

**INOSITOL TETRAKISPHOSPHATE LIBERATES STORED Ca^{2+} IN
XENOPUS OOCYTES AND FACILITATES RESPONSES TO INOSITOL
TRISPHOSPHATE**

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SUMMARY

1. The actions of the putative second messenger inositol 1,3,4,5-tetrakisphosphate ($\text{Ins}(1,3,4,5)\text{P}_4$) were studied by injecting it into voltage-clamped oocytes while recording Ca^{2+} -dependent chloride membrane currents and, in some experiments, fluorescence signals from Ca^{2+} indicators.

2. $\text{Ins}(1,3,4,5)\text{P}_4$ evoked a rise in intracellular Ca^{2+} and associated chloride current in oocytes bathed in normal or Ca^{2+} -free Ringer solutions. The fluorescence Ca^{2+} signal showed a prolonged rise with superimposed oscillations, whereas the current reflected only the oscillatory component.

3. Injections of inositol 1,4,5-trisphosphate ($\text{Ins}(1,4,5)\text{P}_3$) evoked currents showing an initial transient, followed by oscillations. $\text{Ins}(1,3,4,5)\text{P}_4$ evoked similar oscillations, but the transient component was usually small or absent. $\text{Ins}(1,3,4,5)\text{P}_4$ was about 20-fold less potent than $\text{Ins}(1,4,5)\text{P}_3$, as measured by comparing doses required to elicit currents with the same integral. The most sensitive oocytes responded to about 1 fmol $\text{Ins}(1,3,4,5)\text{P}_4$ and 0.1 fmol $\text{Ins}(1,4,5)\text{P}_3$.

4. Injections of $\text{Ins}(2,4,5)\text{P}_4$ evoked oscillatory currents, with a potency about three times greater than $\text{Ins}(1,4,5)\text{P}_3$. $\text{Ins}(1,3,4)\text{P}_4$ was ineffective in some oocytes even at doses of several picomoles, but in other oocytes evoked small transient and oscillatory currents with a potency 100 times or more less than $\text{Ins}(1,3,4,5)\text{P}_4$.

5. Injections of $\text{Ins}(1,3,4,5)\text{P}_4$ made into the animal hemisphere of the oocyte evoked larger currents than injections into the vegetal hemisphere.

6. Photo-release of $\text{Ins}(1,4,5)\text{P}_3$ from caged $\text{Ins}(1,4,5)\text{P}_4$ loaded into the oocyte was used to examine interactions between $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$. Injection of low (ca 1 fmol) doses of $\text{Ins}(1,3,4,5)\text{P}_4$ shortly before a light flash greatly facilitated currents evoked by photo-release of near-threshold amounts of $\text{Ins}(1,4,5)\text{P}_3$. This facilitation was unaffected by removal of extracellular Ca^{2+} and arose because $\text{Ins}(1,3,4,5)\text{P}_4$ reduced the threshold amount of $\text{Ins}(1,4,5)\text{P}_3$ required to evoke a response.

7. Larger amounts (several femtomoles) of $\text{Ins}(1,3,4,5)\text{P}_4$ depressed responses evoked by photo-release of $\text{Ins}(1,4,5)\text{P}_3$. This may arise because Ca^{2+} liberated by $\text{Ins}(1,3,4,5)\text{P}_4$ inhibits the ability of $\text{Ins}(1,4,5)\text{P}_3$ to release further Ca^{2+} .

8. We conclude that $\text{Ins}(1,3,4,5)\text{P}_4$ liberates intracellular Ca^{2+} in the oocyte in a

manner similar to that of $\text{Ins}(1,4,5)\text{P}_3$, and suggest that a physiological role for $\text{Ins}(1,3,4,5)\text{P}_4$ may be to facilitate responses to $\text{Ins}(1,4,5)\text{P}_3$.

INTRODUCTION

The role of inositol 1,4,5-trisphosphate ($\text{Ins}(1,4,5)\text{P}_3$) as a second messenger is now well established in many cell types, where it functions by releasing Ca^{2+} from intracellular stores (see Berridge & Irvine, 1989, for recent review). However, the $\text{Ins}(1,4,5)\text{P}_3$ which is formed by receptor-mediated break-down of phosphatidylinositol bisphosphate is subsequently metabolized to create a bewildering array of other inositol phosphates (Berridge & Irvine, 1989), the physiological roles of which are as yet unclear. Among these compounds, much interest has focused on inositol 1,3,4,5-tetrakisphosphate ($\text{Ins}(1,3,4,5)\text{P}_4$), because several features make it a likely candidate as an intracellular messenger with functions discrete from those of $\text{Ins}(1,4,5)\text{P}_3$ (Berridge & Irvine, 1989; Irvine, 1989*a, b*). In particular, $\text{Ins}(1,3,4,5)\text{P}_4$ is formed from $\text{Ins}(1,4,5)\text{P}_3$ by a Ca^{2+} -regulated 3-kinase (Irvine, Letcher, Heslop & Berridge, 1986*a*; Irvine, Moor, Pollock, Smith & Wreggett, 1988), and is produced rapidly on cell stimulation with kinetics similar to that of $\text{Ins}(1,4,5)\text{P}_4$ (Batty, Nahorski & Irvine, 1985; Biden & Wollheim, 1986; Hawkins, Stephens & Downes, 1986).

To determine what, if any, functions $\text{Ins}(1,3,4,5)\text{P}_4$ may have in the cell, many experiments have been made in which it is introduced into the cytoplasm of intact or permeabilized cells, or applied to isolated membrane preparations. Several studies have found that, by itself, $\text{Ins}(1,3,4,5)\text{P}_4$ is almost ineffective in mobilizing Ca^{2+} from intracellular stores (Irvine, Letcher, Lander & Berridge, 1986*b*; Irvine & Moor, 1986; Morris, Gallacher, Irvine & Petersen, 1987; Changya, Gallacher, Irvine, Potter & Petersen, 1989), but may stimulate the entry of Ca^{2+} across the plasma membrane (Irvine & Moor, 1986, 1987) or act synergistically to modulate the ability of $\text{Ins}(1,4,5)\text{P}_3$ to liberate Ca^{2+} (Morris *et al.* 1987; Changya *et al.* 1989; Irvine, 1989*a, b*). Different to this, other experiments have shown that $\text{Ins}(1,3,4,5)\text{P}_4$ evokes responses that appear to arise through the liberation of Ca^{2+} from intracellular stores in intact cells (Parker & Miledi, 1987; Crossley, Swann, Chambers & Whitaker, 1988; Snyder, Krause & Welsh, 1988; Stith & Proctor, 1989), and there is direct evidence for Ca^{2+} release from microsomes (Joseph, Hansen & Williamson, 1989).

The present paper follows on from our original observation (Parker & Miledi, 1987) that injection of $\text{Ins}(1,3,4,5)\text{P}_4$ into *Xenopus* oocytes generates an oscillatory chloride current which is dependent upon intracellular but not extracellular Ca^{2+} . The most likely explanation for this was that $\text{Ins}(1,3,4,5)\text{P}_4$ caused the cyclical liberation of Ca^{2+} from intracellular stores, and that the resulting fluctuations in cytoplasmic free Ca^{2+} concentration were reflected in the opening of Ca^{2+} -dependent chloride channels in the plasma membrane (Miledi & Parker, 1984). However, other explanations remained possible. For example, $\text{Ins}(1,3,4,5)\text{P}_4$ might modulate that sensitivity of the chloride channels, so as to give a fluctuating current in the presence of a steady, resting level of free Ca^{2+} . By the use of fluorescent Ca^{2+} indicators we now show that the current responses to $\text{Ins}(1,3,4,5)\text{P}_4$ are accompanied by elevations of intracellular free Ca^{2+} . Furthermore, we explore other properties of the responses to $\text{Ins}(1,3,4,5)\text{P}_4$

which may cast light on its physiological role, including the spatial distribution of sensitivity across the oocyte and interactions with responses to Ins(1,4,5)P₃.

METHODS

Experiments were done on ovarian oocytes of *Xenopus laevis*, obtained after killing the donor frogs by decerebration and pithing. Oocytes at stages V and VI (Dumont, 1972) were treated with collagenase to remove enveloping cells (Sumikawa, Parker & Miledi, 1989). Membrane currents were recorded using a two-electrode voltage clamp (Sumikawa *et al.* 1989), with the membrane potential held at -60 mV. Records of membrane current were photographed from the screen of an analog storage oscilloscope, or stored on floppy discs by a digital oscilloscope. During recording, oocytes were continually superfused with frog Ringer solution at room temperature ($21-24$ °C). Normal Ringer solution had the composition (in mM): NaCl, 120; KCl, 2; CaCl₂, 1.8; HEPES, 5; at pH about 7.0. Zero Ca²⁺ Ringer solution was made by omitting CaCl₂ and adding 1 mM-EGTA and 5 mM-MgCl₂. Intracellular injections were made by applying pneumatic pressure pulses to a glass micropipette (Sumikawa *et al.* 1989), and the volumes of fluid injected were estimated by measuring the diameter of the droplets expelled when the pipette tip was in the air. During experiments involving optical techniques (caged InsP₃ or fluorescent Ca²⁺ indicators) injections were made into the vegetal hemisphere, close to the equator, so as to avoid light absorption by the pigment in the animal hemisphere. In other experiments, injections were made into the animal hemisphere, at latitudes of 20–40 deg, so as to maximize Ca²⁺-activated chloride currents (Miledi & Parker, 1984). Compounds for injection were dissolved in aqueous solutions including 5 mM-HEPES (at pH 7.0) and 50 μ M-EDTA, which were passed through a 0.22 μ m Millipore filter. Caged InsP₃ (*myo*-inositol 1,4,5-trisphosphate P^{4,5}-1-(2-nitrophenyl) ethyl ester) was obtained from Calbiochem (La Jolla, CA, USA) and injected as a 1 mM solution. Samples of Ins(1,4,5)P₃, Ins(1,3,4)P₃, Ins(2,4,5)P₃ and Ins(1,3,4,5)P₄ were each obtained from Calbiochem, and as generous gifts from Dr R. F. Irvine (AFRC Institute of Animal Physiology, Babraham, Cambridge). All had been purified by HPLC. They were injected as 1 mM or 100 μ M solutions. Except for Ins(1,3,4)P₃ (see Results), samples from the two different sources gave essentially the same results. InsP₃ was obtained from Sigma, and Ins(1)P from Boehringer Mannheim Biochemicals (Indianapolis, IN, USA).

Procedures for light-flash photolysis of caged InsP₃ and for fluorescence monitoring of intracellular free Ca²⁺ were as described previously (Parker & Miledi, 1989; Ivorra & Parker, 1990a; Parker & Ivorra, 1990). Briefly, oocytes were loaded with 1–10 pmol of caged InsP₃, and allowed to rest for about 1 h before recording. Ultraviolet light was focused onto the oocyte surface as a square of about 100 μ m sides, positioned close to the equator on the vegetal hemisphere. The light source was a continuous xenon arc lamp, and flashes of varying duration were set by an electronic shutter. Except for experiments in which the oocyte was stimulated by repetitive trains of light flashes, intervals of at least 90 s were allowed between trials. The pipette used to inject Ins(1,3,4,5)P₄ was inserted into the oocyte with its tip at the centre of the light spot.

To monitor intracellular Ca²⁺, oocytes were loaded with a few picomoles of the fluorescent Ca²⁺ indicators Fluo-3 or Rhod-2 (Minta, Kao & Tsien, 1989) (Molecular Probes, Eugene, OR, USA). Recordings were made after allowing at least 20 min for the dyes to diffuse in the oocyte. The amounts injected correspond to final intracellular concentrations of a few micromolar, assuming even distribution of the dyes throughout the cell. Fluorescence measurements were made with an epifluorescence microscope (Zeiss), fitted with standard filter sets for fluorescein (used with Fluo-3) or rhodamine (with Rhod-2). The excitation light was focused on the oocyte as a spot of 75–150 μ m diameter, centred on the tip of the Ins(1,3,4,5)P₄ injection pipette. Fluorescence emission was monitored by a photomultiplier, and increases in free Ca²⁺ correspond to increasing fluorescence. However, because neither of the indicators show appreciable shifts in excitation or emission spectra with Ca²⁺ (Minta *et al.* 1989), we were unable to calibrate the fluorescence signals in terms of free Ca²⁺ concentration.

RESULTS

Ca²⁺ and membrane current responses to Ins(1,3,4,5)P₄

Figure 1A shows simultaneous records of membrane current and Ca²⁺-dependent fluorescence, obtained following injection of various amounts of Ins(1,3,4,5)P₄ into

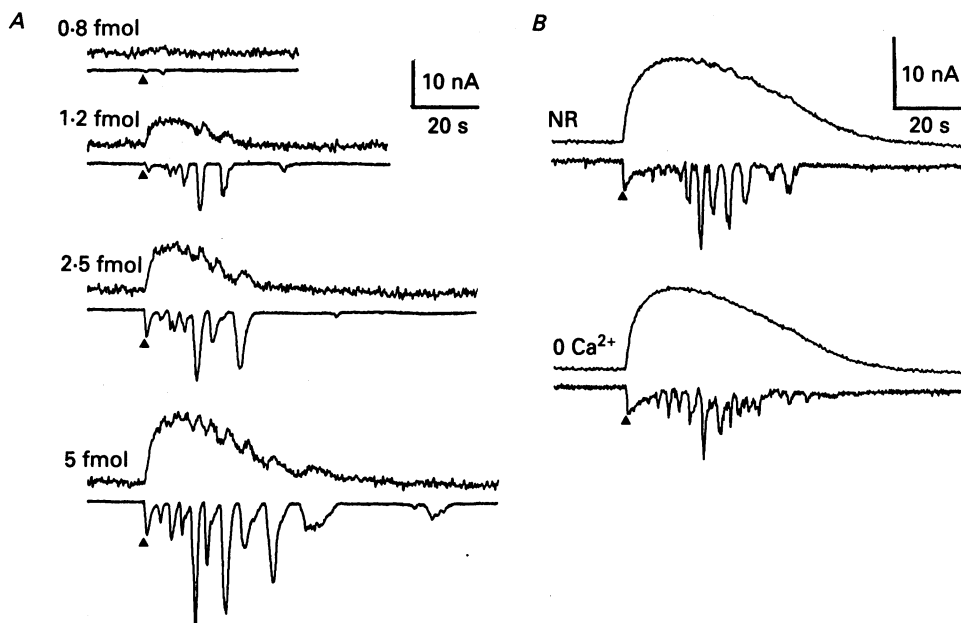


Fig. 1. Membrane current and Ca²⁺ signals evoked by injections of Ins(1,3,4,5)P₄ into *Xenopus* oocytes. In each frame the lower trace shows clamp current at a clamp potential of -60 mV, and the upper trace shows fluorescence of Rhod-2 loaded into the oocyte. Fluorescence signals were low-pass filtered at 2 Hz. Increases in fluorescence are shown as upward deflections, and correspond to increases in free Ca²⁺. The amplitudes of the fluorescence signals are not calibrated. In this, and other figures, downward deflections of the current trace correspond to inward membrane currents. Injections of Ins(1,3,4,5)P₄ were given when indicated by the arrow-heads. *A*, responses evoked by injections of various amounts of Ins(1,3,4,5)P₄ (indicated in femtomoles next to each record) into an oocyte bathed in normal Ringer solution (NR). *B*, membrane current and intracellular Ca²⁺ signals evoked by Ins(1,3,4,5)P₄ injections into a different oocyte bathed in normal (1.8 mM-Ca²⁺) solution (upper) and in Ca²⁺-free solution (lower).

an oocyte previously loaded with the calcium indicator dye Rhod-2. Injections of Ins(1,3,4,5)P₄ evoked oscillatory chloride currents (Parker & Miledi, 1987), and these were accompanied by rises in intracellular free Ca²⁺ as detected by the fluorescent indicator. In the oocyte illustrated, both the current and fluorescence signals first became detectable with injection of about 0.9 fmol Ins(1,3,4,5)P₄, and grew progressively in magnitude and duration as larger amounts were injected.

A striking feature was that the membrane current responses did not simply mirror the Ca²⁺ signals. Injections of Ins(1,3,4,5)P₄ evoked discrete oscillations in current,

which usually returned to the baseline between each 'spike'. In contrast, the Ca²⁺ signal displayed a more maintained rise, on which were superimposed oscillations corresponding to the spikes in current. A further difference was that the Ca²⁺ signals rose rapidly following injection, and were nearly maximal within about 10 s, whereas the largest membrane current oscillations were not seen until later. Ins(1,3,4,5)P₄ was injected into more than a dozen oocytes that had been loaded with either Rhod-2 or Fluo-3 to monitor intracellular Ca²⁺. All showed prolonged Ca²⁺ signals but, in several cases (e.g. Fig. 1B), oscillations in the signals were small or undetectable.

Neither the membrane current responses nor the rise in intracellular Ca²⁺ depended upon the presence of Ca²⁺ ions in the extracellular medium. Figure 1B shows records from an oocyte that was injected with Ins(1,3,4,5)P₄ while bathed in normal Ringer solution (1.8 mM-Ca²⁺), and then after changing to Ca²⁺-free solution. Currents and Ca²⁺ signals of similar sizes were obtained in both solutions. A further five oocytes examined in Ca²⁺-free solution all gave current and intracellular Ca²⁺ responses to Ins(1,3,4,5)P₄.

Patterns of currents evoked by Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄

The oscillatory currents evoked by Ins(1,3,4,5)P₄ resemble those generated by Ins(1,4,5)P₃ (Oron, Dascal, Nadler & Lupu, 1985; Parker & Miledi, 1986, 1987). However, it already appeared that each compound may give a characteristic pattern of response (Parker & Miledi, 1987), and we were interested to examine these differences in more detail.

Responses to Ins(1,4,5)P₃ usually comprise two or more components (Parker & Miledi, 1986; Gillo, Lass, Nadler & Oron, 1987; Berridge, 1988). Typical examples are shown in Fig. 2A. After injection of Ins(1,4,5)P₃ a transient current developed almost immediately, and was then followed by a series of more slowly developing and decaying oscillations. The peak size of the transient current varied only slightly with dose (mean increase of 2.6 ± 0.5 times increase per 10-fold increase in dose), but its decay slowed as more Ins(1,4,5)P₃ was injected. The oscillatory current showed marked increases in peak size and duration with increasing dose. Considerable variation was seen between oocytes in the relative sizes of the transient and oscillatory components. For example, the oocyte in Fig. 2B responded with a prominent transient current, but showed little oscillatory current, even when injected with relatively high doses of Ins(1,4,5)P₃. A third, relatively maintained component has been described in the response to Ins(1,4,5)P₃, which arises due to influx of Ca²⁺ ions from the external solution (Snyder *et al.* 1988; Ivorra, Miledi & Parker, 1989). That component was not prominent in our experiments, probably because the Ca²⁺ concentration in the bathing solution was not elevated, and because injections were restricted to relatively small (usually < 10 fmol) amounts of Ins(1,4,5)P₃.

In contrast to the biphasic response to Ins(1,4,5)P₃, injections of Ins(1,3,4,5)P₄ gave responses with prominent oscillations, but the rapid transient current was usually (though not always, e.g. Fig. 1B) small or absent. This difference between the tris- and tetrakisphosphates remained even when their actions were compared in the same oocytes (e.g. Fig. 2A, B). The relative magnitudes of the transient and oscillatory currents evoked by Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ were estimated by

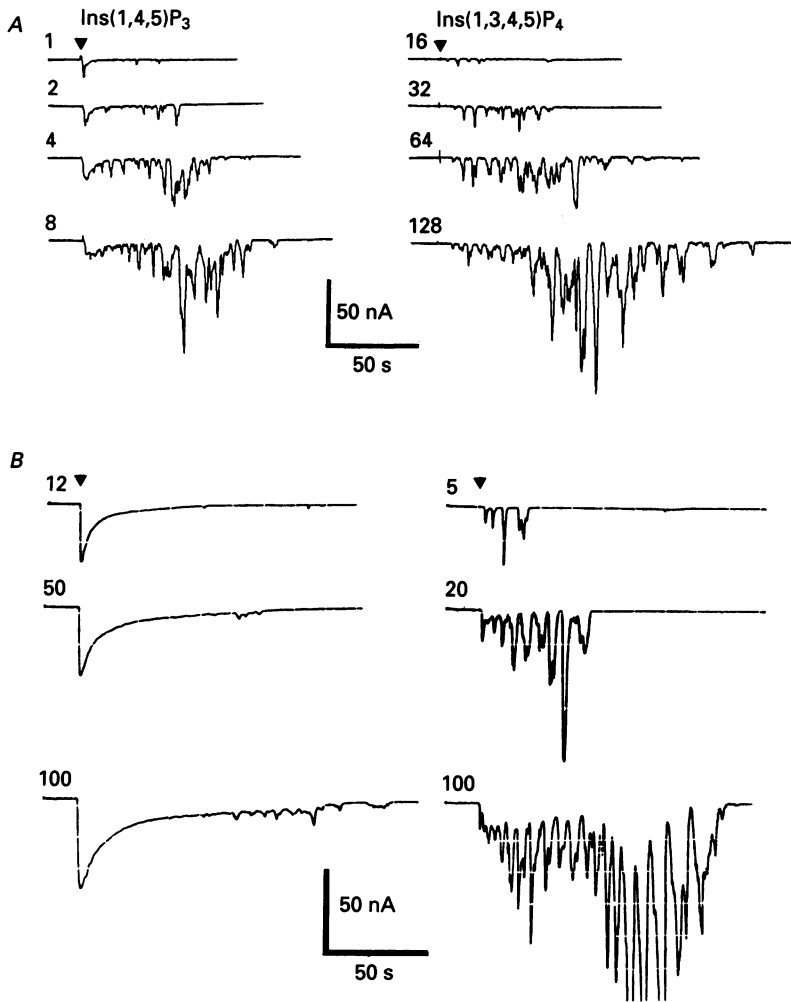


Fig. 2. Examples of membrane currents evoked by intracellular injections of $\text{Ins}(1,4,5)\text{P}_3$ (left) and $\text{Ins}(1,3,4,5)\text{P}_4$ (right). Records in *A* and *B* are from two oocytes, which were both impaled at about the same latitude in the animal hemisphere by two micropipettes containing each of the compounds. Injections were made when indicated by the arrowheads, and the amounts injected are given in femtomoles next to each trace. The oocyte in *A* showed 'typical' responses to $\text{Ins}(1,4,5)\text{P}_3$, while that in *B* showed only a small oscillatory response. The three largest peaks in the lower right trace are off-scale.

measuring the ratio of the peak sizes of the transient current and the largest oscillation produced during each response. Means were then calculated from the ratios derived from experiments on sixteen oocytes, which were each injected with a range of doses of $\text{Ins}(1,4,5)\text{P}_3$ or $\text{Ins}(1,3,4,5)\text{P}_4$. The mean ratio (transient current/peak oscillation) for $\text{Ins}(1,4,5)\text{P}_3$ was 3.0 ± 0.78 (s.e.m.; thirty-nine observations), and for $\text{Ins}(1,3,4,5)\text{P}_4$, 0.14 ± 0.03 (thirty-four observations).

Relative potencies of Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄

To compare the potency of Ins(1,3,4,5)P₄ to evoke membrane currents in relation to Ins(1,4,5)P₃, oocytes were injected with various amounts of each, so as to be able to construct dose-response relationships. Injections were made in order of increasing

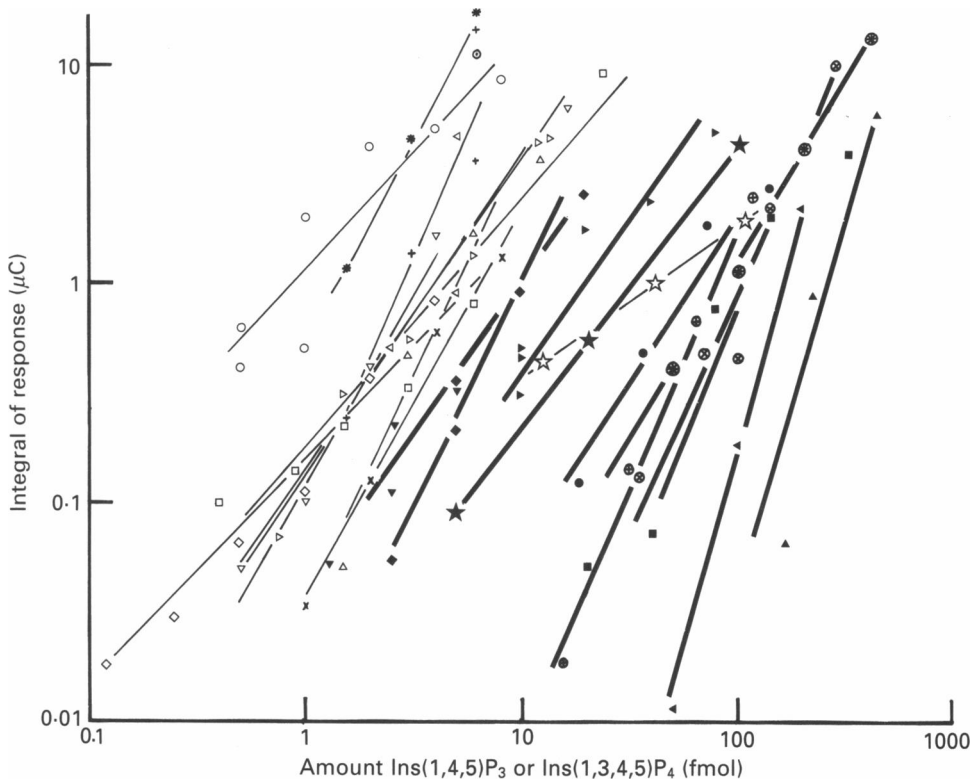


Fig. 3. Magnitudes of current responses of varying amounts of Ins(1,4,5)P₃ (open symbols and thin lines) and Ins(1,3,4,5)P₄ (filled or enclosed symbols and thick lines). The vertical axis shows the integral under the current response (i.e. total charge displacement in coulombs). Data are from ten runs with Ins(1,4,5)P₃ and eleven runs with Ins(1,3,4,5)P₄; different symbols denote different runs with each compound. Measurements from the oocyte in Fig. 2B are shown by open and filled stars. Lines are drawn by eye through the points from individual runs.

dose, and intervals of about 5 min were allowed after a response had ceased before the next injection was made. Because of the fluctuating nature of the responses, and because the responses became longer as well as larger with increasing doses, it seemed more appropriate to quantify the currents by their integrals (i.e. total charge displacement), rather than by measuring peak current sizes. A double-logarithmic plot of dose-response relationships measured in this way is shown in Fig. 3. Lines are drawn by eye through data points for each of ten trials with Ins(1,4,5)P₃ and eleven trials with Ins(1,3,4,5)P₄. A comparison of the potencies of the compounds was made

by interpolating the amounts required to evoke responses with an integral of $1 \mu\text{C}$. For $\text{Ins}(1,4,5)\text{P}_3$ the mean amount was $6.1 \pm 2.6 \text{ fmol}$ ($\pm \text{s.e.m.}$) and for $\text{Ins}(1,3,4,5)\text{P}_4$, $120 \pm 40 \text{ fmol}$. Thus, in these experiments, $\text{Ins}(1,3,4,5)\text{P}_4$ was on average about 20 times less effective than $\text{Ins}(1,4,5)\text{P}_3$.

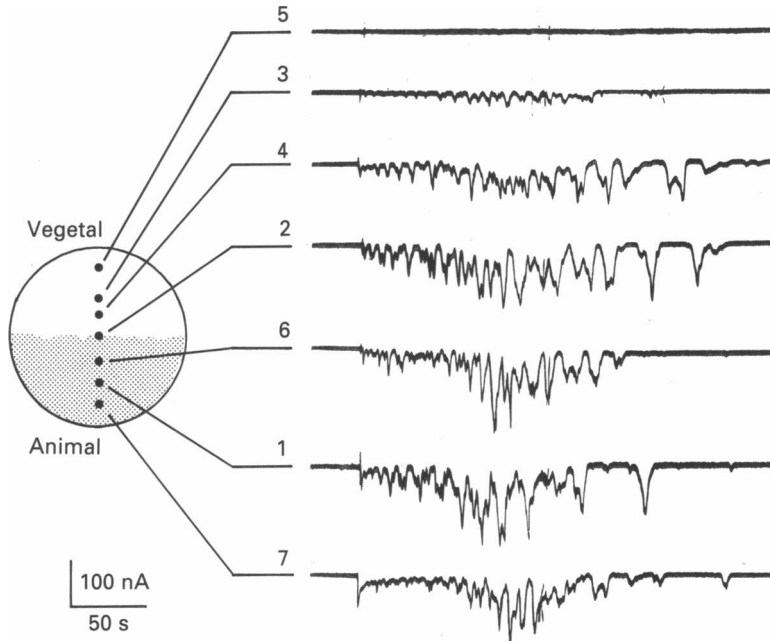


Fig. 4. Spatial variation in sensitivity to $\text{Ins}(1,3,4,5)\text{P}_4$ across the oocyte. Traces show currents evoked by injections of about 200 fmol $\text{Ins}(1,3,4,5)\text{P}_4$ at different locations, as indicated by the diagram on the left. A single injection pipette was repeatedly re-inserted at different locations, in the order indicated by the numbers next to each trace.

Seasonal variations have proved to be a problem in studying the actions of $\text{Ins}(1,3,4,5)\text{P}_4$ in sea urchin eggs (Irvine *et al.* 1988), but the properties of the *Xenopus* oocyte did not appear to change in this way. The present study is based on recordings from forty-seven oocytes, examined at various times throughout the year, with the exceptions of the months of May, August, September and December. All responded to injection of $\text{Ins}(1,3,4,5)\text{P}_4$ with oscillatory currents.

Variation in sensitivity to $\text{Ins}(1,3,4,5)\text{P}_4$ across the oocyte

Injections of $\text{Ins}(1,4,5)\text{P}_3$ evoke larger membrane responses when made near the animal rather than the vegetal pole of the oocyte (Berridge, 1988). Figure 4 shows the results of an experiment to examine whether a similar regional variation in sensitivity is seen for membrane currents evoked by injection of $\text{Ins}(1,3,4,5)\text{P}_4$. A single injection pipette was repeatedly removed and re-inserted at various sites across the oocyte, and the size of the fluid droplet expelled by the pressure pulse was measured between each trial to ensure that the pipette did not become plugged. Also, the insertions were made in random order as a further precaution that any change

in volume of fluid expelled by a constant pressure pulse would not bias the results. Injections of Ins(1,3,4,5)P₄ at various positions in the animal hemisphere evoked responses of similar sizes. However, injection into the vegetal hemisphere near the equator gave a smaller response, and this decreased to become almost undetectable

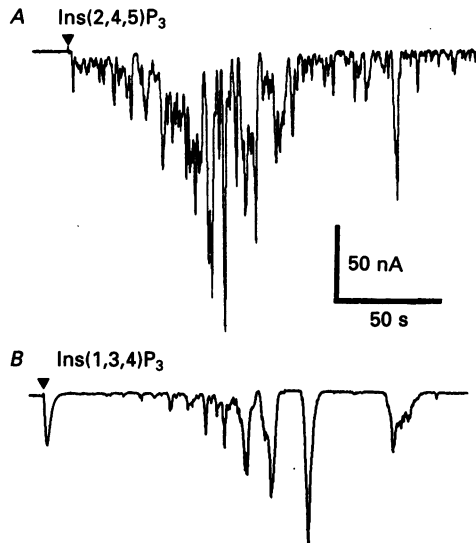


Fig. 5. Currents evoked by injections of 5 fmol Ins(2,4,5)P₃ (A) and 300 fmol Ins(1,3,4,)P₃ (B). Injections were made at the arrow-heads. Records are from separate oocytes.

when the pipette was inserted near the vegetal pole. Experiments on a further six oocytes, which were each injected with a similar amount of Ins(1,3,4,5)P₄ near the vegetal and animal poles, gave a similar result. Peak currents of between 6 and 57 nA were evoked by injections near the animal pole, but in five of the oocytes corresponding injections near the vegetal pole evoked no detectable current. The remaining oocyte gave a response to injection near the vegetal pole that was about one-sixth of that near the animal pole.

Responses to Ins(2,4,5)P₃ and Ins(1,3,4)P₃

Interpretation of responses evoked by injection of inositol phosphates is complicated by the metabolism of these compounds in the cell (Berridge & Irvine, 1989). In particular, Ins(1,4,5)P₃ is phosphorylated to Ins(1,3,4,5)P₄, which in turn is dephosphorylated to form Ins(1,3,4)P₃. Questions thus arise as to whether a part of the response to injection of Ins(1,4,5)P₃ might arise through its conversion to Ins(1,3,4,5)P₃, and whether the response to injection of Ins(1,3,4,5)P₄ might similarly involve Ins(1,3,4,)P₃.

To answer the first of these questions we injected oocytes with Ins(2,4,5)P₃, a synthetic analogue of Ins(1,4,5)P₃ which is able to liberate Ca²⁺ from intracellular stores (Burgess, Irvine, Berridge, McKinney & Putney, 1984), but which is not metabolized in the cell to InsP₄ (Irvine & Moor, 1986). As illustrated in Fig. 5A, injections of a few femtomoles of Ins(2,4,5)P₃ evoked oscillatory membrane currents,

but the short-latency, transient current observed with $\text{Ins}(1,4,5)\text{P}_3$ was absent or small. The potency of $\text{Ins}(2,4,5)\text{P}_3$ was determined in the same way as illustrated in Fig. 3, by estimating the amount required to evoke a response with an integral of $1 \mu\text{C}$. The mean value from four oocytes was $2.0 \pm 0.7 \text{ fmol}$. Thus, $\text{Ins}(2,4,5)\text{P}_3$ was roughly three times more potent than $\text{Ins}(1,4,5)\text{P}_3$.

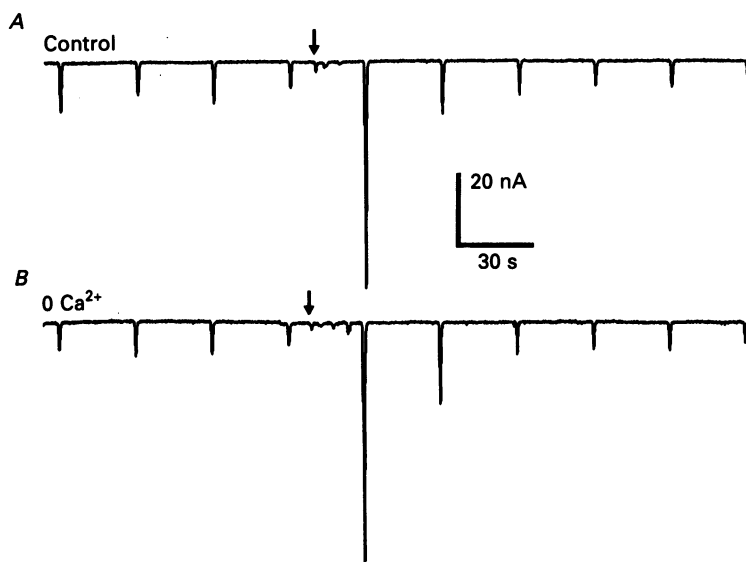


Fig. 6. Responses evoked by photo-release of $\text{Ins}(1,4,5)\text{P}_3$ are facilitated by injection of low doses of $\text{Ins}(1,3,4,5)\text{P}_4$. Traces show membrane current at a clamp potential of -60 mV , in an oocyte loaded with caged $\text{Ins}(1,4,5)\text{P}_3$. Repetitive light flashes were given at 30 s intervals. The intensity and duration of the flashes were constant, and were set so that each flash was just above threshold to evoke a detectable response. Injections of $\text{Ins}(1,3,4,5)\text{P}_4$ (about 1 fmol) were given when indicated by the arrows. *A*, record obtained with the oocyte bathed in normal Ringer solution. *B*, record obtained in the same oocyte, 10 min after changing to zero Ca^{2+} Ringer solution.

Injections of $\text{Ins}(1,3,4)\text{P}_3$ supplied by Dr R. F. Irvine were made into a total of ten oocytes from three donors. Three of these oocytes failed to show any clear responses, even when injected with doses of 2–6 pmol. The remaining oocytes all responded with a brief transient current to doses of a few tens of femtomoles, and at higher doses (several hundred femtomoles) sometimes showed oscillatory currents beginning after a long delay. An example is shown in Fig. 5*B* from an oocyte which showed high sensitivity to $\text{Ins}(1,3,4)\text{P}_3$. Injection of 300 fmol $\text{Ins}(1,3,4)\text{P}_3$ evoked a transient current followed by a few oscillations, whereas injection of 10 fmol $\text{Ins}(1,3,4,5)\text{P}_4$ into the same oocyte evoked a larger oscillatory response. Overall, $\text{Ins}(1,3,4)\text{P}_3$ appeared to be at least 100 times less potent than $\text{Ins}(1,3,4,5)\text{P}_4$.

In contrast to the low potency of $\text{Ins}(1,3,4)\text{P}_3$ supplied by Dr Irvine, $\text{Ins}(1,3,4)\text{P}_3$ obtained from Calbiochem evoked oscillatory currents with a potency similar to that of $\text{Ins}(1,3,4,5)\text{P}_4$ (five oocytes examined). It is possible that these responses arose because of contamination by InsP_3 isomers in the Calbiochem product.

As a further check that the responses described above did not arise from some non-specific effect of the highly charged phosphate groups, we also injected oocytes with inositol 1-phosphate (Ins(1)P) and with phytic acid (InsP₆). No oscillatory currents were observed even at the highest doses examined (1 pmol Ins(1)P and 5 pmol InsP₆), although that dose of InsP₆ did evoke a small (20 nA) transient current.

Ins(1,3,4,5)P₄ facilitates responses evoked by Ins(1,4,5)P₃

Light-flash photolysis of caged Ins(1,4,5)P₃ loaded into oocytes provides a means by which precisely controlled amounts of Ins(1,4,5)P₃ can be released into the cytoplasm. By the use of this technique, we have demonstrated that a threshold amount of Ins(1,4,5)P₃ is required before any Ca²⁺ is liberated from intracellular stores (Parker & Miledi, 1989; Ivorra & Parker, 1990*b*). Associated with this threshold phenomenon, a marked facilitation of the response to a light flash is seen when it is preceded by injection of a low dose of Ins(1,4,5)P₃, or by a subthreshold conditioning light flash (Parker & Miledi, 1989; Parker & Ivorra, 1990). The question thus arose of whether injection of Ins(1,3,4,5)P₄ would facilitate a subsequent response to photo-release of Ins(1,4,5)P₃.

An experiment to test this point is shown in Fig. 6*A*. A regular train of brief light flashes were applied to an oocyte previously loaded with caged Ins(1,4,5)P₃. The intensity and duration of the flashes were set so that they evoked small and roughly constant membrane current responses. After the fourth flash, about 1 fmol Ins(1,3,4,5)P₄ was injected into the oocyte through a pipette centred in the photolysis light spot. This evoked only a few small (3 nA) oscillations in current, but greatly (6-fold) potentiated the response to a light flash given 20 s later. Facilitation was still evident 50 s after the injection, but the light flash response had returned to about the control level after 80 s.

Results like that illustrated were consistently obtained in a total of six oocytes examined. The most sensitive oocyte showed clear facilitation following injection of as little as 0.1 fmol Ins(1,3,4,5)P₄. By comparison, injection of about 0.025 fmol Ins(1,4,5)P₃ into the same oocyte gave a similar potentiation.

The facilitatory effect of Ins(1,3,4,5)P₄ did not require the presence of Ca²⁺ ions in the bathing solution. Figure 6*B* shows an exact repetition of the experiment in Fig. 6*A*, except that the bathing solution was changed about 10 min before beginning recording to a Ringer solution in which the free Ca²⁺ level was reduced to a very low value with EGTA. Injection of Ins(1,3,4,5)P₄ produced a facilitation almost identical to that seen in normal Ringer solution.

Facilitation and depression produced by Ins(1,3,4,5)P₄

Figure 7 shows membrane current records from an oocyte in which the effects of injection of various amounts of Ins(1,3,4,5)P₄ were examined on caged InsP₃ responses evoked by two different durations of light flash. Traces on the left were obtained with trains of brief (22 ms) flashes, which were just suprathreshold and evoked small (1–2 nA) currents. Traces on the right were obtained with longer (40 ms) flashes, evoking currents of about 13 nA.

In the oocyte illustrated, injection of 1 fmol Ins(1,3,4,5)P₄ failed directly to

generate any membrane current response, but it produced a large (360%) facilitation of the response to a brief light flash. The current evoked by the longer flash was also facilitated, but to a much smaller extent (about 20%).

As the injection was increased, an oscillatory response to the $\text{Ins}(1,3,4,5)\text{P}_4$ was first detected to a dose of 4 fmol. This amount did not produce any greater

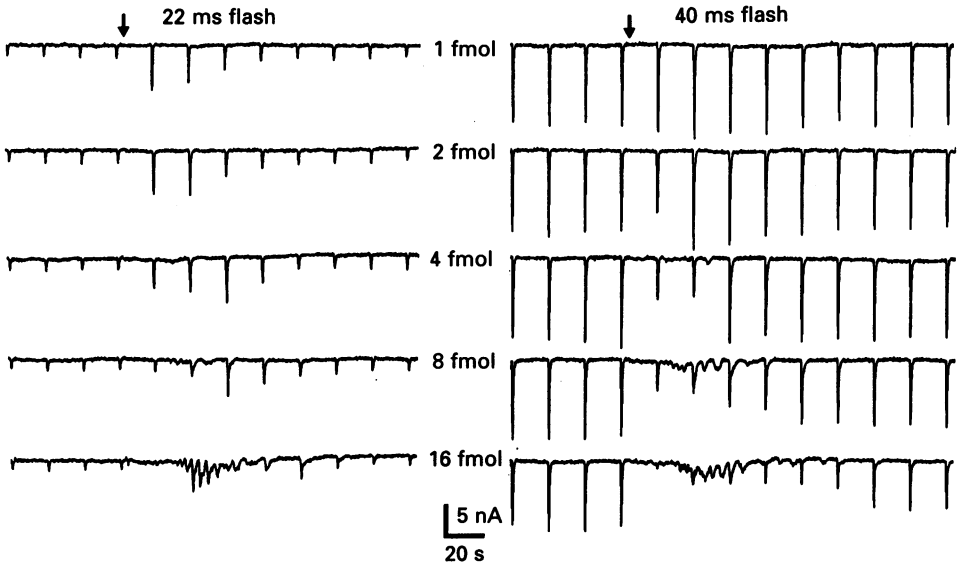


Fig. 7. Injections of $\text{Ins}(1,3,4,5)\text{P}_4$ both facilitate and depress caged $\text{Ins}(1,4,5)\text{P}_3$ responses. All traces show membrane currents evoked by repetitive light flashes at 20 s intervals in an oocyte loaded with caged $\text{Ins}(1,4,5)\text{P}_3$. Traces on the left were obtained with a flash duration (22 ms) which was just suprathreshold. Those on the right were obtained with a flash duration (40 ms) roughly twice the threshold. Various amounts of $\text{Ins}(1,3,4,5)\text{P}_4$ (indicated in femtomoles next to each pair of traces) were injected when indicated by the arrows.

facilitation of currents evoked by the short light flash than that seen with a dose of 1 fmol, but the maximal facilitation was delayed and did not occur until the third flash following the injection. In marked contrast to this facilitation of responses to the short light flash, responses to the longer flash were clearly depressed by 4 fmol $\text{Ins}(1,3,4,5)\text{P}_4$. Further increases in dose of $\text{Ins}(1,3,4,5)\text{P}_4$ evoked progressively larger and longer oscillatory responses, associated with less facilitation of the brief light flash responses and greater depression of the long flash responses. Injection of 16 fmol produced no facilitation of the response to the brief flash and, instead, it was abolished or depressed for about 1 min. The responses to the longer flash was also abolished shortly following the injection, and had not recovered completely 3 min later.

Dose dependence of facilitation and depression

Figure 8 shows measurements of peak sizes of membrane currents evoked by brief (20 ms) and long (50 ms) light flashes preceded by injections of different amounts of $\text{Ins}(1,3,4,5)\text{P}_4$. Facilitation of the response to the brief flash was already apparent

with injection of 0.4 fmol Ins(1,3,4,5)P₄, and was maximal (about 400 % potentiation) with 0.8 fmol. Injection of increasing amounts of Ins(1,3,4,5)P₄ then caused the light flash response to decline and, with doses greater than about 3 fmol, it became smaller than the control level seen without injection. Facilitation of the response to the

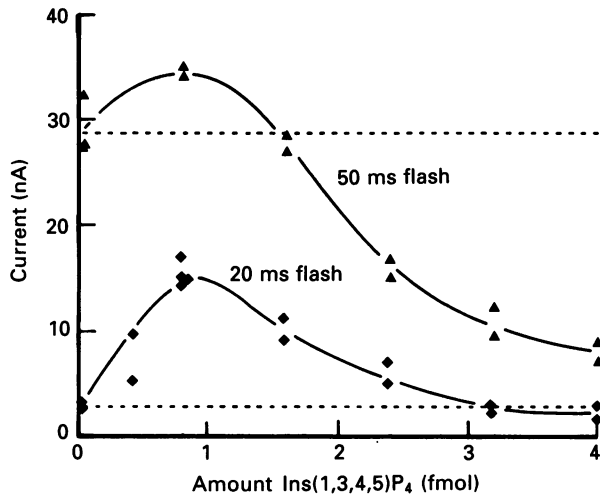


Fig. 8. Effects of injecting different amounts of Ins(1,3,4,5)P₄ on the sizes of membrane currents by short (20 ms) and long (50 ms) light flashes applied to an oocyte loaded with caged Ins(1,4,5)P₃. Measurements were made of peak sizes of currents evoked by flashes delivered 15 s following injections of various amounts of Ins(1,3,4,5)P₄, and are plotted against amount of Ins(1,3,4,5)P₄ injected. ▲, currents evoked by 50 ms flashes; ◆, currents evoked by 20 ms flashes. Dashed lines indicate the mean control responses evoked by each light flash without prior injection of Ins(1,3,4,5)P₄.

longer flash was also maximal with injection of about 0.8 fmol Ins(1,3,4,5)P₄, but the extent of the facilitation (about 20 %) was much less than for the brief flash. As the amount injected was further increased the light flash response reduced to the control level at a dose of about 1.6 fmol, and declined to one-third of the control level with 4 fmol Ins(1,3,4,5)P₄.

Low doses of Ins(1,3,4,5)P₄ reduce the threshold for Ins(1,4,5)P₃ action

Because the potentiation of caged InsP₃ responses by Ins(1,3,4,5)P₄ was proportionally greater for small responses, it seemed likely that this effect might arise because Ins(1,3,4,5)P₄ reduced the threshold level of Ins(1,4,5) required for Ca²⁺ liberation (Parker & Miledi, 1989). To test further this idea, we measured responses evoked by light flashes of different durations, applied either alone, or following injection of a low dose of Ins(1,3,4,5)P₄.

Figure 9A shows sample traces of membrane currents evoked by light flashes of various durations. In this oocyte, a flash of 10 ms duration failed to evoke a response, but a response was obtained when the same flash was preceded by an injection of

about 1 fmol Ins(1,3,4,5)P₄. Potentiation of the light flash response by Ins(1,3,4,5)P₄ was also observed for longer flash durations, but became less pronounced as the flash was lengthened.

Measurements of peak current responses are plotted in Fig. 9B as a function of flash duration. Injection of about 1 fmol Ins(1,3,4,5)P₄ reduced the threshold

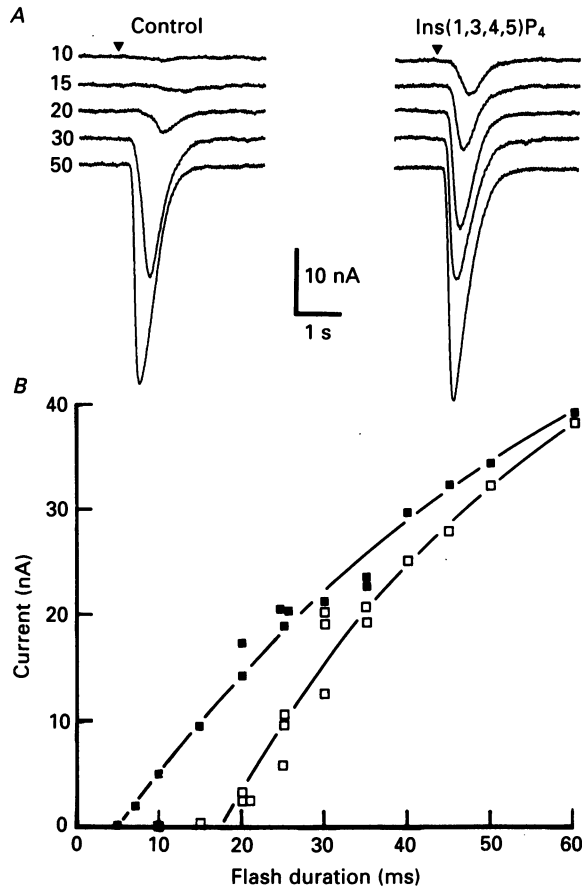


Fig. 9. Ins(1,3,4,5)P₄ reduces the threshold amount of Ins(1,4,5)P₃ required to evoke a Ca²⁺-activated chloride current. *A*, membrane current responses evoked in an oocyte loaded with caged Ins(1,4,5)P₃ by light flashes of various durations (indicated in milliseconds next to each pair of traces). Records on the left are controls, those on the right show responses to the corresponding light flashes given 15 s after injecting about 1 fmol Ins(1,3,4,5)P₄. Intervals of 100 s were allowed between each trial. *B*, measurements of peak currents evoked by light flashes of various durations in the same oocyte as *A*. □, control responses; ■, currents evoked by light flashes 15 s after injecting about 1 fmol Ins(1,3,4,5)P₄.

duration from about 15 ms to about 5 ms. Above threshold, the curves with and without Ins(1,3,4,5)P₄ were not parallel but converged, so that with a 60 ms flash, prior injection of Ins(1,3,4,5)P₄ produced little change in the response. A low dose of Ins(1,3,4,5)P₄ was used in this experiment to minimize any depression. Nevertheless,

it remains possible that the convergence of the curves in Fig. 9B arose because responses to the longer flashes were depressed by the Ins(1,3,4,5)P₄, rather than because the facilitation disappeared with longer flashes.

Facilitation does not arise through inhibition of 5-phosphomonoesterase

A possible explanation for the facilitation of the caged InsP₃ response by Ins(1,3,4,5)P₄ is that the tetrakisphosphate competes for a 5-phosphomonoesterase enzyme that degrades both compounds (Connolly, Bansal, Bross, Irvine & Majerus, 1987), and thus enhances and prolongs the elevation in Ins(1,4,5)P₃ level which follows a light flash. We therefore injected oocytes with 2,3-diphosphoglyceric acid, which has been shown to inhibit 5-phosphomonoesterase activity (Rana, Chandra Sekar, Hokin & MacDonald, 1986). Experiments were done in the same way as in Fig. 6A, by injecting various doses of 2,3-diphosphoglyceric acid at intervals of between 4 and 30 s before light flashes. In a total of thirty-one trials in five oocytes we never observed any clear facilitation of the caged InsP₃ responses. Instead, injections of less than about 50 fmol were without effect, whereas higher doses produced an inhibition. The response was reduced to a mean of about 70% of the control level (six trials) by 100 fmol 2,3-diphosphoglyceric acid, and to about 25% by 200 fmol.

DISCUSSION

Ca²⁺ liberation by Ins(1,3,4,5)P₄

Microinjection of Ins(1,3,4,5)P₄ into oocytes produced a rise in intracellular free Ca²⁺ and the associated generation of a Ca²⁺-activated chloride current. Both of these effects persisted after removal of extracellular Ca²⁺ and, therefore, presumably arise because of liberation of Ca²⁺ from intracellular stores. In comparison to Ins(1,4,5)P₃, Ins(1,3,4,5)P₄ was about twenty times less potent in evoking a current response, so we were concerned that the responses might have been due to contamination of the tetrakisphosphate by Ins(1,4,5)P₃ or other trisphosphate isomers. However, this is most unlikely, because similar results were obtained with two HPLC-purified preparations of Ins(1,3,4,5)P₄: one obtained commercially (Calbiochem) and the other provided by Dr R. F. Irvine. The latter sample is estimated to contain no more than 0.1% inositol trisphosphates (R. F. Irvine, personal communication), and Ins(1,3,4,5)P₄ prepared by the same procedure has previously been found to be ineffective in liberating intracellular Ca²⁺ in Swiss 3T3 cells (Irvine *et al.* 1986*b*), in sea urchin eggs (Irvine & Moor, 1986) and in lacrimal cells (Changya *et al.* 1989). Furthermore, the different patterns of current response evoked by Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄, as well as their different effects on modulating Ca²⁺ influx through the plasma membrane (Parker & Miledi, 1987; Snyder *et al.* 1988), all argue against the response to Ins(1,3,4,5)P₄ arising because of contaminating Ins(1,4,5)P₃. We can also eliminate the possibility that responses to Ins(1,3,4,5)P₄ arise through its metabolism in the cell to Ins(1,3,4)P₃, because injections of Ins(1,3,4)P₃ were much less potent.

The literature on the actions of Ins(1,3,4,5)P₄ is sharply divided into those reports which describe it as being (by itself) almost ineffective in liberating intracellular Ca²⁺ (for example: Irvine *et al.* 1986*b*; Irvine & Moor, 1986, 1987; Morris *et al.* 1987;

Changya *et al.* 1989), and others (Parker & Miledi, 1987; Crossley *et al.* 1988; Snyder *et al.* 1988; Joseph *et al.* 1989; Stith & Proctor, 1989) which indicate a Ca^{2+} -liberating action, albeit with a potency 20–40 times lower than $\text{Ins}(1,4,5)\text{P}_3$. Reasons for this difference are not yet obvious. One reason for the negative results may be that many of those studies were made on permeabilized or perfused cell preparations, in which a soluble cytoplasmic factor required for the action of $\text{Ins}(1,3,4,5)\text{P}_4$ could have been washed out. However, the recent demonstration that $\text{Ins}(1,3,4,5)\text{P}_4$ liberates Ca^{2+} from cerebellum microsomes (Joseph *et al.* 1989) is inconsistent with this idea. A different explanation is suggested by the demonstration of an intracellular $\text{Ins}(1,3,4,5)\text{P}_4$ -binding protein, distinct from that which binds $\text{Ins}(1,4,5)\text{P}_3$ (Bradford & Irvine, 1987; Theibert, Supattapone, Worley, Baraban, Meek & Snyder, 1987; Enyedi & Williams, 1988). The numbers of binding sites for $\text{Ins}(1,3,4,5)\text{P}_4$ found in different tissues vary widely (Theibert *et al.* 1987), so that the differing efficacy of $\text{Ins}(1,3,4,5)\text{P}_4$ to liberate Ca^{2+} in various cells may result from differences in the numbers of functional intracellular receptors.

Although $\text{Ins}(1,3,4,5)\text{P}_4$ evokes oscillatory currents, it is unlikely that more than a small fraction of the oscillatory response to $\text{Ins}(1,4,5)\text{P}_3$ arises because it is phosphorylated in the cell to form $\text{Ins}(1,3,4,5)\text{P}_4$. Firstly, the potency of $\text{Ins}(1,3,4,5)\text{P}_4$ was lower than that of $\text{Ins}(1,4,5)\text{P}_3$. Secondly, prominent oscillations were evoked by injection of $\text{Ins}(2,4,5)\text{P}_3$, an inositol trisphosphate isomer that is not a substrate for the kinase that converts $\text{Ins}(1,4,5)\text{P}_3$ to $\text{Ins}(1,3,4,5)\text{P}_4$ (Irvine & Moor, 1986).

Injections of $\text{Ins}(1,3,4,5)\text{P}_4$ were more effective when made into the animal, rather than the vegetal, hemisphere of the oocyte. A similar regional variation has previously been described for responses evoked by injections of $\text{Ins}(1,4,5)\text{P}_3$ (Berridge, 1988) and by local extracellular applications of various Ca^{2+} -mobilizing agonists (Kusano, Miledi & Stinnakre, 1982; Miledi & Parker, 1989). The asymmetrical distribution of Ca^{2+} -activated chloride channels across the oocyte surface (Miledi & Parker, 1984) probably accounts for much of the variation in sensitivity to all these agents, although it remains possible that other factors, such as variations in density of intracellular Ca^{2+} release site, may also contribute to the differences in sensitivity to $\text{Ins}(1,3,4,5)\text{P}_4$.

An interesting feature of the $\text{Ins}(1,3,4,5)\text{P}_4$ -evoked Ca^{2+} signals monitored by Fluo-3 and Rhod-2 was that they appeared to comprise two components; a prolonged elevation that began with short latency, on which superimposed brief (about 4–5 s) oscillations could often be seen. Only the oscillatory component was reflected in the Ca^{2+} -activated chloride membrane current, which often returned to the baseline between oscillations even though the Ca^{2+} signal remained elevated (Fig. 1). We have previously seen similar discrepancies between Ca^{2+} signals and chloride currents activated in the oocyte by $\text{Ins}(1,4,5)\text{P}_3$ (Parker & Ivorra, 1990) and by agonist application (Miledi & Parker, 1989). For several reasons it is unlikely that the failure of the chloride current to track the sustained rise in Ca^{2+} can be due to inactivation or desensitization of the chloride channels. Most strikingly, some oocytes showed little membrane current response several seconds after injection of $\text{Ins}(1,3,4,5)\text{P}_4$, even though the Ca^{2+} signal was nearly maximal at this time. Also, current responses to injections of Ca^{2+} were not depressed during the time when the

Ca²⁺ signal was elevated (I. Ivorra and I. Parker, unpublished data). Instead, the differences between the fluorescence and membrane current Ca²⁺ signals may arise from spatial differences in Ca²⁺ liberation and sequestration in the oocyte.

Interactions of Ins(1,3,4,5)P₄ and Ins(1,4,5)P₃

Calcium-dependent membrane currents evoked by flash photolysis of caged Ins(1,4,5)P₃ were facilitated by a preceding injection of low (*ca* 1 fmol) doses of Ins(1,3,4,5)P₄, but were depressed by higher doses. Both of these effects have been observed also with prior injections of Ins(1,4,5)P₃ (Parker, 1989; Parker & Miledi, 1989; Parker & Ivorra, 1990), suggesting that Ins(1,3,4,5)P₄ and Ins(1,4,5)P₃ might act by similar, or the same mechanisms. An alternative explanation for the facilitation may be that Ins(1,3,4,5)P₄ facilitates responses to Ins(1,4,5)P₃ by inhibiting its phosphorylation by the 3-kinase. However, this seems unlikely, because the time-to-peak of the responses to photo-released Ins(1,4,5)P₃ is short compared to the expected time course of its metabolism (Irvine *et al.* 1986*a*).

The facilitation of responses to Ins(1,4,5)P₃ by low doses of Ins(1,3,4,5)P₄ resulted from a reduction in the amount of Ins(1,4,5)P₃ required to evoke a response. We have previously shown (Parker & Miledi, 1989; Ivorra & Parker, 1990*b*) that a threshold level of Ins(1,4,5)P₃ is required in the oocyte to trigger Ca²⁺ liberation, and proposed that this may explain the facilitation seen between paired responses to Ins(1,4,5)P₃. The finding that Ins(1,3,4,5)P₄ is able also to reduce the threshold for Ins(1,4,5)P₃ thus suggests that both compounds exert similar actions on the Ca²⁺-liberating mechanism, a view which is supported by the finding that higher doses of Ins(1,3,4,5)P₄ were able directly to evoke Ca²⁺ liberation.

Another action that has been proposed for Ins(1,3,4,5)P₄ is that it may promote the filling of Ins(1,4,5)P₃-sensitive stores by Ca²⁺, derived either from the extracellular solution (Irvine & Moor, 1987) or from Ins(1,4,5)P₃-insensitive intracellular stores (Irvine *et al.* 1988; Berridge & Irvine, 1989). Because the facilitatory effect of Ins(1,3,4,5)P₄ that we describe was unaffected by removal of extracellular Ca²⁺, it seems that it cannot arise through the first of the above mechanisms. Enhanced filling from Ins(1,4,5)P₃-insensitive stores remains a possibility. Although one might expect this to result only in a proportionally greater liberation of Ca²⁺ by Ins(1,4,5)P₃, the observed shift in threshold could be accounted for if the properties of the Ins(1,4,5)P₃ receptor are modulated by the intraluminal Ca²⁺ concentration (Irvine, 1990).

Higher doses of Ins(1,3,4,5)P₄ depressed, rather than enhanced, responses to Ins(1,4,5)P₃. A likely explanation for this is that when sufficient Ins(1,3,4,5)P₄ is injected to cause Ca²⁺ liberation, the resulting rise in cytoplasmic free Ca²⁺ inhibits the ability of Ins(1,4,5)P₃ to release further Ca²⁺ (Payne, Walz, Levy & Fein, 1988; Parker & Ivorra, 1990). An alternative possibility, that Ins(1,3,4,5)P₄ causes appreciable depletion of Ca²⁺ from Ins(1,4,5)P₃-sensitive stores, is less attractive because low doses of Ins(1,3,4,5)P₄, which themselves produced no or very small currents, were already able to depress subsequent large responses evoked by Ins(1,4,5)P₃.

Mechanism of Ca²⁺ liberation by Ins(1,3,4,5)P₄

The results are consistent with the idea that Ins(1,3,4,5)P₄ causes liberation of intracellular Ca²⁺ in the oocyte; but how does this occur? We may consider several possibilities. (i) Ins(1,3,4,5)P₄ releases Ca²⁺ by direct interaction with a Ca²⁺-mobilizing receptor. (ii) Ins(1,3,4,5)P₄ enhances the ability of Ins(1,4,5)P₃ to liberate Ca²⁺, so that the resting level of Ins(1,4,5)P₃ on the cell becomes sufficient to evoke a response. (iii) By competing with a 5-phosphomonoesterase enzyme that breaks down both Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ (Irvine *et al.* 1988), or by inhibiting the conversion of Ins(1,4,5)P₃ to Ins(1,3,4,5)P₄, Ins(1,3,4,5)P₄ causes the resting level of Ins(1,4,5)P₃ to rise above threshold. (iv) A 3-phosphatase is present in the oocyte, that forms Ins(1,4,5)P₃ from the injected Ins(1,3,4,5)P₄ (Cullen, Irvine, Drøbak & Dawson, 1989).

We cannot yet rule out any of the above possibilities, but arguments can be made against several of them. Mechanisms (iii) and (iv) seem unlikely, in view of the rapid (200 ms or less) onset of the Ca²⁺ rise following injection of Ins(1,3,4,5)P₄. If the Ca²⁺ signal arises through the formation of Ins(1,4,5)P₄ this conversion would have to occur very rapidly, and would imply a massive futile cycling in the cell between the tris- and tetrakisphosphates. Similarly, if competition for the 5-phosphomonoesterase or inhibition of conversion to Ins(1,3,4,5)P₄ were to sufficiently elevate the resting level of Ins(1,4,5)P₃, the normal rate of break-down of Ins(1,4,5) would have to occur with a time constant of a few hundred milliseconds, whereas other measurements in the oocyte indicate a value of tens of seconds (Parker & Miledi, 1989) or several minutes (Irvine *et al.* 1986*a*). Furthermore, we did not observe facilitation of the caged InsP₃ response following injection of 2,3-diphosphoglyceric acid, which is known to inhibit the degradation of Ins(1,4,5)P₃ by 5-phosphomonoesterase enzymes (Rana *et al.* 1986). Additional evidence against mechanisms (ii), (iii) and (iv) is provided by the finding (Joseph *et al.* 1989) that Ins(1,3,4,5)P₄ mobilizes Ca²⁺ from a microsomal preparation in which there was no background Ins(1,4,5)P₃ and no detectable 3-phosphatase activity.

Thus, we favour the idea that Ins(1,3,4,5)P₄ liberates sequestered Ca²⁺ by directly interacting with an intracellular receptor. The identity of this is presently unclear. The simplest explanation for our results is that Ins(1,3,4,5)P₄ and Ins(1,4,5)P₃ both bind to a common site, and that the different patterns of responses activated by the two compounds result from factors such as differences in residence times at the receptor or in rates of enzymatic degradation. Alternatively, the actions of Ins(1,3,4,5)P₄ might arise through distinct and specific receptors (Bradford & Irvine, 1987; Theibert *et al.* 1987; Enyedi & Williams, 1988). However, if distinct receptors mediate the actions of Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄, they must both be linked to a common Ca²⁺-release system, so as to give rise to the non-linear facilitation of Ca²⁺ liberation.

Physiological function of Ins(1,3,4,5)P₄

The steady-state levels of Ins(1,3,4,5)P₄ in agonist-stimulated cells may be higher or lower than those for Ins(1,4,5)P₃, depending upon the type of cell and agonist (Joseph & Williamson, 1989), and remain elevated for longer after removal of agonist

(Pittet, Schlegel, Lew, Monod & Mayr, 1989). The intracellular concentrations of Ins(1,3,4,5)P₄ attained under physiological conditions may, therefore, be sufficient to play a role in Ca²⁺ mobilization. For example, Ca²⁺-activated currents were detected in our experiments following injection of about 1 fmol Ins(1,3,4,5)P₄, and facilitation of Ins(1,4,5)P₃ responses was evident with smaller doses. If a quantity of 1 fmol were to be distributed evenly throughout the oocyte, the resulting intracellular concentration would be 1–2 nM. Even though the local concentrations near the injection site were undoubtedly higher, this value is very low in comparison to concentrations of a few micromolar which have been measured in other cells in response to stimulation (Pittet *et al.* 1989).

If Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ both liberate Ca²⁺, why do cells go to the trouble of making two intracellular messengers that both function in the same way? One answer might be that there are important differences in the responses to each; for example, in the different patterns of responses mediated by release of stored Ca²⁺, and in their differing actions on the entry of extracellular Ca²⁺ (Parker & Miledi, 1987; Snyder *et al.* 1988). Another possibility arises from the finding that low doses of Ins(1,3,4,5)P₄ can potentiate responses to Ins(1,4,5)P₃ and might serve to produce a relatively long-lasting facilitation of phosphoinositide signalling. For example, a brief stimulus that causes a transient formation of Ins(1,4,5)P₃ will evoke Ca²⁺ release that declines as the Ins(1,4,5)P₃ is metabolized. Some of the Ins(1,4,5)P₃ will be converted into Ins(1,3,4,5)P₄ but, because of its lower potency, sufficient amounts may not be formed to allow further Ca²⁺ liberation. However, subthreshold levels of Ins(1,3,4,5)P₄ could still be important in facilitating responses to subsequent stimuli which evoke Ins(1,4,5)P₃ formation.

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