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$InsP_3$ and $Ins(1,3,4,5)P_4$ act in synergy to stimulate influx of extracellular Ca^{2+} in *Xenopus* oocytes

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DeLisle, Sylvain, Didier Pittet, Barry V. L. Potter, P. Daniel Lew, and Michael J. Welsh. $InsP_3$ and $Ins(1,3,4,5)P_4$ act in synergy to stimulate influx of extracellular Ca^{2+} in *Xenopus* oocytes. *Am. J. Physiol.* 262 (*Cell Physiol.* 31): C1456–C1463, 1992.—To investigate the role of D-*myo*-inositol 1,3,4,5-tetrakisphosphate [$Ins(1,3,4,5)P_4$] in the regulation of Ca^{2+} influx, we injected inositol phosphates into *Xenopus* oocytes and measured Ca^{2+}-gated Cl^- current to assay intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). To assess Ca^{2+} influx, we removed extracellular Ca^{2+} or added the inorganic Ca^{2+} channel blocker Mn^{2+} to the extracellular bath and measured the resulting change in Cl^- current. $Ins(1,3,4,5)P_4$ did not cause Ca^{2+} influx when injected alone or when preceded by an injection of Ca^{2+}. In contrast, $Ins(1,3,4,5)P_4$ stimulated Ca^{2+} influx when injected after the poorly metabolized inositol trisphosphate ($InsP_3$) analogues D-*myo*-inositol 1,4,5-trisphosphorothioate [$Ins(1,4,5)P_3S_3$] or D-*myo*-inositol 2,4,5-trisphosphate [$Ins(2,4,5)P_3$]. These results indicate that $Ins(1,3,4,5)P_4$ is not sufficient to stimulate Ca^{2+} influx but acts in synergy with $InsP_3$s to cause Ca^{2+} influx. We also studied the effect of Ca^{2+} influx on the immediate metabolism of D-*myo*-inositol 1,4,5-trisphosphate [$Ins(1,4,5)P_3$] in single oocytes. Ca^{2+} influx shunted the metabolism of $Ins(1,4,5)P_3$ toward the formation of $Ins(1,3,4,5)P_4$ and away from D-*myo*-inositol 1,4-bisphosphate [$Ins(1,4)P_2$]. These results suggest that there is a positive feedback regulatory mechanism in which Ca^{2+} influx stimulates $Ins(1,3,4,5)P_4$ production and $Ins(1,3,4,5)P_4$ stimulates further Ca^{2+} influx.

inositol trisphosphate; D-*myo*-inositol 1,3,4,5-tetrakisphosphate; extracellular calcium influx; intracellular calcium concentration

STIMULATION OF MANY plasma membrane receptors increases the intracellular concentration of the second messenger D-*myo*-inositol 1,4,5-trisphosphate [$Ins(1,4,5)P_3$]. By opening Ca^{2+} channels, $Ins(1,4,5)P_3$ releases Ca^{2+} from intracellular stores (2, 15, 34). $Ins(1,4,5)P_3$ also causes a sustained influx of extracellular Ca^{2+} into the cell (2). We do not know how $Ins(1,4,5)P_3$ regulates Ca^{2+} influx (33).

Intracellular injection of $Ins(1,4,5)P_3$ can stimulate Ca^{2+} influx (7, 11, 21, 22, 27, 30, 36). However, because $Ins(1,4,5)P_3$ is extensively metabolized by the cell, inositol phosphates (InsP) other than $Ins(1,4,5)P_3$ may control Ca^{2+} influx. D-*Myo*-inositol 1,3,4,5-tetrakisphosphate [$Ins(1,3,4,5)P_4$] is a direct metabolite of $Ins(1,4,5)P_3$ that may also release intracellular Ca^{2+} (16, 36). $Ins(1,3,4,5)P_4$ has been implicated in the regulation of Ca^{2+} influx in some cellular sytems (7, 21, 22, 27, 31) but not in others (30, 36). The controversy surrounding the requirement of $Ins(1,3,4,5)P_4$ for Ca^{2+} influx has recently been emphasized when two groups of investigators using similar techniques in mouse lacrimal acinar cells came to opposite conclusions; the results of one study suggested that $Ins(1,3,4,5)P_4$ was required for Ca^{2+} influx (27), and the results of the other study suggested it was not (5). In the present studies, we investigate how inositol trisphosphates ($InsP_3$) and $Ins(1,3,4,5)P_4$ control Ca^{2+} influx in *Xenopus* oocytes. We show that $Ins(1,3,4,5)P_4$ stimulates Ca^{2+} influx in the presence but not in the absence of poorly metabolizable $InsP_3$s. In addition, we tested the possibility that Ca^{2+} influx may stimulate the phosphorylation of $Ins(1,4,5)P_3$ into $Ins(1,3,4,5)P_4$. Our observations suggest a positive feedback mechanism, whereby $Ins(1,3,4,5)P_4$ stimulates Ca^{2+} influx and Ca^{2+} influx promotes formation of $Ins(1,3,4,5)P_4$.

MATERIALS AND METHODS

Electrophysiology and intracellular injections. We obtained *Xenopus* oocytes as described previously (11, 36) and defolliculated them manually. To assay intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), we measured Ca^{2+}-activated Cl^- currents with the two-electrode voltage-clamp technique. This assay has been validated using both Ca^{2+} electrodes and fluorescent calcium indicators (10). Oocytes were impaled with two microelectrodes (3 M KCl filled, resistance = 0.5–1.0 MΩ), and membrane voltage was maintained at −50 mV with a voltage-clamp amplifier (Axoclamp 2A, Axon Instruments, Burlingame, CA). The current necessary to maintain this membrane voltage was recorded on-line (MacLab, World Precision Instruments, New Haven, CT) and analyzed by a microcomputer (Macintosh SE/30, Apple Computer, Cupertino, CA). A third micropipette (tip diameter 2–5 μm) was attached to a pneumatic microinjector (PLI-100, Medical Systems, Greenvale, NY) and used for intracellular injections. When sequential intracellular injections involving different substances were required, the first injection pipette was withdrawn from the cell and a second pipette was reinserted at the same site. Although removal of the injection pipette from the cell did not impair subsequent cellular responses, it caused a transient inward current; after 30–60 s, current returned to values similar to those obtained just before pipette removal. For the sake of clarity, this transient current was blanked from the tracings. Injection pipettes were backfilled with $Ins(1,3,4,5)P_4$ (Calbiochem, San Diego, CA), $Ins(1,4,5)P_3$ (Amersham, Arlington Heights, IL), ^3H-labeled $Ins(1,4,5)P_3$ (Amersham), D-*myo*-inositol 2,4,5-trisphosphate [$Ins(2,4,5)P_3$] (Boehringer Mannheim Biochemicals, Indianapolis, IN), D-*myo*-inositol 3,4,5,6-tetrakisphosphate [$Ins(3,4,5,6)P_4$] (Boehringer Mannheim), D-*myo*-inositol 1,4,5-trisphosphorothioate [$Ins(1,4,5)P_3S_3$] [prepared as previously described (9)], or $CaCl_2$ (Sigma Chemicals, St. Louis, MO). Each injection had a volume of 10 nl or ~1% of the oocyte volume. Before and after each experiment, we calibrated the injection pipette by expelling a drop of solution in paraffin oil and calculating volume from the measured diameter of the drop.

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Ca^{2+} influx measurements. For most experiments, oocytes were initially stimulated in a bath solution containing (in mM) 116 NaCl, 2 KCl, 6 $CaCl_2$, 1 $MgCl_2$, and 5 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.4. We assessed Ca^{2+} influx by measuring the change in Cl^- current induced by either removing external Ca^{2+} or adding the inorganic Ca^{2+} channel blocker Mn^{2+} (4 mM) to the bath solution. For most experiments, we added Mn^{2+} rather than removing bath Ca^{2+} because membrane electrical resistance often decreased with time in the absence of external Ca^{2+} (10). Because we have previously found that Ca^{2+} influx usually occurs after the disappearance of $[Ca^{2+}]_i$ oscillations (11, 36), our standard procedure was to add Mn^{2+} during the nonoscillatory current that followed the disappearance of $[Ca^{2+}]_i$ oscillations. Occasionally, the injected InsP caused prolonged $[Ca^{2+}]_i$ oscillations. In such cases, we added Mn^{2+} when the baseline current (the average current around which the oscillations occurred) had stabilized. To determine if the addition of Mn^{2+} (or the removal of extracellular Ca^{2+}) caused a change in Cl^- current, we extracted the digitized current values for the 60-s time period immediately preceding the addition of Mn^{2+} and the current values for the 60-s time period beginning 1 min after the addition of Mn^{2+}. Because we acquired data at a rate of 4 Hz, each 60-s time period contained 240 values. We compared the two groups of values (i.e., immediately before and 1 min after Mn^{2+}) using the Student's *t* test for paired data. We interpreted a statistically significant decrease in the mean Cl^- current caused by Mn^{2+} as positive evidence of Ca^{2+} influx (11, 36). The absolute difference between the means was taken as a semiquantitative index of Ca^{2+} influx.

Ins(1,4,5)P_3 metabolism in single oocytes. To measure the metabolism of Ins(1,4,5)P_3, we injected [^{3}H]Ins(1,4,5)P_3 into single oocytes and arrested InsP metabolism at fixed time points. The injectate contained 5 μCi of lyophilized [^{3}H]Ins(1,4,5)P_3 (sp act 20–60 Ci/mmol) mixed with 10 μl of cold Ins(1,4,5)P_3 (10^{-4} M). Total calculated concentration of Ins(1,4,5)P_3 (cold and ^{3}H-labeled) in the injection pipette was ~0.11 mM. Given an injection volume of 10 nl and an oocyte volume of 1 μl, the calculated intracellular Ins(1,4,5)P_3 concentration was ~1.1 μM, i.e., comparable with what we have used in the past to cause Ca^{2+} influx (36). At predetermined time points (0.25, 0.5, 1, 2, 3, 5, 10, 20, and 40 min), InsP metabolism was arrested by crushing the oocyte in 1 ml of ice-cold trichloroacetic acid solution (15%). The sample was then centrifuged (4°C, 800 *g*) for 10 min, and the supernatant was transferred into a glass tube. Three successive diethyl ether extractions (5:1, vol/vol) were performed to remove lipids and phospholipids, and the aqueous phase was frozen at −70°C. When feasible, i.e., time points of 1 min or more, we measured the cellular response to the [^{3}H]Ins(1,4,5)P_3/Ins(1,4,5)P_3 injection mixture by recording Cl^- current. In these cases, recording electrodes were withdrawn from the cell 30 s before InsP metabolism was stopped.

High-performance liquid chromatography (HPLC) separation of InsPs. After addition of ATP (10 μM) and EDTA (1 mM), the thawed samples were chromatographed on two sequential anion exchange columns, SAX-10, 0.4 × 25 cm (Technicol, Stockport, Cheshire, UK), at a flow rate of 1.6 ml/min as previously described (31). An initial 5-min washing period in H_2O was followed by a discontinuous gradient of phosphate buffer prepared from phosphoric acid and adjusted to pH 4.2 with NaOH. Elution occurred over a 60-min period. The gradient was held at a concentration of 0.36 M from 12 to 20 min, at 0.84 M from 24 to 31 min, and at 1.8 M from 54 to 59 min; changes between these isocratic steps were linear gradients. Fractions were collected at 0.2-min intervals and radioactivity assessed by liquid scintillation counting. This HPLC procedure was optimized for the separation of the three distinct inositol tetrakisphosphates: D-*myo*-inositol 1,3,4,6-tetrakisphosphate [Ins(1,3,4,6)P_4], Ins(1,3,4,5)P_4, and Ins(3,4,5,6)P_4 (31). ^{3}H-labeled D-*myo*-inositol 1,4-bisphosphate [Ins(1,4)P_2], Ins(1,4,5)P_3, and Ins(1,3,4,5)P_4 standards were purchased from Amersham.

Statistical analysis. Means were compared using the Student's *t* test for unpaired data. Differences in current within single cells were compared using the Student's *t* test for paired data. Difference between the outcome in two groups of cells (intervention vs. control) was assessed with Fisher's exact test (two-tailed). Significance was assumed when $P < 0.05$.

RESULTS AND DISCUSSION

Effect of Ins(1,3,4,5)P_4 on Ca^{2+} influx. Injection of 1 pmol of Ins(1,4,5)P_3 (estimated intracellular concentration of 1 μM) into *Xenopus* oocytes caused a biphasic increase in $[Ca^{2+}]_i$ (Fig. 1*A*). We have previously shown that the initial, transient increase in $[Ca^{2+}]_i$ represents the release of Ca^{2+} from intracellular stores and is not affected by extracellular $[Ca^{2+}]$ (36). The more sustained increase in $[Ca^{2+}]_i$ that follows can be blocked either by removing extracellular Ca^{2+} or by adding Mn^{2+} (4 mM) to the external bath (Fig. 1*A*). This result indicates that the sustained increase in $[Ca^{2+}]_i$ is due to the influx of extracellular Ca^{2+}. Thus, as previously reported in *Xenopus* oocytes (25, 36), Ins(1,4,5)P_3 causes Ca^{2+} influx.

To assess the role of Ins(1,3,4,5)P_4 in Ins(1,4,5)P_3-induced Ca^{2+} influx, we injected 10 pmol of Ins(1,3,4,5)P_4 (estimated intracellular concentration of 10 μM). Ins(1,3,4,5)P_4 caused $[Ca^{2+}]_i$ oscillations (12/12 cells) (Fig. 1, *B* and *C*). In previous experiments, we (11, 36) and others (28, 29) have found that either removing extracellular Ca^{2+} or adding Mn^{2+} to the bath solution had no effect on Ins(1,3,4,5)P_4-induced $[Ca^{2+}]_i$ oscillations (for an example, qualitatively compare Fig. 1*B* with Fig. 1*C*). Because Ca^{2+} influx could possibly occur following completion of the intracellular Ca^{2+} release phase [as occurs with Ins(1,4,5)P_3; Fig. 1*A*], we also added Mn^{2+} during the steady-state current that followed the $[Ca^{2+}]_i$ oscillations (Fig. 1*C*). Mn^{2+} did not alter Ca^{2+}-gated Cl^- current under these conditions (no change in 11/12 cells, current decreased by 22 nA in one cell). Thus, under these conditions, Ins(1,3,4,5)P_4 is not sufficient to cause Ca^{2+} influx. These results agree with previous reports in *Xenopus* oocytes (11, 28, 29, 36).

The synthetic Ins(1,4,5)P_3 analogue, Ins(1,4,5)P_3S_3 (37), is a poor substrate for the Ins(1,4,5)P_3 3-kinase (38), the enzyme responsible for the conversion of Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4. As previously observed (11, 37), injection of 10 pmol of Ins(1,4,5)P_3S_3 (estimated intracellular concentration of 10 μM) caused prolonged $[Ca^{2+}]_i$ oscillations (30/30 cells) (Fig. 1*D*). Removal of Ca^{2+} from the bath or addition of Mn^{2+} (4 mM) did not change the average Cl^- current response (14/14 cells) (Fig. 1*D*). This result indicates that Ins(1,4,5)P_3S_3 does not cause Ca^{2+} influx.

To mimic the physiological sequence where Ins(1,4,5)P_3 is metabolized to Ins(1,3,4,5)P_4, we first injected Ins(1,4,5)P_3S_3 (10 pmol) and then Ins(1,3,4,5)P_4 (10 pmol) in the same cell. When injected after Ins(1,4,5)P_3S_3, Ins(1,3,4,5)P_4 stimulated a Cl^- current that could be inhibited by removing extracellular Ca^{2+} or by adding Mn^{2+} (4 mM) to the bath solution (11/13 cells) (Fig. 1*E*). These results suggest that the combination of

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Ins(1,4,5)P_3S_3 and Ins(1,3,4,5)P_4 stimulates Ca^{2+} influx. This contrasts with results obtained when either compound was injected alone. The magnitude of Ins(1,4,5)P_3S_3/Ins(1,3,4,5)P_4-induced Ca^{2+} influx varied considerably from cell to cell [191 ± 46 (SE) nA, range = 57–440 nA]. There was no relationship between the amplitude of Ins(1,3,4,5)P_4-induced Ca^{2+} influx and the time interval between Ins(1,4,5)P_3S_3 and Ins(1,3,4,5)P_4 injections (from 5 to 30 min).

To ensure that Ca^{2+} influx did not simply result from a greater total amount of injected InsP, we performed two types of experiments. First, we injected twice the amount (20 pmol) of either Ins(1,3,4,5)P_4 (7 cells) or Ins(1,4,5)P_3S_3 (3 cells). This failed to cause Ca^{2+} influx. Second, we injected Ins(1,4,5)P_3S_3 (10 pmol) twice, sequentially. This also did not cause Ca^{2+} influx (2 cells). These results suggest that Ca^{2+} influx does not result from doubling the total amount of injected InsPs.

To determine whether the potential to cause Ca^{2+} influx following Ins(1,4,5)P_3S_3 was specific to Ins(1,3,4,5)P_4, we injected Ins(3,4,5,6)P_4, the predominant $InsP_4$ in nonstimulated *Xenopus* oocytes (24). Ins(3,4,5,6)P_4 (10 pmol) did not cause $[Ca^{2+}]_i$ oscillations (12/13 cells) when injected alone and did not cause Ca^{2+} influx when injected after Ins(1,4,5)P_3S_3 (5/5 cells). These results suggest that the ability to cause Ca^{2+} influx following an injection of Ins(1,4,5)P_3S_3 is relatively specific to the Ins(1,3,4,5)P_4 isomer.

Ins(2,4,5)P_3 is another $InsP_3$ analogue not expected to be metabolized to Ins(1,3,4,5)P_4 (26). Ins(2,4,5)P_3, like Ins(1,4,5)P_3S_3, releases Ca^{2+} from intracellular stores (11, 13, 36). In contrast to Ins(1,4,5)P_3S_3, however, Ins(2,4,5)P_3 causes Ca^{2+} influx (Fig. 2*A* and Ref. 36). Although we do not know why the response to Ins(2,4,5)P_3 differs from Ins(1,4,5)P_3S_3, we hypothesized that Ins(2,4,5)P_3 could also enable Ins(1,3,4,5)P_4 to stimulate Ca^{2+} influx. To test this hypothesis, we first injected Ins(2,4,5)P_3 and waited until there was no increase in the nonoscillatory Cl^- current for a period of at least 3 min. We then injected Ins(1,3,4,5)P_4. Compared with the average amount of current present during the 1-min time period immediately preceding Ins(1,3,4,5)P_4 injection, Ins(1,3,4,5)P_4 increased the current by 115 ± 21 nA (the increase in current was statistically significant in 24/26 cells; Fig. 2*B*). Injecting water instead of Ins(1,3,4,5)P_4 did not produce a significant increase in

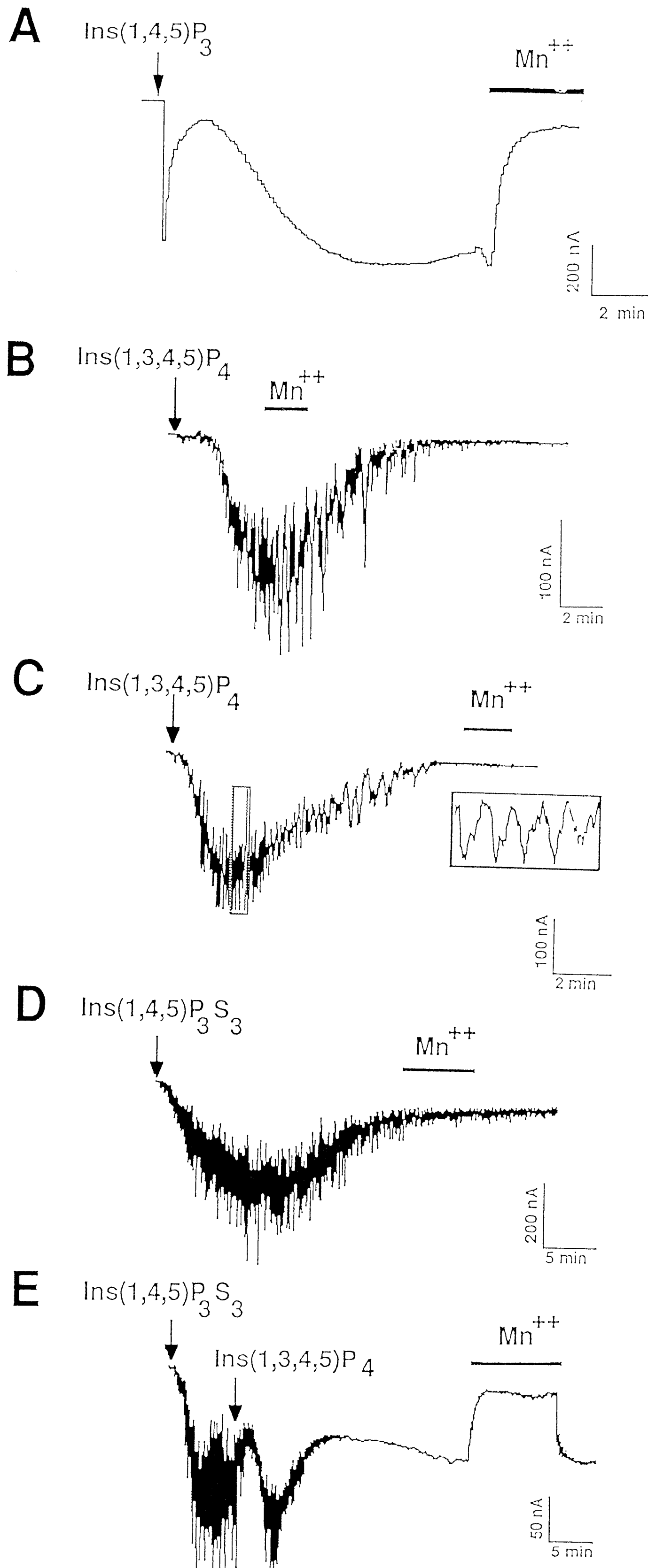

Fig. 1. Cl^- current response to intracellular injection of inositol phosphates (InsP) as a function of time. Inward current (downward deflection) represents an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Bar, addition of Mn^{2+} (4 mM) to extracellular bath. *A*: injection of D-*myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] (1 pmol) caused a brief increase in $[Ca^{2+}]_i$ followed by a more sustained increase in $[Ca^{2+}]_i$. Mn^{2+} (bar) blocked sustained increase in $[Ca^{2+}]_i$. Thus Ins(1,4,5)P_3 caused Ca^{2+} influx. *B* and *C*: injection of D-*myo*-inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P_4] (10 pmol). To show resulting $[Ca^{2+}]_i$ oscillations more clearly, we have expanded time scale of boxed area (insert in *C*). Addition of Mn^{2+} during $[Ca^{2+}]_i$ oscillations (*B*) or following the disappearance of $[Ca^{2+}]_i$ oscillations (*C*) did not change the average Cl^- current. Thus Ins(1,3,4,5)P_4 alone did not cause Ca^{2+} influx. *D*: injection of Ins(1,4,5)P_3S_3 (10 pmol) caused prolonged $[Ca^{2+}]_i$ oscillations. Addition of Mn^{2+} (bar) did not cause any change in average current. Thus D-*myo*-inositol 1,4,5-trisphosphorothioate [Ins(1,4,5)P_3S_3] alone did not cause Ca^{2+} influx. *E*: injection of Ins(1,4,5)P_3S_3 (10 pmol) followed by Ins(1,3,4,5)P_4 (10 pmol). In this case, a nonoscillatory current developed after $[Ca^{2+}]_i$ oscillations had stopped. Addition of Mn^{2+} (bar) caused a reversible decrease in Cl^- current. Thus Ins(1,3,4,5)P_4 stimulated influx of extracellular Ca^{2+} in the presence of Ins(1,4,5)P_3S_3.

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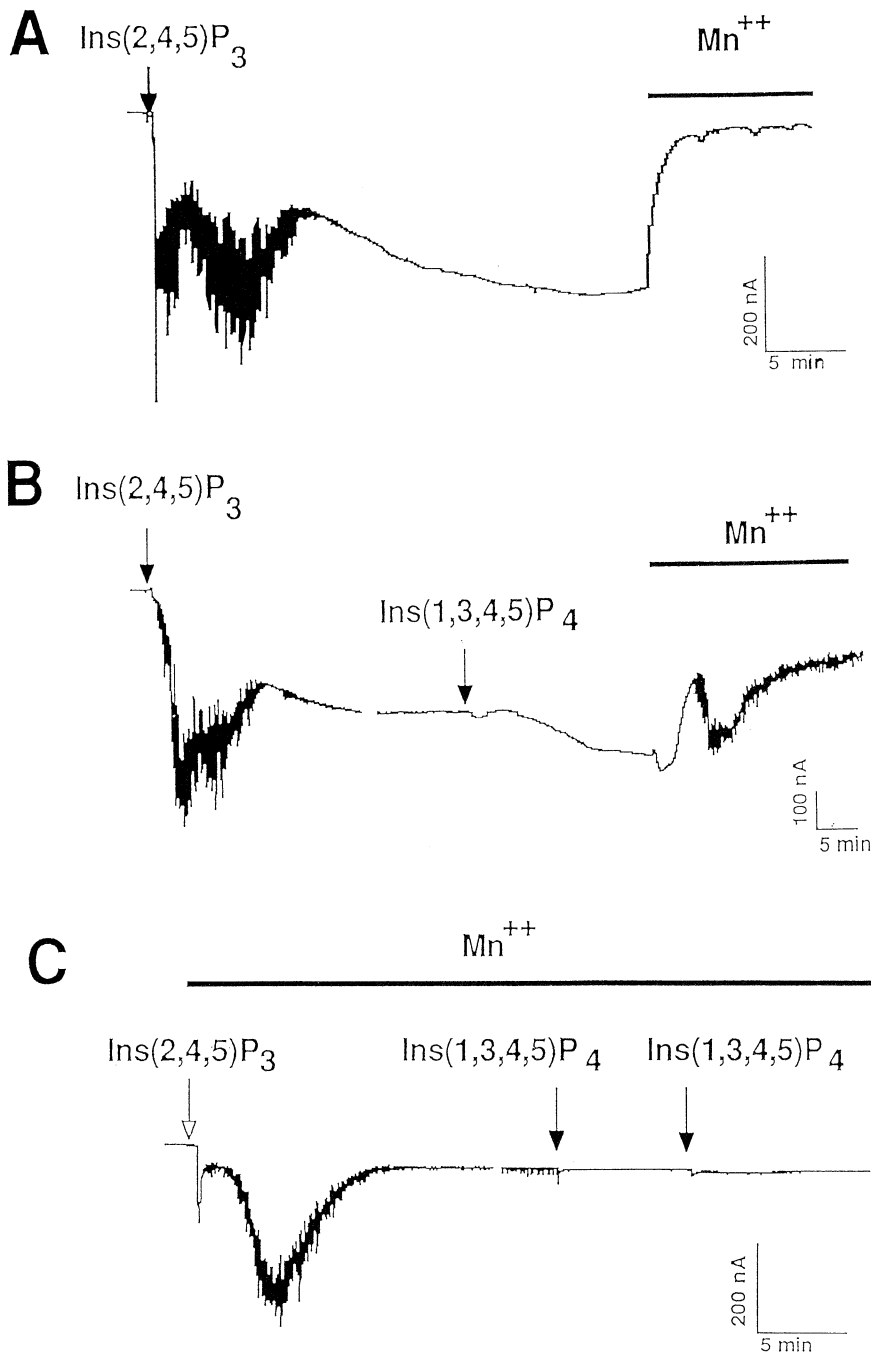

Fig. 2. *A*: injection of D-*myo*-inositol 2,4,5-trisphosphate [Ins(2,4,5)P_3] (10 pmol) caused $[Ca^{2+}]_i$ oscillations followed by a gradual increase in nonoscillatory current. This current could be blocked by adding Mn^{2+} (bar). Thus Ins(2,4,5)P_3 caused Ca^{2+} influx. *B*: injection of Ins(2,4,5)P_3 (10 pmol) followed by Ins(1,3,4,5)P_4 (10 pmol). Ins(1,3,4,5)P_4 caused further increase in Cl^- current. Mn^{2+} (bar) blocked nonoscillatory current, and $[Ca^{2+}]_i$ oscillations resumed. Because resulting oscillatory current did not go back to baseline, this experiment did not rule out the possibility that Ins(1,3,4,5)P_4 released intracellular Ca^{2+} instead of causing Ca^{2+} influx. *C*: same experiment as *B* except that Mn^{2+} was present throughout experiment, preventing Ca^{2+} influx. After Ins(2,4,5)P_3 (10 pmol), Ins(1,3,4,5)P_4 (10 pmol) had no further effect on $[Ca^{2+}]_i$. This indicated that Ins(1,3,4,5)P_4-induced increase in Cl^- current seen in *B* was due to Ca^{2+} influx (see text).

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Cl^- current ($n = 5$). The difference between the effect of Ins(1,3,4,5)P_4 and control injections was statistically significant ($P < 0.001$). These results contrast with previous work in rat hepatocytes that suggested that a predominant effect of Ins(1,3,4,5)P_4 was to stimulate reuptake of free Ca^{2+} back into intracellular stores (18). If that had been the case, Ins(1,3,4,5)P_4 would have reduced the Cl^- current caused by Ins(2,4,5)P_3; we found the opposite.

To determine whether the Ins(1,3,4,5)P_4-induced increase in Cl^- current was due to intracellular Ca^{2+} release and/or to Ca^{2+} influx, we used the same experimental protocol [i.e., injection of Ins(2,4,5)P_3 followed by an injection of Ins(1,3,4,5)P_4] but this time in the continuous presence of extracellular Mn^{2+}. Under these conditions, the Ins(1,3,4,5)P_4-induced increase in Cl^- current would be inhibited if it was due to Ca^{2+} influx. Conversely, if Ins(1,3,4,5)P_4 released Ca^{2+} from intracellular stores, Cl^- current would increase. As previously reported (11, 36), Mn^{2+} did not inhibit the initial Ins(2,4,5)P_3-induced $[Ca^{2+}]_i$ oscillations but abolished Ins(2,4,5)P_3-induced Ca^{2+} influx (compare the tracings shown in Fig. 2*A* and Fig. 2*C*). As shown in Fig. 2*C*, subsequent injection of Ins(1,3,4,5)P_4 did not increase Cl^- current (4/4 cells). This indicates that Ins(1,3,4,5)P_4 does not release intracellular Ca^{2+} when injected after Ins(2,4,5)P_3; instead, it causes Ca^{2+} influx.

In four experiments, we injected increasing doses of Ins(1,3,4,5)P_4 after Ins(2,4,5)P_3. As the dose of injected Ins(1,3,4,5)P_4 increased, the magnitude of Ca^{2+} influx increased (Fig. 3). These results suggest that when pre-

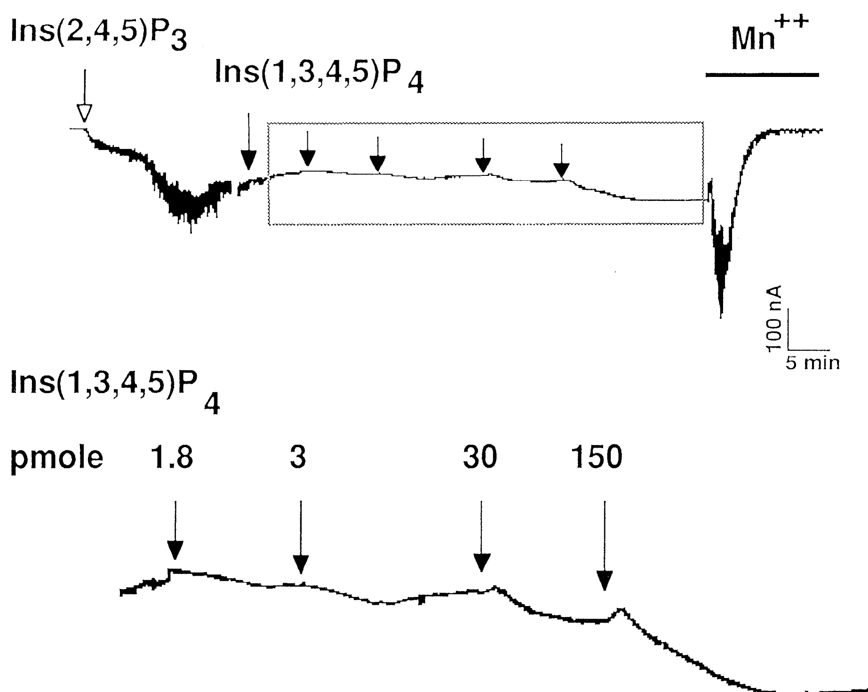

Fig. 3. Injection of $Ins(2,4,5)P_3$ (10 pmol) followed by five sequential injections of $Ins(1,3,4,5)P_4$ (doses of 0.3, 1.8, 3, 30, and 150 pmol at closed arrows) (*top trace*). First $Ins(1,3,4,5)P_4$ injection inhibited $[Ca^{2+}]_i$ oscillations. Magnitude of $Ins(1,3,4,5)P_4$-induced extracellular Ca^{2+} influx increased with amount of $Ins(1,3,4,5)P_4$ injected. Note that Mn^{2+} caused a recurrence of $[Ca^{2+}]_i$ oscillations before blocking current. Area enclosed by box is enlarged in *bottom trace*.

ceded by $Ins(2,4,5)P_3$, $Ins(1,3,4,5)P_4$ stimulates Ca^{2+} influx in a dose-dependent manner.

We considered the possibility that it is the increase in $[Ca^{2+}]_i$ produced by $Ins(1,4,5)P_3S_3$ and $Ins(2,4,5)P_3$ that provides $Ins(1,3,4,5)P_4$ with the ability to stimulate Ca^{2+} influx. We did not think that this possibility was very likely because $Ins(1,3,4,5)P_4$ itself increases $[Ca^{2+}]_i$ but does not cause Ca^{2+} influx. To test this possibility further, we injected $CaCl_2$ in doses expected to increase the average $[Ca^{2+}]_i$ to 25 μM [in *Xenopus* oocytes, $Ins(1,4,5)P_3$ stimulation increases $[Ca^{2+}]_i$ to 0.25–1 μM (6, 17)]. Subsequent injection of $Ins(1,3,4,5)P_4$ caused $[Ca^{2+}]_i$ oscillations but failed to cause Ca^{2+} influx (8 cells) (Fig. 4). Thus an increase in $[Ca^{2+}]_i$ is not sufficient to induce $Ins(1,3,4,5)P_4$-dependent Ca^{2+} influx. The presence of $InsP_3$ appears to be required.

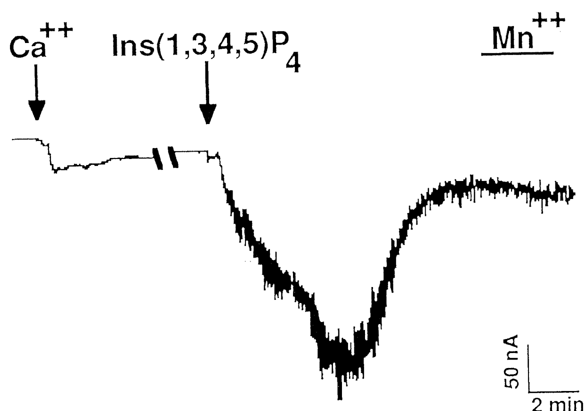

Fig. 4. Injection of $CaCl_2$ (5 μM) followed by an injection of $Ins(1,3,4,5)P_4$. Absence of Mn^{2+}-inhibitable current indicates that an increase in $[Ca^{2+}]_i$ was not sufficient to confer $Ins(1,3,4,5)P_4$ with ability to cause Ca^{2+} influx. Changes in Ca^{2+}-gated Cl^--current induced by injections of a given volume and concentration of $CaCl_2$ varied widely from cell to cell [218 ± 78 (SE) nA, range from 28 to 503 nA, $n = 8$; Ref. 10]. Discrepancy between magnitude of current responses to $CaCl_2$ and to $Ins(1,3,4,5)P_4$ was not consistent.

Effect of Ca^{2+} influx on $Ins(1,3,4,5)P_4$ generation. Once formed following membrane-receptor activation, $Ins(1,4,5)P_3$ follows two main metabolic pathways. In one pathway, it is dephosphorylated to $Ins(1,4)P_2$ by the $Ins(1,4,5)P_3$ 5-phosphatase (12). In a second pathway, it is phosphorylated to $Ins(1,3,4,5)P_4$ by the Ca^{2+}/calmodulin-dependent $Ins(1,4,5)P_3$ 3-kinase (4, 8, 39). The second pathway might be favored by the cell, in part, because the $Ins(1,4,5)P_3$-induced increase in $[Ca^{2+}]_i$ would activate the 3-kinase and not the 5-phosphatase (3, 32). Because Ca^{2+} influx increases $[Ca^{2+}]_i$, we hypothesized that it might shunt the metabolism of $Ins(1,4,5)P_3$ toward the formation of $Ins(1,3,4,5)P_4$. To examine the effect of Ca^{2+} influx on the metabolism of $Ins(1,4,5)P_3$, we injected $[^3H]Ins(1,4,5)P_3$ into single *Xenopus* oocytes ($2–20 \times 10^3$ counts · min^{-1} · $cell^{-1}$) incubated in the presence or absence of extracellular Ca^{2+}. We then stopped InsP metabolism at predetermined time points. In cells with time points ≥1 min ($n = 56$), we recorded the electrophysiological response and found typical changes in $[Ca^{2+}]_i$ (Fig. 1*A*). In cells incubated in the absence of extracellular Ca^{2+}, the sustained increase in $[Ca^{2+}]_i$ shown in Fig. 1*A* was not present. HPLC separation of the $[^3H]InsP$ was performed for each individual cell ($n = 72$).

By 40 min after injection, $[^3H]Ins(1,4,5)P_3$ was almost completely metabolized (Fig. 5*A*). The rate of $Ins(1,4,5)P_3$ metabolism was comparable to that reported by Irvine et al. (20) and was not significantly influenced by extracellular Ca^{2+} (Fig. 5*A*).

The predominant $InsP_4$ isomer recovered was $Ins(1,3,4,5)P_4$. In the presence of extracellular Ca^{2+}, the maximal accumulation of $[^3H]Ins(1,3,4,5)P_4$ counts [11.9% of total disintegrations/min (dpm) injected] occurred 3 min after $[^3H]Ins(1,4,5)P_3$ injection (Fig. 5*B*, open circles). In the absence of extracellular Ca^{2+} (Fig. 5*B*, closed circles), the maximum increase in

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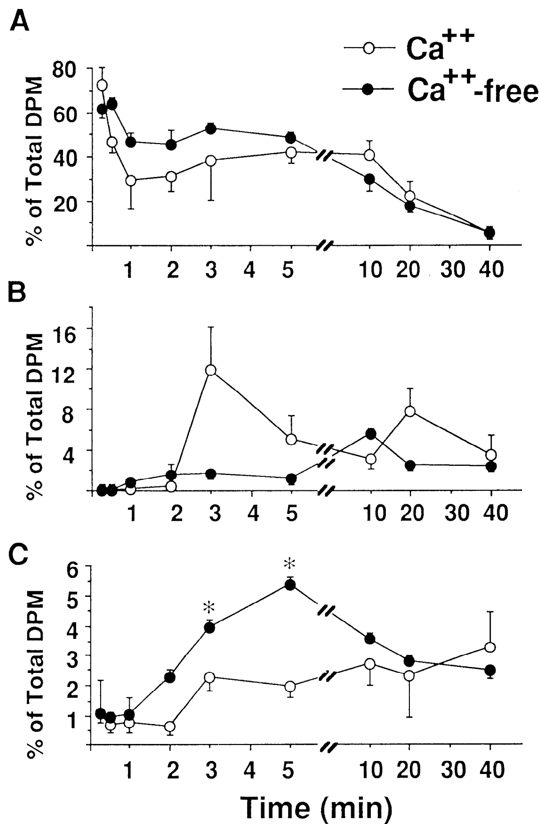

Fig. 5. Effect of Ca^{2+} influx on immediate metabolism of Ins(1,4,5)P_3 in single *Xenopus* oocytes. Cells were injected with [^{3}H]Ins(1,4,5)P_3 [2,000–20,000 disintegrations/min (dpm)] in the presence (open circles) or absence (closed circles) of extracellular Ca^{2+}. Cellular metabolism was then arrested at various time points and InsPs were separated by high-pressure liquid chromatography (see MATERIALS AND METHODS). For each time point, 3–5 individual cells were studied in presence of extracellular Ca^{2+} and 2–3 cells in absence of extracellular Ca^{2+} (total of 72 cells). Radioactivity specific to each InsP was expressed as a percentage of total injected dpm. Value recorded at each time point represented mean ± SE of radioactivity attributable to Ins(1,4,5)P_3 (*A*), Ins(1,3,4,5)P_4 (*B*), or Ins(1,4)P_2 (*C*). In *A*, extracellular [Ca^{2+}] had no effect on gradual metabolism of Ins(1,4,5)P_3. In *B*, generation of Ins(1,3,4,5)P_4 was increased in presence of extracellular Ca^{2+} (open circles). In *C*, production of Ins(1,4)P_2 was decreased in presence of extracellular Ca^{2+} (open circles). * Statistically different ($P < 0.05$). These results suggest that Ca^{2+} influx shunted metabolism of Ins(1,4,5)P_3 toward formation of Ins(1,3,4,5)P_4 (see text).

[^{3}H]Ins(1,3,4,5)P_4 was smaller (5.6% of total dpm injected) and occurred later (10 min). These results suggest that more Ins(1,3,4,5)P_4 is formed from Ins(1,4,5)P_3 in the presence of extracellular Ca^{2+}.

We also recovered [^{3}H]Ins(1,3,4)P_3, the direct metabolite of Ins(1,3,4,5)P_4. [^{3}H]Ins(1,3,4)P_3 counts began to increase 3 min after the [^{3}H]Ins(1,4,5)P_3 injection, peaked at 20 min and decreased thereafter (not shown). [^{3}H]Ins(1,3,4,6)P_4 was recovered in two cells incubated in the presence of extracellular Ca^{2+}, one 10 min and the other 20 min after injection of [^{3}H]Ins(1,4,5)P_3. In these two cells, there was also maximal accumulation of Ins(1,3,4)P_3, the proposed precursor of Ins(1,3,4,6)P_4 (1, 35). We did not recover [^{3}H]Ins(3,4,5,6)P_4; to do so would have required long-term labeling of inositol phospholipids (20, 35). These data are nevertheless consistent with the notion that Ins(3,4,5,6)P_4 is not an immediate intracellular second messenger (24).

The product of the Ins(1,4,5)P_3 5′-phosphatase, Ins(1,4)P_2, was the only InsP_2 isomer recovered. The effect of extracellular Ca^{2+} on [^{3}H]Ins(1,4)P_2 levels was opposite to its effect on [^{3}H]Ins(1,3,4,5)P_4 levels. In the presence of extracellular Ca^{2+}, [^{3}H]Ins(1,4)P_2 counts began to rise at 3 minutes and gradually increased to a maximum (3.3% of initial dpm) throughout the 40-min time course (Fig. 5*C*, open circles). In the absence of extracellular Ca^{2+}, [^{3}H]Ins(1,4)P_2 counts reached their maximum (5.3% of initial dpm) 5 min after injection and then gradually decreased (Fig. 5*C*, closed circles). These results suggest that less Ins(1,4)P_2 is formed from Ins(1,4,5)P_3 in the presence of extracellular Ca^{2+}.

The data represent the proportion of the initial radioactivity recovered as [^{3}H]Ins(1,3,4,5)P_4 and [^{3}H]Ins-(1,4)P_2 at given points in time and not the overall mass of these metabolites. Within individual cells, however, the ratios of Ins(1,3,4,5)P_4/Ins(1,4)P_2 counts were different in the presence or absence of extracellular Ca^{2+} (Table 1). The presence of Ca^{2+} produced a 10-fold increase in the [^{3}H]Ins(1,3,4,5)P_4/Ins(1,4,5)P_3 ratio. Despite the limits of our method, these results suggest that Ins(1,4,5)P_3 is preferentially metabolized to Ins(1,3,4,5)P_4 in the presence of extracellular Ca^{2+}.

Summary and implications. Our results indicate that Ins(1,3,4,5)P_4 does not cause Ca^{2+} influx by itself but does stimulate Ca^{2+} influx when preceded by poorly metabolizable InsP_3. These results suggest a synergistic action of InsP_3 and Ins(1,3,4,5)P_4 to cause Ca^{2+} influx (7, 21, 22, 27).

Our results show that two poorly metabolizable InsP_3s had different effects; Ins(2,4,5)P_3 stimulated Ca^{2+} influx, whereas Ins(1,4,5)P_3S_3 did not. A previous report has suggested that the two analogues also generate different patterns of intracellular Ca^{2+} release (14). We do not think that the difference between Ins(2,4,5)P_3 and Ins(1,4,5)P_3S_3 is caused by a different metabolism because we have previously shown that neither compound is metabolized significantly in the oocyte (11). Because Ins(2,4,5)P_3 alone causes Ca^{2+} influx, we previously argued that Ins(1,3,4,5)P_4 is not required to cause Ca^{2+} influx (36). The finding that Ins(1,4,5)P_3S_3 does not cause Ca^{2+} influx could lead to the opposite interpretation, i.e., that Ins(1,3,4,5)P_4 is required for Ca^{2+} influx. Until we know which poorly metabolizable compound best reproduces the action of Ins(1,4,5)P_3, we may have to

Table 1. *Effect of Ca^{2+} influx on the metabolism of Ins(1,4,5)P_3 in single Xenopus oocytes: ratios of Ins(1,3,4,5)P_4 to Ins(1,4)P_2 within individual cells*

Time, min	[Ca^{2+}]$_o$	InsP_4/InsP_2 ratios	*n*
3	nominal	0.4±0.1*	3
3	6 mM	4.8±0.1*	4
5	nominal	0.25±0.05	2
5	6 mM	2.1±0.6	3

Three and five minutes following the injection of [^{3}H]Ins(1,4,5)P_3, ratio of recovered [^{3}H]Ins(1,3,4,5)P_4 and [^{3}H]Ins(1,4)P_2 was calculated for each *Xenopus* oocyte. Oocytes were bathed with high (6 mM) or low (nominal) extracellular Ca^{2+} concentration ([Ca^{2+}]$_o$). Values are expressed as means ± SE. * Value at 6 mM [Ca^{2+}]$_o$ statistically different ($P < 0.05$) from value at nominal [Ca^{2+}]$_o$.

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use alternative strategies to resolve the controversy about the absolute requirement of Ins(1,3,4,5)P_4 for Ca^{2+} influx (19). Nevertheless, we observed that Ins(1,3,4,5)P_4 could stimulate Ca^{2+} influx when preceded by either Ins(2,4,5)P_3 or Ins(1,4,5)P_3S_3. Thus our data suggest that Ins(1,3,4,5)P_4 could regulate Ca^{2+} influx, even if it was not absolutely required.

Because of the unavailabilty of nonmetabolizable $InsP_4$, we cannot rule out the possibility that a metabolite of Ins(1,3,4,5)P_4 is also involved in stimulating Ca^{2+} influx. Ivorra et al. (23) have recently shown that Ins(1,3,4,6)P_4 causes both intracellular Ca^{2+} release and Ca^{2+} influx in *Xenopus* oocytes. We have recovered Ins(1,3,4,6)P_4 only in cells incubated in the presence of extracellular Ca^{2+}. Therefore, the effect of Ca^{2+} influx on the metabolism of Ins(1,4,5)P_3 may promote the formation of more than one compound active in Ca^{2+} homeostasis.

Our data also suggest that Ca^{2+} influx can shunt the metabolism of Ins(1,4,5)P_3 toward the formation of Ins(1,3,4,5)P_4 and away from dephosphorylation to Ins(1,4)P_2. Because we performed our experiments in intact cells, our data suggest that the known Ca^{2+} dependence of the Ins(1,4,5)P_3 3-kinase enzyme is physiologically relevant. Ca^{2+} influx increased Ins(1,3,4,5)P_4 levels over and above those found in cells where Ins(1,4,5)P_3-induced release of intracellular Ca^{2+} had occurred. Thus the Ins(1,4,5)P_3 3-kinase may require sustained high levels of $[Ca^{2+}]_i$ to remain activated. There may therefore be a positive feedback regulatory mechanism by which Ca^{2+} influx increases the amounts of Ins(1,3,4,5)P_4 produced from Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 stimulates Ca^{2+} influx. However, the physiological contribution of this mechanism to the regulation of Ca^{2+} influx remains to be determined.

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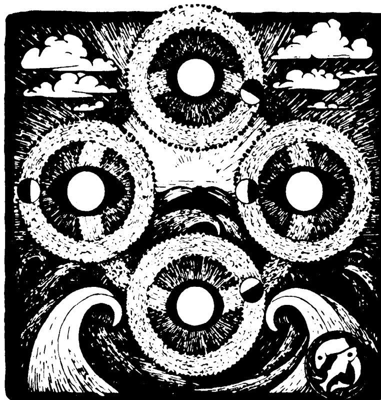

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