T_{in} current arises from the entire surface of the oocyte, whereas the photolysis light exposed only the hemisphere facing the microscope lens. Thus, if Ca^{2+} influx were globally activated by loading the cell with 3-F-InsP_3, the maximum inhibition that could result from photorelease of InsP_3 would be only 50%. To circumvent this limitation, we induced a local activation of Ca^{2+} influx by exposing the oocyte to photolysis light of low intensity for several minutes (Yao & Parker, 1992*a*). Figure 5*A* shows records obtained beginning about 1 min after the end of an 800 s exposure to UV light attenuated (to about 0·1%) by neutral density filters. Hyperpolarizing pulses failed to evoke any T_{in} current prior to the photolysis exposure, but T_{in} currents and associated Ca^{2+} signals were evoked after the exposure. Brief light flashes at full intensity were then applied to cause transient photorelease of InsP_3, which evoked intracellular Ca^{2+} signals and associated membrane current responses. The T_{in} currents and Ca^{2+} signals evoked by hyperpolarization were strongly depressed for several seconds following a light flash. Conversely, responses evoked by photoreleased InsP_3 were depressed for several seconds following hyperpolarizing pulses (data not shown).

T_{in} currents were also inhibited following photorelease of Ca^{2+} from a caged precursor loaded into the oocyte. Measurements in two oocytes loaded with 3-F-InsP_3 together with DM-nitrophen showed that the T_{in} current reduced to 55% of the control value (mean of 4 trials) when hyperpolarizing pulses were preceded at intervals between 0·5 and 2 s by a light flash causing photorelease of Ca^{2+}. This indicates a strong inhibition by photoreleased Ca^{2+} because, in this experiment, global Ca^{2+} influx was induced following loading of 3-F-InsP_3, whereas photolysis of caged Ca^{2+} was restricted to the exposed half of the cell.

Regarding the time course of inhibition, T_{in} currents evoked by the second hyperpolarizing pulse in a pair are reduced in size at short intervals, but recover to full size as the interpulse interval is lengthened to about 10 s (Parker *et al.* 1985). Similarly, membrane currents evoked by photorelease of InsP_3 using paired light flashes are depressed for several seconds (Parker & Ivorra, 1990*a*). Figure 6*A* and *B* shows an experiment to compare the time course of recovery of both responses in a single oocyte that was loaded with both 3-F-InsP_3 (to induce a stable T_{in} current) and

with caged InsP_3. Measurements of peak sizes of the currents evoked by hyperpolarization (□) and by light flashes (■) for the second stimulus in each pair are plotted in Fig. 6*C* as a percentage of the corresponding control responses evoked by the first stimuli. T_{in} currents and InsP_3-evoked currents recovered with increasing interflash interval along very similar time courses, returning to 50% of the control level after about 6 s.

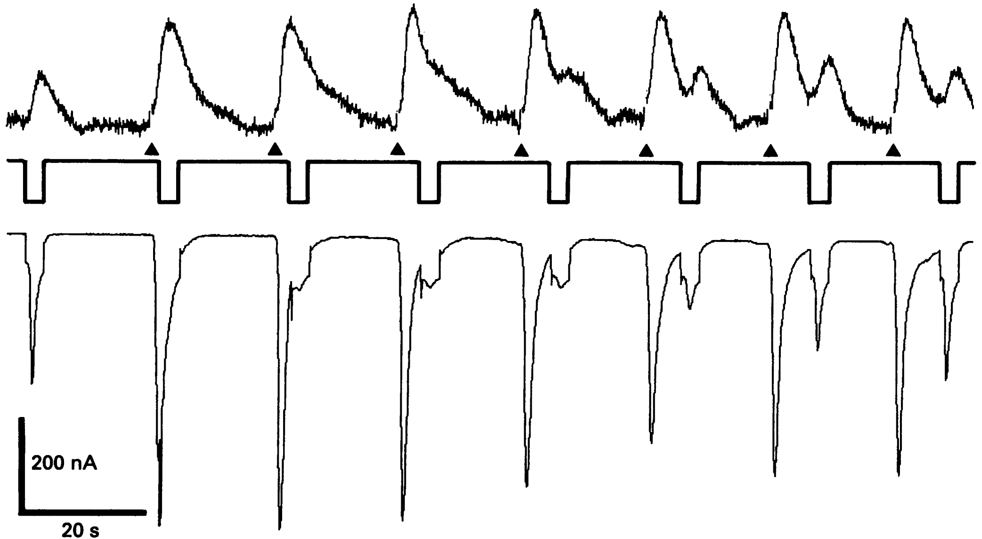

Fig. 5. The T_{in} current activated by hyperpolarization is inhibited by prior InsP_3-mediated liberation of intracellular Ca^{2+}. The upper trace shows fluorescence Ca^{2+} signals, the middle trace indicates steps in membrane potential, and the lower trace shows membrane current. Records are from an oocyte loaded with caged InsP_3 and calcium green-5N, and begin about 1 min after induction of Ca^{2+} influx by prolonged exposure to low intensity UV light. Arrowheads mark times when light flashes (40 ms duration) were applied to cause transient photorelease of InsP_3. The fluorescence traces are blanked out during and shortly after the flash artifacts. Records show responses to a hyperpolarizing pulse alone (to −130 mV from a holding potential of −50 mV), and then to the same pulse delivered at different intervals after photolysis light flashes.

Dissection of transient and sustained Ca^{2+} components

As illustrated in Fig. 7, conditioning hyperpolarizations selectively inhibited the transient Ca^{2+} signal evoked by subsequent test pulses, thus providing a means to separate the two components of the Ca^{2+} signals evoked by hyperpolarization. Figure 7*A* shows Ca^{2+} signals and membrane currents evoked by a test pulse to −80 mV from a holding potential of +20 mV. In Fig. 7*B* the same test pulse was preceded by a brief conditioning pulse to −120 mV, whereas Fig. 7*C* shows responses to the conditioning pulse alone. Subtraction of *C* from *B* then revealed those responses to the test pulse that remained following the conditioning pulse (Fig. 7*D*). The Ca^{2+} signal rose slowly during the pulse to reach a steady level after about 5 s, and subsequently declined over a similar period when the potential was returned to the holding level. In contrast, the membrane current showed almost no active response, but only a passive leakage current. Finally, Fig. 7*E* shows the inactivating

components of the test responses, derived by subtracting the traces in D from the control responses evoked in A by the test pulse alone. The inactivating Ca^{2+} signal was comprised primarily of a transient spike, which reached a peak within about 1 s of the onset of hyperpolarization, and then decayed within about 5 s even though the

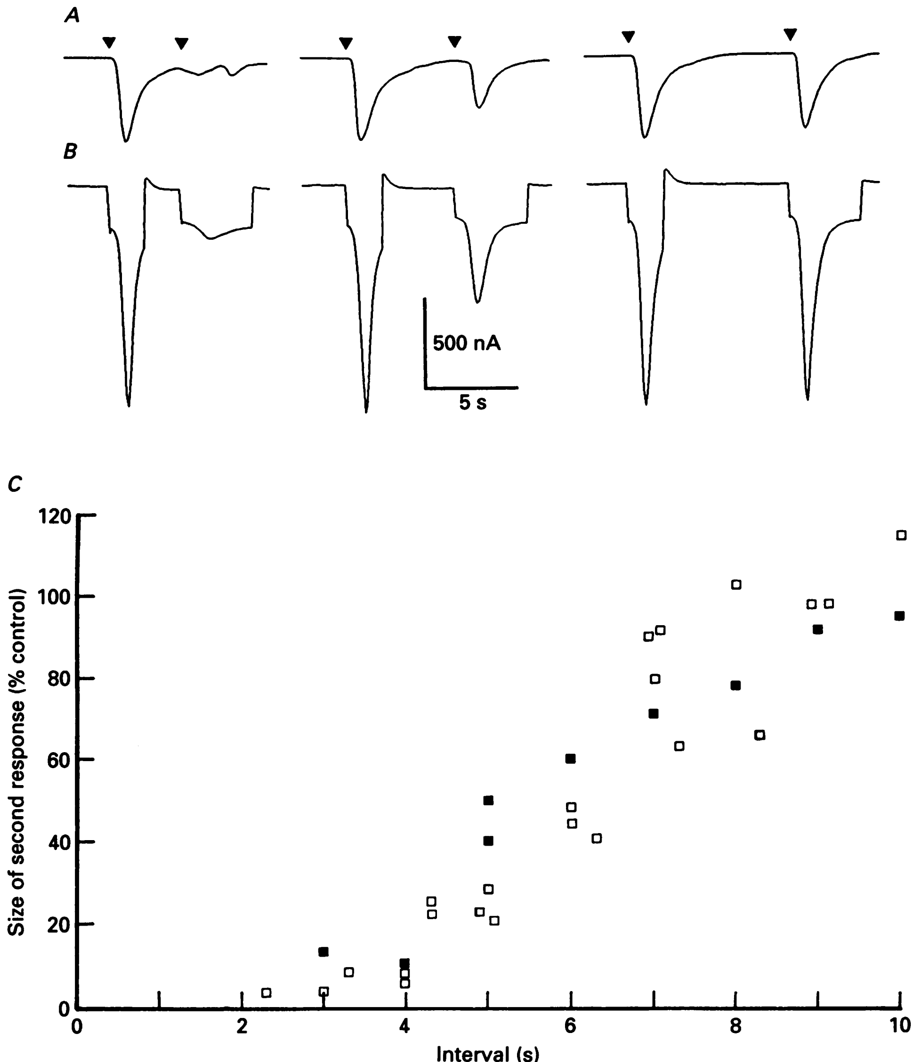

Fig. 6. $InsP_3$-evoked currents and T_{in} current recover from inactivation along similar time courses. A, currents evoked by paired photolysis flashes (250 ms duration) delivered at various intervals, as indicated by the arrowheads. B, currents evoked by paired hyperpolarizing pulses at intervals corresponding to those in A. The holding potential was -50 mV and was stepped to -130 mV during the pulses. C, measurements showing peak sizes of currents evoked by test photolysis flashes (■) and hyperpolarizing pulses (□) as a function of interval following a conditioning stimulus. Responses are scaled as a percentage of that evoked by the initial conditioning stimulus (light flash or hyperpolarization) in each pair. Data are from a single oocyte loaded with caged $InsP_3$ and 3-F-$InsP_3$.

potential was maintained at -80 mV. Following this was a much smaller gradual rise in Ca^{2+}, but it is not clear whether that truly represents an inactivating component of the Ca^{2+} signal, or arose artifactually through variability in responses to the successive stimuli in Fig. 7*A* and *B*. Associated with the transient Ca^{2+} signal was a T_{in} current which peaked during the rise in Ca^{2+} and decayed to the baseline before the Ca^{2+} level had fallen to about one-half of its peak level. In contrast to the lack

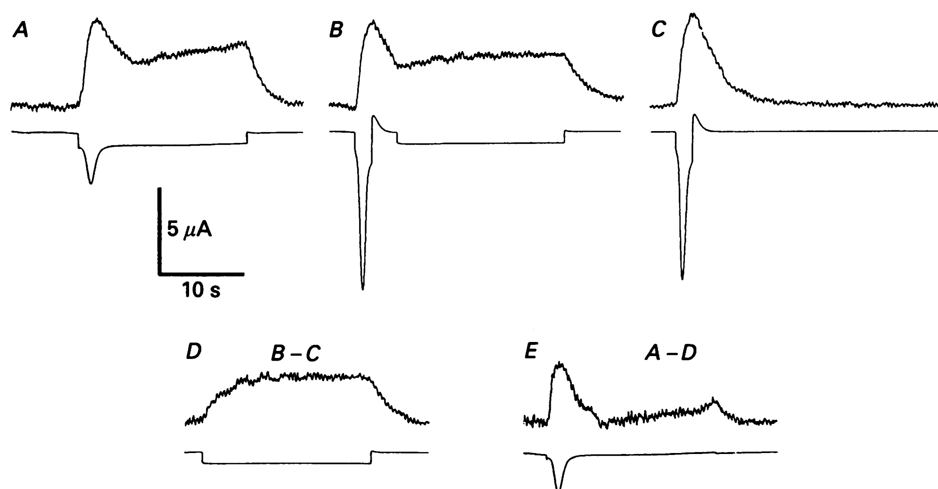

Fig. 7. Conditioning hyperpolarizing pulses selectively inhibit the transient component of the Ca^{2+} signal. In each frame, the upper trace shows Ca^{2+}-dependent fluorescence and the lower trace shows membrane currents evoked by negative-going steps from a holding potential of $+20$ mV. Traces in *A–C* are experimental data, whereas those in *D* and *E* show a dissection of inactivating and non-inactivating components of responses to the test pulse derived by computer subtractions. *A*, responses evoked by a test pulse of 20 s duration to -80 mV. *B*, the test pulse was preceded at an interval of 3 s by a conditioning pulse of 2 s duration to -120 mV. *C*, responses evoked by the conditioning pulse alone. *D*, subtraction of traces in *C* from those in *B*, showing responses to the test pulse that remained following the conditioning pulse. *E*, subtraction of traces in *D* from those in *A*, revealing responses that were inactivated by the conditioning pulse.

of Ca^{2+}-activated current during the slow component of the Ca^{2+} signal (Fig. 7*D*), the T_{in} current in Fig. 7*E* was over 2 μA, even though the associated transient Ca^{2+} signal was only slightly larger than the slow component.

The inactivation of the transient Ca^{2+} component following Ca^{2+} entry is consistent with it arising through triggered liberation of intracellular Ca^{2+}. On the other hand, the slow component may reflect a rise in cytosolic free Ca^{2+} resulting directly from entry of extracellular Ca^{2+}. This appears to show little inactivation, and the time course of the slow Ca^{2+} signal suggests that Ca^{2+} entry is maintained during hyperpolarization, so that cytosolic Ca^{2+} levels approach a steady state when the rate of influx is matched by the rate of removal of Ca^{2+} from the cytosol.

Photolysis of caged Ca^{2+} evokes delayed liberation of intracellular Ca^{2+}

As a final approach to exclude the possibility that the T_{in} current was generated

directly as a result of an inactivating Ca^{2+} influx, we attempted to mimic the response to hyperpolarization by transiently elevating cytosolic free Ca^{2+} levels while influx was suppressed in Ca^{2+}-free extracellular solution.

Figure 8 shows membrane currents evoked by photorelease of Ca^{2+} from DM-

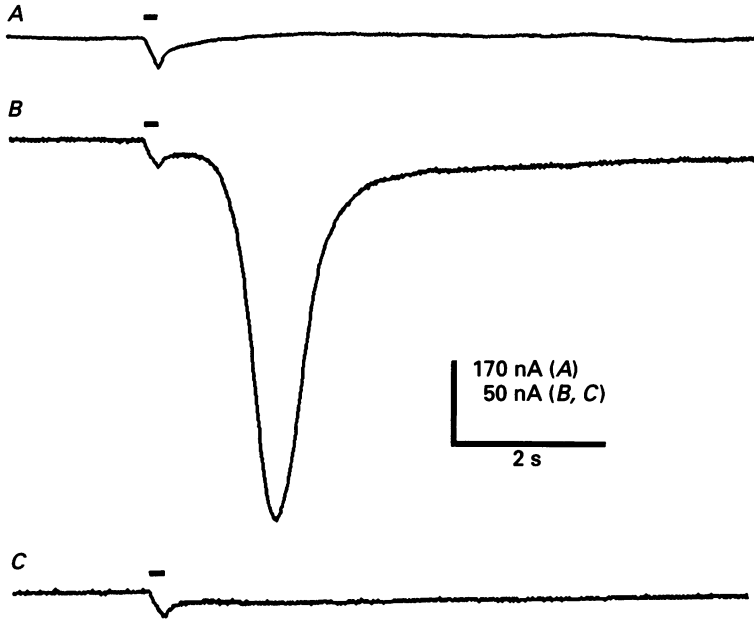

Fig. 8. Flash photolysis of caged Ca^{2+} loaded in the oocyte evokes a delayed liberation of intracellular Ca^{2+}. All traces show recordings of Ca^{2+}-activated membrane current at a clamp potential of −60 mV, and the stimuli were light flashes of 200 ms duration applied when indicated by the bars. The oocyte was bathed in Ca^{2+}-free Ringer solution including 1 mM EGTA. *A*, control response evoked in an oocyte loaded with DM-nitrophen alone. *B*, delayed current response in an oocyte loaded with DM-nitrophen together with 3-F-InsP_3. *C*, a second light flash delivered 20 s after that in *B* failed to elicit any delayed response.

nitrophen ('caged Ca^{2+}') loaded into oocytes. In oocytes loaded with DM-nitrophen alone, the photolysis flash evoked small inward currents (at −60 mV) that grew abruptly during the flash, and then decayed monotonically over a few hundred milliseconds (Fig. 8*A*; and see Parker & Ivorra, 1992). In contrast to this, oocytes that were injected with DM-nitrophen after preloading with 3-F-InsP_3 showed a biphasic current response (Fig. 8*B*). The flash evoked a small transient current like that in oocytes without 3-F-InsP_3, but this was followed by a much larger spike of current with a time course similar to that of the T_{in} current evoked by hyperpolarization. Although the latency of the current spike was quite long (about 500 ms), it was comparable to that of T_{in} currents of similar size evoked by small hyperpolarizing steps (Fig. 2*B*). Recordings in a further two oocytes loaded with 3-F-InsP_3 and DM-nitrophen showed a similar delayed current following light flashes.

Because the response in Fig. 8*B* was obtained while the oocyte was bathed in Ca^{2+}-free solution, the delayed current could not have arisen from Ca^{2+} influx and, instead, probably arose through Ca^{2+}-induced Ca^{2+} release from InsP_3-sensitive stores. This conclusion is strengthened by the observation that it was suppressed following prior activation by a conditioning flash (Fig. 8*C*). Since flash-evoked currents evoked in oocytes loaded with DM-nitrophen alone show almost no inactivation at any interval between 0·1 and 20 s (I. Parker & Y. Yao; unpublished data), this effect was not due to depletion of DM-nitrophen or inactivation of the Ca^{2+}-activated Cl^- channels, but is more consistent with the inactivation of InsP_3-evoked Ca^{2+} liberation as described above. A difficulty is that the inactivation was more prolonged than would be expected from the results in Fig. 6, but this might have arisen if the presence of unphotolysed DM-nitrophen buffered changes in intracellular free Ca^{2+} and slowed their kinetics.

DISCUSSION

Ca^{2+} entry into oocytes triggers release of intracellular Ca^{2+}

The main conclusion of this paper is that Ca^{2+} ions entering the oocyte via an InsP_3-sensitive pathway evoke changes in intracellular Ca^{2+} and associated Ca^{2+}-activated membrane currents that arise, not only as a direct consequence of the Ca^{2+} influx, but also through effects on the liberation of Ca^{2+} ions from intracellular stores. A dramatic demonstration of the latter phenomenon is seen when oocytes that are activated by InsP_3 are hyperpolarized so as to produce a step increase in driving force for Ca^{2+} influx. This results in fast, transient increases in intracellular Ca^{2+} level, accompanied by large Ca^{2+}-activated T_{in} currents (Fig. 2; and see Parker *et al.* 1985). Because similar currents are evoked when Ca^{2+} is readmitted to the bathing solution while the oocyte is clamped at a fixed potential, generation of the T_{in} current does not require a voltage change *per se*, but instead is triggered by Ca^{2+} ions entering the cell.

The Ca^{2+} ions underlying the transient fluorescence signal and the T_{in} current could, in principle, arise either from a rapidly inactivating entry of extracellular Ca^{2+} or from transient liberation of Ca^{2+} from intracellular stores triggered by Ca^{2+} entry. Taken together, the results provide convincing support for the second mechanism. Firstly, the transient Ca^{2+} signal continues to rise almost unchanged after triggering by a brief hyperpolarization, even when the potential is stepped back to more positive levels to reduce the driving force for subsequent Ca^{2+} influx (Fig. 4). A second argument is based on the observation that elevated levels of cytosolic Ca^{2+} produce a delayed inhibition of InsP_3-mediated liberation of Ca^{2+} from intracellular stores (Parker & Ivorra, 1990*a*). Thus, the present experiments demonstrate that the T_{in} current evoked by hyperpolarization is inhibited by prior liberation of Ca^{2+} from intracellular stores (Fig. 5) and that it recovers from inhibition with a time course like that of recovery of InsP_3-mediated Ca^{2+} liberation (Fig. 6). Thirdly, delayed membrane current responses like those evoked on hyperpolarization can be mimicked in oocytes bathed in Ca^{2+}-free solution by direct photochemical manipulation of cytosolic Ca^{2+} levels using caged Ca^{2+} (Fig. 8). Finally, circumstantial evidence that the T_{in} current arises through intracellular Ca^{2+} liberation is given by the similarity of its time course to that of currents evoked by photorelease of InsP_3 (Fig. 6*B*) and by photorelease of Ca^{2+} in 3-F-InsP_3-loaded oocytes (Fig. 8).

Mechanism underlying modulation of intracellular Ca^{2+} liberation

Activation of the plasma membrane Ca^{2+} permeability by InsP_3 is necessarily accompanied by activation of InsP_3 receptors in intracellular organelles. Thus, a likely explanation for our results is that Ca^{2+} ions entering the cytosol from the extracellular space modulate the intracellular InsP_3-gated release channels. Ca^{2+} has been shown to function as a co-agonist together with InsP_3 in promoting Ca^{2+} channel opening, acting with a bell-shaped dose–response curve to cause a rapid facilitation at low concentrations and a slower inhibition at higher concentrations (Iino, 1990; Bezprozvanny *et al.* 1991; Finch *et al.* 1991). Consistent with this, transient entry of Ca^{2+} through voltage-gated channels expressed in the oocyte membrane evokes release of Ca^{2+} from InsP_3-sensitive stores (Yao & Parker, 1992*a*), whereas InsP_3-mediated Ca^{2+} release is inhibited for several seconds after Ca^{2+} injections (Parker & Ivorra, 1990*a*). A dual positive and negative feedback of cytosolic Ca^{2+} ions on InsP_3-mediated Ca^{2+} liberation may, therefore, underlie the generation of repetitive Ca^{2+} spikes and propagating waves in the presence of a steady level of InsP_3 (Parker & Yao, 1991; DeLisle & Welsh, 1992; Lechleiter & Clapham, 1992; Yao & Parker, 1992*a*; Miyazaki *et al.* 1992). Changes in cytosolic Ca^{2+} imposed by step increases in influx of extracellular Ca^{2+} are expected to have complex time-dependent effects on these patterns of activity. For example, increased influx will lead to rapid triggering of Ca^{2+} liberation through facilitation of InsP_3 action, followed by an inhibition lasting for several seconds.

As well as actions on the InsP_3 receptor, two other mechanisms have been proposed by which elevations of cytosolic Ca^{2+} level may stimulate Ca^{2+} liberation from intracellular stores. The first derives from a Ca^{2+} sensitivity of phospholipase C, leading to stimulated formation of InsP_3, and hence release of Ca^{2+} from InsP_3-sensitive stores (Harootunian, Kao, Paranjape & Tsien, 1991). Secondly, Ca^{2+}-induced Ca^{2+} release may be triggered from InsP_3-insensitive stores (Endo, Tanaka & Ogawa, 1970; Goldbeter, Dupont & Berridge, 1990). We consider that neither of these schemes is likely to account for our results, since both predict that elevating intracellular Ca^{2+} levels should trigger Ca^{2+} liberation in the absence of InsP_3. In contrast to this, there is little evidence for such InsP_3-independent Ca^{2+}-induced Ca^{2+} release in the *Xenopus* oocyte (Fig. 8*A*; and see Miledi & Parker, 1984; DeLisle & Walsh, 1992; Parker & Ivorra, 1992; Yao & Parker, 1992*a*; Lechleiter & Clapham, 1992), although this mechanism may contribute to InsP_3-evoked responses in other cells (Wakui, Osipchuk & Petersen, 1990).

Friel & Tsien (1992) recently described modulation of the effects of Ca^{2+} entry through voltage-gated channels by a caffeine-sensitive store in sympathetic neurons. After this store was depleted, changes in intracellular Ca^{2+} resulting from Ca^{2+} entry were depressed because the store acted as a Ca^{2+} sink while it was refilling. An analogous mechanism may contribute to the depression of Ca^{2+} signals evoked by Ca^{2+} influx that we observed following InsP_3-activated Ca^{2+} liberation (Fig. 5). However, a large part of this effect is likely to arise, instead, through inhibition of further Ca^{2+} release from the stores, since a similar inhibition of InsP_3-mediated Ca^{2+} release is seen following injections of Ca^{2+} into the oocyte (Parker & Ivorra, 1990*a*).

Finally, a very different form of interaction between Ca^{2+} influx and liberation from intracellular stores has been reported, whereby Ca^{2+} entry is stimulated by

depletion of Ca^{2+} in intracellular pools (Putney, 1990). To account for this result it has been proposed that Ca^{2+} ions entering the cell may first pass into intracellular stores before being liberated into the cytosol (Putney, 1990). It is difficult to evaluate whether this mechanism contributes to our results, as several key details, particularly the way in which intracellular pools communicate with the plasma membrane, remain unclear. The demonstration (Fig. 8) that changes in cytosolic Ca^{2+} modulate Ca^{2+} liberation is, however, consistent with the simpler interpretation that the effects of Ca^{2+} influx arise because Ca^{2+} ions enter directly into the cytosol.

Role of $InsP_4$ in Ca^{2+} influx

Inositol 1,3,4,5-tetrakisphosphate ($InsP_4$) has been proposed to be the second messenger that controls Ca^{2+} entry (Irvine, 1991) and, in some cell types, there is good evidence that $InsP_3$ alone cannot stimulate Ca^{2+} entry, but absolutely requires the presence also of $InsP_4$ (see, for example, Changya, Gallacher, Irvine, Potter & Petersen, 1989). We had previously shown that injections of Ins(1,4,5)P_3 ($InsP_3$) into *Xenopus* oocytes caused both release of Ca^{2+} from intracellular stores and influx of extracellular Ca^{2+} (Parker & Miledi, 1987) but, in those experiments it was likely that $InsP_4$ was formed by phosphorylation of $InsP_3$ through action of Ins(1,4,5)P_3 3-kinase. However, the present experiments show that injections of 3-F-$InsP_3$ into *Xenopus* oocytes cause a long-lasting Ca^{2+} entry, even though this $InsP_3$ analogue cannot be metabolized into $InsP_4$ (Kozikowski *et al.* 1990). This result is in agreement with other experiments where Ins(2,4,5)P_3 was shown to induce Ca^{2+} entry in oocytes (Snyder, Krause & Welsh, 1988) and lacrimal acinar cells (Bird, Rossier, Hughes, Shears, Armstrong & Putney, 1991), despite the fact that this $InsP_3$ isomer is phosphorylated slowly, if at all, by the 3-kinase (Bird *et al.* 1991). Although it is clear, therefore, that exogenous $InsP_4$ is not needed for Ca^{2+} influx, a synergistic role for $InsP_4$ cannot be entirely ruled out. For example, the resting level of $InsP_4$ in the cell may already be sufficient for activation of Ca^{2+} influx, or Ca^{2+} released from intracellular stores may stimulate the formation of endogenous $InsP_3$ (Harootunian *et al.* 1991), which then becomes phosphorylated to form $InsP_4$.

Relation between T_{in} current and fluorescence Ca^{2+} signals

As demonstrated in Fig. 2, there is no simple relationship between the T_{in} current and calcium green-5N fluorescence signals, even though both arise from changes in intracellular free Ca^{2+} concentrations. Most obviously, the peak of the T_{in} current occurs while the initial phase of the Ca^{2+} signal is still rising, yet the current is small at later times during the pulse even when the Ca^{2+} level is higher. These discrepancies may arise because the amplitude of the Ca^{2+}-activated Cl^- current reflects the rate of rise of intracellular free Ca^{2+} (as monitored by fluorescent dyes), rather than the absolute level of Ca^{2+} (Parker & Ivorra, 1992; Parker & Yao, 1992). Thus, large currents are expected shortly after hyperpolarization, when the Ca^{2+} signal rises rapidly, but little current will be associated with the subsequent, more slowly rising Ca^{2+} signal, even though it may grow to be much larger than the initial transient. The mechanism by which the current signals the differential of intracellular Ca^{2+} remains to be determined, but probably does not involve inactivation or desensitization of the Cl^- channels, since little depression is seen with paired photorelease of Ca^{2+} from caged Ca^{2+} (Parker & Yao, 1992).

Physiological significance of Ca^{2+} liberation stimulated by Ca^{2+} entry

Xenopus oocytes are enormous cells (1 mm diameter) in which responses mediated by intracellular Ca^{2+} liberation can be sustained for many minutes or hours even when Ca^{2+} is absent from the extracellular fluid. This is not so in most cells of 'normal' dimensions, where the duration of Ca^{2+} mobilization is short in Ca^{2+}-free medium, because Ca^{2+} ions released into the cytosol are rapidly pumped out across the plasma membrane (Irvine, 1992). An essential function of Ca^{2+} influx in small cells is, therefore, to prevent depletion of internal stores, and it may also be necessary as a supplementary source of Ca^{2+} to drive the physiological end response (Yamagami, Nishimura & Sorimachi, 1991). In addition, the present results show that a further action of Ca^{2+} influx is to modulate the liberation of intracellular Ca^{2+}, so it is interesting to consider what roles this mechanism may play in different cells.

The triggering of Ca^{2+} liberation by changes in Ca^{2+} influx is analogous to Ca^{2+}-induced Ca^{2+} release (Endo *et al.* 1970), but with the important difference that $InsP_3$ must be present both to enhance the plasma membrane permeability to Ca^{2+} and to allow activation of intracellular stores. Also, in contrast to 'classical' Ca^{2+}-induced Ca^{2+} release, which is evoked when cells are depolarized to cause the opening of voltage-gated membrane Ca^{2+} channels, $InsP_3$-mediated Ca^{2+} signals are evoked by hyperpolarization, which increases the driving force for Ca^{2+} entry via a second messenger-gated pathway. The Ca^{2+}-dependent Cl^- currents triggered in the oocyte by hyperpolarization can be large (many microamps), and are often greater than currents resulting directly from $InsP_3$-evoked Ca^{2+} liberation (e.g. Fig. 1*A*), and those resulting from the Ca^{2+} influx *per se* (Fig. 7*D*). A major function of $InsP_3$-mediated Ca^{2+} entry may, therefore, be to regulate the liberation of intracellular Ca^{2+}, as well as simply providing a supplementary source of Ca^{2+}. This role is likely to be of particular importance in electrically excitable cells which undergo rapid and large excursions in membrane potential, but could also be significant in inexcitable cells such as glia, which may experience voltage changes resulting from changes in extracellular K^+ or the opening of hormone- and neurotransmitter-gated ion channels.

Finally, oscillations in membrane potential may contribute to the generation of repetitive Ca^{2+} spiking. It is clear from experiments in voltage-clamped oocytes that Ca^{2+} spikes can be generated by an internal biochemical oscillator without any need for changes in membrane potential (Parker & Ivorra, 1990*b*, 1993). However, an extra feedback path will be added if the membrane potential is free to change as a result of the opening of Ca^{2+}-activated ion channels, so that Ca^{2+} influx then becomes modulated by voltage changes arising from intracellular Ca^{2+} liberation. This will introduce further complexity in the generation of repetitive Ca^{2+} spiking, and will also tend to synchronize activity within a cell and within populations of electrically coupled cells. For example, such a mechanism may underlie recent findings that coupled endothelial cells show synchronous Ca^{2+} spikes and membrane potential oscillations that are dependent upon extracellular Ca^{2+} (Laskey, Adams, Cannell & van Breemen, 1992).

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REFERENCES

BERRIDGE, M. J. & IRVINE, R. F. (1989). Inositol phosphates and cell signalling. *Nature* **241**, 197–205.

BEZPROZVANNY, I., WATRAS, J. & ERLICH, B. E. (1991). Bell-shaped calcium-response curves of Ins(1,4,5)P_3- and calcium-gated channels from endoplasmic reticulum of cerebellum. *Nature* **351**, 751–754.

BIRD, G. ST J., ROSSIER, M. F., HUGHES, A. R., SHEARS, S. B., ARMSTRONG, D. L. & PUTNEY, J. W. JR (1991). Activation of Ca^{2+} entry into acinar cells by a non-phosphorylatable inositol trisphosphate. *Nature* **352**, 162–165.

CHANGYA, L., GALLACHER, D. V., IRVINE, R. F., POTTER, B. V. L. & PETERSEN, O. H. (1989). Inositol 1,3,4,5-tetrakisphosphate is essential for the sustained activation of the Ca^{2+}-dependent K^+ current in single internally perfused mouse lacrimal acinar cells. *Journal of Membrane Biology* **109**, 85–93.

DELISLE, S. & WELSH, M. J. (1992). Inositol trisphosphate is required for the propagation of calcium waves in *Xenopus* oocytes. *Journal of Biological Chemistry* **267**, 7963–7966.

ECKERT, R. & CHAD, J. E. (1984). Inactivation of Ca^{2+} channels. *Progress in Biophysics and Molecular Biology* **44**, 215–267.

ENDO, M., TANAKA, M. & OGAWA, Y. (1970). Calcium induced release of calcium from the sarcoplasmic reticulum of skinned skeletal muscle fibres. *Nature* **228**, 34–36.

FERRIS, C. D. & SNYDER, S. H. (1992). Inositol phosphate receptors and calcium disposition in the brain. *Journal of Neuroscience* **12**, 1567–1574.

FINCH, E. A., TURNER, T. J. & GOLDIN, S. M. (1991). Calcium as a coagonist of inositol 1,4,5-trisphosphate-induced calcium release. *Science* **254**, 443–446.

FRIEL, D. D. & TSIEN, R. W. (1992). A caffeine- and ryanodine-sensitive Ca^{2+} store in bullfrog sympathetic neurones modulates effects of Ca^{2+} entry on $[Ca^{2+}]_i$. *Journal of Physiology* **450**, 217–246.

GOLDBETER, A., DUPONT, G. & BERRIDGE, M. J. (1990). Minimal model for signal-induced Ca^{2+} oscillations and for their frequency encoding through protein phosphorylation. *Proceedings of the National Academy of Sciences of the USA* **87**, 1461–1465.

HAROOTUNIAN, A. T., KAO, J. P. Y., PARANJAPE, S. & TSIEN, R. Y. (1991). Generation of calcium oscillations in fibroblasts by positive feedback between calcium and IP_3. *Science* **251**, 75–78.

IINO, M. (1990). Biphasic Ca^{2+}-dependence of inositol 1,4,5-trisphosphate-induced Ca^{2+} release in smooth muscle cells of the guinea pig *Taenia Caeci*. *Journal of General Physiology* **95**, 1103–1122.

IRVINE, R. F. (1991). Inositol tetrakisphosphate as a second messenger: Confusions, contradictions and a potential resolution. *BioEssays* **13**, 419–427.

IRVINE, R. F. (1992). Inositol phosphates and Ca^{2+} entry: toward a proliferation or a simplification? *FASEB Journal* **6**, 3085–3091.

JACOB, R. (1990). Agonist-stimulated divalent cation entry into single cultured human umbilical vein endothelial cells. *Journal of Physiology* **421**, 55–77.

KAPLAN, J. H. & ELLIS-DAVIES, G. C. R. (1988). Photolabile chelators for the rapid release of divalent cations. *Proceedings of the National Academy of Sciences of the USA* **85**, 6571–6575.

KOZIKOWSKI, A. P., FAUQ, A. H., AKSOY, I. A., SEEWALD, M. J. & POWIS, G. (1990). Synthesis of the first optically pure, fluorinated inositol 1,4,5-trisphosphate of *myo*-inositol. Stereochemistry and its effect on Ca^{2+} release in Swiss 3T3 cells. *Journal of the American Chemical Society* **112**, 7403–7404.

KUSANO, K., MILEDI, R. & STINNAKRE, J. (1982). Cholinergic and catecholaminergic receptors in the *Xenopus* oocyte membrane. *Journal of Physiology* **328**, 143–170.

LASKEY, R. E., ADAMS, D. J., CANNELL, M. & VAN BREEMEN, C. (1992). Calcium entry-dependent oscillations of cytoplasmic calcium concentration in cultured endothelial cell monolayers. *Proceedings of the National Academy of Sciences of the USA* **89**, 1690–1694.

LECHLEITER, J. D. & CLAPHAM, D. E. (1992). Molecular mechanisms of intracellular calcium excitability in X. laevis oocytes. *Cell* **69**, 283–294.

MCCRAY, J. A. & TRENTHAM, D. R. (1989). Properties and uses of photoreactive caged compounds. *Annual Reviews of Biophysics and Biophysical Chemistry* **18**, 239–270.

MATTHEWS, G., NEHER, E. & PENNER, R. (1989). Second messenger-activated calcium influx in rat peritoneal mast cells. *Journal of Physiology* **418**, 105–130.

MILEDI, R. & PARKER, I. (1984). Chloride current induced by injection of calcium into *Xenopus* oocytes. *Journal of Physiology* **357**, 173–183.

MIYAZAKI, S.-I., YUZAKI, M., NAKADA, K., SHIRAKAWA, H., NAKANISHI, S., NAKADE, S. & MIKOSHIBA, K. (1992). Block of Ca^{2+} wave and Ca^{2+} oscillation by antibody to the inositol 1,4,5-trisphosphate receptor in fertilized hamster eggs. *Science* **257**, 251–255.

PARKER, I. (1992). Caged intracellular messengers and the inositol phosphate signalling pathway. *Neuromethods* **20**, 369–393.

PARKER, I., GUNDERSEN, G. B. & MILEDI, R. (1985). A transient inward current elicited by hyperpolarization during serotonin activation in *Xenopus* oocytes. *Proceedings of the Royal Society* B **223**, 279–292.

PARKER, I. & IVORRA, I. (1990*a*). Inhibition by Ca^{2+} of inositol trisphosphate-mediated Ca^{2+} liberation: A possible mechanism for oscillatory release of Ca^{2+}. *Proceedings of the National Academy of Sciences of the USA* **87**, 260–264.

PARKER, I. & IVORRA, I. (1990*b*). Localized all-or-none calcium liberation by inositol trisphosphate. *Science* **250**, 977–979.

PARKER, I. & IVORRA, I. (1992). Characteristics of membrane currents evoked by photorelease of inositol trisphosphate in *Xenopus* oocytes. *American Journal of Physiology* **263**, C154–165.

PARKER, I. & IVORRA, I. (1993). Confocal microfluorimetry of Ca^{2+} signals evoked in *Xenopus* oocytes by photoreleased inositol trisphosphate. *Journal of Physiology* **461**, 133–165.

PARKER, I. & MILEDI, R. (1986). Changes in intracellular calcium and in membrane currents evoked by injections of inositol trisphosphate into *Xenopus* oocytes. *Proceedings of the Royal Society* B **228**, 307–315.

PARKER, I. & MILEDI, R. (1987). Inositol trisphosphate activates a voltage-dependent calcium influx in *Xenopus* oocytes. *Proceedings of the Royal Society* B **231**, 27–36.

PARKER, I. & MILEDI, R. (1989). Non-linearity and facilitation in phosphoinositide signalling studied by the use of caged inositol trisphosphate in *Xenopus* oocytes. *Journal of Neuroscience* **9**, 4068–4077.

PARKER, I. & YAO, Y. (1991). Regenerative release of calcium from functionally discrete subcellular stores by inositol trisphosphate. *Proceedings of the Royal Society* B **246**, 269–274.

PARKER, I. & YAO, Y. (1992). Inositol trisphosphate-mediated currents in *Xenopus* oocytes reflect spatial and temporal derivatives of intracellular calcium. *Society for Neuroscience Abstracts* **18**, 800.

PARKER, I. & YAO, Y. (1993). Inositol trisphosphate-mediated Ca^{2+} entry into *Xenopus* oocytes evokes transient release of Ca^{2+} from intracellular stores. *Journal of Physiology* **459**, 348*P*.

PUTNEY, J. W. JR. (1990). Capacitative calcium entry revisited. *Cell Calcium* **11**, 611–624.

SNYDER, P. M., KRAUSE, K.-H. & WELSH, M. J. (1988). Inositol trisphosphate isomers, but not inositol 1,3,4,5-tetrakisphosphate, induce calcium influx in *Xenopus laevis* oocytes. *Journal of Biological Chemistry* **263**, 11048–11051.

SUMIKAWA, K., PARKER, I. & MILEDI, R. (1989). Expression of neurotransmitter receptors and voltage-activated channels from brain mRNA in *Xenopus* oocytes. *Methods in Neuroscience* **1**, 30–45.

TAYLOR, C. W. & RICHARDSON, A. (1991). Structure and function of inositol trisphosphate receptors. *Pharmacology and Therapeutics* **51**, 97–137.

WAKUI, M., OSIPCHUK, Y. V. & PETERSEN, O. H. (1990). Receptor-activated cytoplasmic Ca^{2+} spiking mediated by inositol trisphosphate is due to Ca^{2+}-induced Ca^{2+} release. *Cell* **63**, 1025–1032.

WALKER, J. W., FEENEY, J. & TRENTHAM, D. R. (1989). Photolabile precursors of inositol phosphates. Preparation and properties of 1(2-nitrophenyl)ethyl esters of *myo*-inositol 1,4,5-trisphosphate. *Biochemistry* **28**, 3272–3280.

YAMAGAMI, K., NISHIMURA, S. & SORIMACHI, M. (1991). Internal Ca^{2+} mobilization by muscarinic stimulation increases secretion from adrenal chromaffin cells only in the presence of Ca^{2+} influx. *Journal of Neurochemistry* **56**, 1681–1689.

YAO, Y. & PARKER, I. (1992*a*). Potentiation of inositol trisphosphate-induced Ca^{2+} mobilization in *Xenopus* oocytes by cytosolic Ca^{2+}. *Journal of Physiology* **458**, 319–338.

YAO, Y. & PARKER, I. (1992*b*). Sustained calcium influx induced by a non-metabolizable inositol trisphosphate in *Xenopus* oocytes. *Society for Neuroscience Abstracts* **18**, 477.