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InsP₃ and Ins(1,3,4,5)P₄ act in synergy to stimulate influx of extracellular Ca²⁺ in Xenopus oocytes

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Delisle, Sylvain, Didier Pittet, Barry V. L. Potter, P. Daniel Lew, and Michael J. Welsh. InsP₃ and Ins(1,3,4,5)P₄ act in synergy to stimulate influx of extracellular Ca²⁺ 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-tetakisphosphate [Ins(1,3,4,5)P₄] in the regulation of Ca²⁺ influx, we injected inositol phosphates into Xenopus oocytes and measured Ca²⁺-gated Cl⁻ current to assay intracellular free Ca²⁺ concentration ([Ca²⁺]ᵢ). To assess Ca²⁺ influx, we removed extracellular Ca²⁺ or added the inorganic Ca²⁺ channel blocker Mn²⁺ to the extracellular bath and measured the resulting change in Cl⁻ current. Ins(1,3,4,5)P₄ did not cause Ca²⁺ influx when injected alone or when preceded by an injection of Ca²⁺. In contrast, Ins(1,3,4,5)P₄ stimulated Ca²⁺ influx when injected after the poorly metabolized inositol trisphosphate (InsP₃) analogues d-myo-inositol 1,4,5-trisphosphorothioate [Ins(1,4,5)P₃S₂] or d-myo-inositol 2,4,5-trisphosphate [Ins(2,4,5)P₃]. These results indicate that Ins(1,3,4,5)P₄ is not sufficient to stimulate Ca²⁺ influx but acts in synergy with InsP₃ to cause Ca²⁺ influx. We also studied the effect of Ca²⁺ influx on the immediate metabolism of d-myo-inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃] in single oocytes. Ca²⁺ influx shunted the metabolism of Ins(1,4,5)P₃ toward the formation of Ins(1,3,4,5)P₄ and away from d-myo-inositol 1,4-bisphosphate [Ins(1,4)P₂]. These results suggest that there is a positive feedback regulatory mechanism in which Ca²⁺ influx stimulates Ins(1,3,4,5)P₄ production and Ins(1,3,4,5)P₄ stimulates further Ca²⁺ influx.

Inositol trisphosphate; d-myo-inositol 1,3,4,5-tetakisphosphate; 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)P₃]. By opening Ca²⁺ channels, Ins(1,4,5)P₃ releases Ca²⁺ from intracellular stores (2, 15, 34). Ins(1,4,5)P₃ also causes a sustained influx of extracellular Ca²⁺ into the cell (2). We do not know how Ins(1,4,5)P₃ regulates Ca²⁺ influx (33).

Intracellular injection of Ins(1,4,5)P₃ can stimulate Ca²⁺ influx (7, 11, 21, 22, 27, 30, 36). However, because Ins(1,4,5)P₃ is extensively metabolized by the cell, inositol phosphates (InsP) other than Ins(1,4,5)P₃ may control Ca²⁺ influx. D-Myo-inositol 1,3,4,5-tetakisphosphate [Ins(1,3,4,5)P₄] is a direct metabolite of Ins(1,4,5)P₃ that may also release intracellular Ca²⁺ (16, 36). Ins(1,3,4,5)P₄ has been implicated in the regulation of Ca²⁺ influx in some cellular systems (7, 21, 22, 27, 31) but not in others (30, 36). The controversy surrounding the requirement of Ins(1,3,4,5)P₄ for Ca²⁺ 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)P₄ was required for Ca²⁺ influx (27), and the results of the other study suggested it was not (5). In the present studies, we investigate how inositol trisphosphates (InsP₃) and Ins(1,3,4,5)P₄ control Ca²⁺ influx in Xenopus oocytes.

We show that Ins(1,3,4,5)P₄ stimulates Ca²⁺ influx in the presence but not in the absence of poorly metabolizable InsP₃. In addition, we tested the possibility that Ca²⁺ influx may stimulate the phosphorylation of Ins(1,4,5)P₃ into Ins(1,3,4,5)P₄. Our observations suggest a positive feedback mechanism, whereby Ins(1,3,4,5)P₄ stimulates Ca²⁺ influx and Ca²⁺ influx promotes formation of Ins(1,3,4,5)P₄.

MATERIALS AND METHODS

Electrophysiology and intracellular injections. We obtained Xenopus oocytes as described previously (11, 36) and defolliculated them manually. To assay intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ), we measured Ca²⁺-activated Cl⁻ currents with the two-electrode voltage-clamp technique. This assay has been validated using both Ca²⁺ electrodes and fluorescent calcium indicators (10). Oocytes were impaled with two microelectrodes (3 M KC1 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 (Maelab, 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 (PL1-100, 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 back-filled with Ins(1,3,4,5)P₄ (Calbiochem, San Diego, CA), Ins(1,4,5)P₃ (Amersham, Arlington Heights, IL), ³H-labeled Ins(1,4,5)P₃ (Amersham), d-myo-inositol 2,4,5-trisphosphate [Ins(2,4,5)P₃] (Boehringer Mannheim Biochemicals, Indianapolis, IN), d-myo-inositol 3,4,5,6-tetakisphosphate [Ins(3,4,5,6)P₄] (Boehringer Mannheim), d-myo-inositol 1,4,5-trisphosphorothioate [Ins(1,4,5)P₃S₂] (prepared as previously described (9)), or CaCl₂ (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\textsuperscript{2+} influx measurements. For most experiments, oocytes were initially stimulated in a bath solution containing (in mM) 116 NaCl, 2 KCl, 6 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, and 5 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.4. We assessed Ca\textsuperscript{2+} influx by measuring the change in Cl\textsuperscript{−} current induced by either removing external Ca\textsuperscript{2+} or adding the inorganic Ca\textsuperscript{2+} channel blocker Mn\textsuperscript{2+} (4 mM) to the bath solution. For most experiments, we added Mn\textsuperscript{2+} rather than removing bath Ca\textsuperscript{2+} because membrane electrical resistance often decreased with time in the absence of external Ca\textsuperscript{2+} (10). Because we have previously found that Ca\textsuperscript{2+} influx usually occurs after the disappearance of [Ca\textsuperscript{2+}]i oscillations (11, 36), our standard procedure was to add Mn\textsuperscript{2+} during the nonoscillatory current that followed the disappearance of [Ca\textsuperscript{2+}]i oscillations. Occasionally, the injected InsP caused prolonged [Ca\textsuperscript{2+}]i oscillations. In such cases, we added Mn\textsuperscript{2+} when the baseline current (the average current around which the oscillations occurred) had stabilized. To determine if the addition of Mn\textsuperscript{2+} (or the removal of extracellular Ca\textsuperscript{2+}) caused a change in Cl\textsuperscript{−} current, we extracted the digitized current values for the 60-s time period immediately preceding the addition of Mn\textsuperscript{2+} and the current values for the 60-s time period beginning 1 min after the addition of Mn\textsuperscript{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\textsuperscript{2+} using the Student’s t test for paired data. We interpreted a statistically significant decrease in the mean Cl\textsuperscript{−} current caused by Mn\textsuperscript{2+} as positive evidence of Ca\textsuperscript{2+} influx (11, 30). The absolute difference between the means was taken as a semiquantitative index of Ca\textsuperscript{2+} influx.

Ins(1,4,5)P\textsubscript{3} metabolism in single oocytes. To measure the metabolism of Ins(1,4,5)P\textsubscript{3}, we injected [H]Ins(1,4,5)P\textsubscript{3} into single oocytes and arrested InsP metabolism at fixed time points. The injectate contained 5 μCi of lyophilized [H]Ins(1,4,5)P\textsubscript{3} (sp act 20–60 Ci/mmol) mixed with 10 μl of cold Ins(1,4,5)P\textsubscript{3} (10−4 M). Total calculated concentration of Ins(1,4,5)P\textsubscript{3} (cold and [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\textsubscript{3} concentration was 1.1 μM, i.e., comparable with what we have used in the past to cause Ca\textsuperscript{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 (14°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., after 40 min, the injection pipette was reinserted into the oocyte and the intracellular InsP concentration was measured a second time. The sum of InsP in the injection pipette and the intracellular pool was taken as the initial intracellular InsP concentration. The ATP used in the injection pipette was depleted during the 5-min time period, and ATP was measured using scintillation counting. This HPLC procedure was optimized for the separation of the three distinct inositol tetrakisphosphates: d-myo-inositol 1,3,4,6-tetrasphosphate [Ins(1,3,4,6)P\textsubscript{4}], Ins(1,3,4,5)P\textsubscript{4}, and Ins(3,4,5,6)P\textsubscript{4} (31). [H]-labeled d-myo-inositol 1,4-bisphosphate [Ins(1,4)P\textsubscript{2}], Ins(1,4,5)P\textsubscript{3}, and Ins(1,3,4,5)P\textsubscript{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\textsubscript{4} on Ca\textsuperscript{2+} influx. Injection of 1 pmol of Ins(1,4,5)P\textsubscript{3} (estimated intracellular concentration of 1 μM) into Xenopus oocytes caused a biphasic increase in [Ca\textsuperscript{2+}]i (Fig. 1A). We have previously shown that the initial, transient increase in [Ca\textsuperscript{2+}]i represents the release of Ca\textsuperscript{2+} from intracellular stores and is not affected by extracellular Ca\textsuperscript{2+} (36). The more sustained increase in [Ca\textsuperscript{2+}]i, that follows can be blocked either by removing extracellular Ca\textsuperscript{2+} or by adding Mn\textsuperscript{2+} (4 mM) to the external bath (Fig. 1A). This result indicates that the sustained increase in [Ca\textsuperscript{2+}]i is due to the influx of extracellular Ca\textsuperscript{2+}. Thus, as previously reported in Xenopus oocytes (25, 36), Ins(1,4,5)P\textsubscript{3} causes Ca\textsuperscript{2+} influx.

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

The synthetic Ins(1,4,5)P\textsubscript{3} analogue, Ins(1,4,5)P\textsubscript{2}S\textsubscript{3} (37), is a poor substrate for the Ins(1,4,5)P\textsubscript{3} 3-kinase (38), the enzyme responsible for the conversion of Ins(1,4,5)P\textsubscript{3} to Ins(1,3,4,5)P\textsubscript{4}. As previously observed (11, 37), injection of 10 pmol of Ins(1,4,5)P\textsubscript{3}S\textsubscript{3} (estimated intracellular concentration of 10 μM) caused prolonged [Ca\textsuperscript{2+}]i oscillations (30/30 cells) (Fig. 1D). Removal of Ca\textsuperscript{2+} from the bath or addition of Mn\textsuperscript{2+} (4 mM) did not change the average Cl\textsuperscript{−} current response (14/14 cells) (Fig. 1D). This result indicates that Ins(1,4,5)P\textsubscript{3}S\textsubscript{3} does not cause Ca\textsuperscript{2+} influx. To mimic the physiological sequence where Ins(1,4,5)P\textsubscript{3}S\textsubscript{3} is metabolized to Ins(1,3,4,5)P\textsubscript{4}, we first injected Ins(1,4,5)P\textsubscript{3}S\textsubscript{3} (10 pmol) and then Ins(1,3,4,5)P\textsubscript{4} (10 pmol) in the same cell. When injected after Ins(1,4,5)P\textsubscript{3}S\textsubscript{3}, Ins(1,3,4,5)P\textsubscript{4} stimulated a Cl\textsuperscript{−} current that could be inhibited by removing extracellular Ca\textsuperscript{2+} or by adding Mn\textsuperscript{2+} (4 mM) to the bath solution (11/13 cells) (Fig. 1E). These results suggest that the combination of...
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 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 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. 2A 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. 2B). 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+} (bar) 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.
**Fig. 2.** A: injection of d-myo-inositol 2,4,5-trisphosphate [Ins(2,4,5)P₃] (10 pmol) caused [Ca²⁺]ᵢ oscillations followed by a gradual increase in nonoscillatory current. This current could be blocked by adding Mn²⁺ (bar). Thus Ins(2,4,5)P₃ caused Ca²⁺ influx. B: injection of Ins(2,4,5)P₃ (10 pmol) followed by Ins(1,3,4,5)P₄ (10 pmol). Ins(1,3,4,5)P₄ caused further increase in Cl⁻ current. Mn²⁺ (bar) blocked nonoscillatory current, and [Ca²⁺]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)P₄ released intracellular Ca²⁺ instead of causing Ca²⁺ influx. C: same experiment as B except that Mn²⁺ was present throughout experiment, preventing Ca²⁺ influx. After Ins(2,4,5)P₃ (10 pmol), Ins(1,3,4,5)P₄ (10 pmol) had no further effect on [Ca²⁺]ᵢ. This indicated that Ins(1,3,4,5)P₄-induced increase in Cl⁻ current seen in B was due to Ca²⁺ influx (see text).

Cl⁻ current (n = 5). The difference between the effect of Ins(1,3,4,5)P₄ 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)P₄ was to stimulate reuptake of free Ca²⁺ back into intracellular stores (18). If that had been the case, Ins(1,3,4,5)P₄ would have reduced the Cl⁻ current caused by Ins(2,4,5)P₃; we found the opposite.

To determine whether the Ins(1,3,4,5)P₄-induced increase in Cl⁻ current was due to intracellular Ca²⁺ release and/or to Ca²⁺ influx, we used the same experimental protocol [i.e., injection of Ins(2,4,5)P₃ followed by an injection of Ins(1,3,4,5)P₄] but this time in the continuous presence of extracellular Mn²⁺. Under these conditions, the Ins(1,3,4,5)P₄-induced increase in Cl⁻ current would be inhibited if it was due to Ca²⁺ influx. Conversely, if Ins(1,3,4,5)P₄ released Ca²⁺ from intracellular stores, Cl⁻ current would increase. As previously reported (11, 36), Mn²⁺ did not inhibit the initial Ins(2,4,5)P₃-induced [Ca²⁺]ᵢ oscillations but abolished Ins(2,4,5)P₃-induced Ca²⁺ influx (compare the tracings shown in Fig. 2A and Fig. 2C). As shown in Fig. 2C, subsequent injection of Ins(1,3,4,5)P₄ did not increase Cl⁻ current (4/4 cells). This indicates that Ins(1,3,4,5)P₄ does not release intracellular Ca²⁺ when injected after Ins(2,4,5)P₃; instead, it causes Ca²⁺ influx.

In four experiments, we injected increasing doses of Ins(1,3,4,5)P₄ after Ins(2,4,5)P₃. As the dose of injected Ins(1,3,4,5)P₄ increased, the magnitude of Ca²⁺ influx increased (Fig. 3). These results suggest that when pre-
INFLUX OF EXTRACELLULAR CA$^{2+}$ IN XENOPUS OOCYTES

The 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.

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 2 min (n = 56), we recorded the electrophysiological response and found typical changes in [Ca$^{2+}$]$_i$ (Fig. 1A). In cells incubated in the absence of extracellular Ca$^{2+}$, the sustained increase in [Ca$^{2+}$]$_i$ shown in Fig. 1A 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. 5A). 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. 5A).

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. 5B, open circles). In the absence of extracellular Ca$^{2+}$ (Fig. 5B, closed circles), the maximum increase in

We considered the possibility that it is the increase in [Ca$^{2+}$]$_i$ produced by Ins(l$$)P& and Ins(2,4,5)P, that provides Ins(l,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 $\mu$M [in Xenopus oocytes, Ins(1,4,5)P$_3$ stimulation increases [Ca$^{2+}$]$_i$ to 0.25-1 $\mu$M (6, 17)]. Subsequent injection of Ins(1,3,4,5)P$_4$ caused [Ca$^{2+}$]$_i$ oscillations but failed to cause Ca$^{2+}$ influx (Fig. 4). Thus an increase in [Ca$^{2+}$]$_i$ is not sufficient to induce Ins(l,3,4,5)P$_4$-dependent Ca$^{2+}$ influx. The presence of InsP$_3$ appears to be required.

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The product of the Ins(1,4,5)P₃ 5’-phosphatase, Ins(1,4,5)P₂, was the only InsP₂ isomer recovered. The effect of extracellular Ca²⁺ on [³H]Ins(1,4,5)P₃ levels was opposite to its effect on [³H]Ins(1,3,4,5)P₄ levels. In the presence of extracellular Ca²⁺, [³H]Ins(1,4,5)P₃ counts began to rise at 3 minutes and gradually increased to a maximum (33% of initial dpm) throughout the 40-min time course (Fig. 5C, open circles). In the absence of extracellular Ca²⁺, [³H]Ins(1,4,5)P₃ counts reached their maximum (53% of initial dpm) 5 min after injection and then gradually decreased (Fig. 5C, closed circles). These results suggest that less Ins(1,4,5)P₃ is formed from Ins(1,4,5)P₃ in the presence of extracellular Ca²⁺.

The data represent the proportion of the initial radioactivity recovered as [³H]Ins(1,3,4,5)P₄ and [³H]Ins(1,4,5)P₃ 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)P₄/Ins(1,4,5)P₃ were different in the presence or absence of extracellular Ca²⁺ (Table 1). The presence of Ca²⁺ produced a 10-fold increase in the [³H]Ins(1,3,4,5)P₄/Ins(1,4,5)P₃ ratio. Despite the limits of our method, these results suggest that Ins(1,4,5)P₃ is preferentially metabolized to Ins(1,3,4,5)P₄ in the presence of extracellular Ca²⁺.

**Summary and implications.** Our results indicate that Ins(1,3,4,5)P₄ does not cause Ca²⁺ influx by itself but does stimulate Ca²⁺ influx when preceded by poorly metabolizable InsP₃. These results suggest a synergistic action of InsP₃ and Ins(1,3,4,5)P₄ to cause Ca²⁺ influx (7, 21, 22, 27).

Our results show that two poorly metabolizable InsP₃ had different effects; Ins(2,4,5)P₃ stimulated Ca²⁺ influx, whereas Ins(1,4,5)P₃ did not. A previous report has suggested that the two analogues also generate different patterns of intracellular Ca²⁺ release (14). We do not think that the difference between Ins(2,4,5)P₃ and Ins(1,4,5)P₃ 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)P₃ alone causes Ca²⁺ influx, we previously argued that Ins(1,3,4,5)P₄ is not required to cause Ca²⁺ influx (36). The finding that Ins(1,4,5)P₃ does not cause Ca²⁺ influx could lead to the opposite interpretation, i.e., that Ins(1,3,4,5)P₄ is required for Ca²⁺ influx. Until we know which poorly metabolizable compound best reproduces the action of Ins(1,4,5)P₃, we may have to

<table>
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<th>Time, min</th>
<th>[Ca²⁺]₀</th>
<th>InsP₄/InsP₃ ratios</th>
<th>n</th>
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<tr>
<td>3</td>
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<td>0.4±0.1*</td>
<td>3</td>
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<tr>
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<td>2</td>
</tr>
<tr>
<td>5</td>
<td>5 mM</td>
<td>2.1±0.6</td>
<td>3</td>
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</table>

Three and five minutes following the injection of [³H]Ins(1,4,5)P₃, ratio of recovered [³H]Ins(1,3,4,5)P₄ and [³H]Ins(1,4,5)P₃ was calculated for each Xenopus oocyte. Oocytes were bathed with high (6 mM) or low (nominal) extracellular Ca²⁺ concentration ([Ca²⁺]₀). Values are expressed as means ± SE. * Value at 6 mM [Ca²⁺]₀, statistically different (P < 0.05) from value at nominal [Ca²⁺]₀.

Table 1. Effect of Ca²⁺ influx on the metabolism of Ins(1,3,4,5)P₃ in single Xenopus oocytes: ratios of Ins(1,3,4,5)P₄ to Ins(1,4,5)P₃ within individual cells

---

A

B

C

Fig. 5. Effect of Ca²⁺ influx on immediate metabolism of Ins(1,4,5)P₃ in single Xenopus oocytes. Cells were injected with [³H]Ins(1,4,5)P₃ [2,000–20,000 disintegrations/min (dpm)] in the presence (open circles) or absence (closed circles) of extracellular Ca²⁺. 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 Ca²⁺ and 2–3 cells in absence of extracellular Ca²⁺ (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)P₃ (A), Ins(1,3,4,5)P₄ (B), or Ins(1,4,5)P₃ (C). In A, extracellular [Ca²⁺] had no effect on gradual metabolism of Ins(1,4,5)P₃. In B, generation of Ins(1,3,4,5)P₄ was increased in presence of extracellular Ca²⁺ (open circles). In C, production of Ins(1,4,5)P₃ was decreased in presence of extracellular Ca²⁺ (open circles). * Statistically different (P < 0.05). These results suggest that Ca²⁺ influx shunted metabolism of Ins(1,4,5)P₃ toward formation of Ins(1,3,4,5)P₄ (see text).

[³H]Ins(1,3,4,5)P₄ was smaller (5.6% of total dpm injected) and occurred later (10 min). These results suggest that more Ins(1,3,4,5)P₄ is formed from Ins(1,4,5)P₃ in the presence of extracellular Ca²⁺.

We also recovered [³H]Ins(1,3,4)P₆, the direct metabolite of Ins(1,3,4,5)P₄. [³H]Ins(1,3,4)P₆ counts began to increase 3 min after the [³H]Ins(1,4,5)P₃ injection, peaked at 20 min and decreased thereafter (not shown). [³H]Ins(1,3,4,6)P₄ was recovered in two cells incubated in the presence of extracellular Ca²⁺, one 10 min and the other 20 min after injection of [³H]Ins(1,4,5)P₃. In these two cells, there was also maximal accumulation of Ins(1,3,4,5)P₆, the proposed precursor of Ins(1,3,4,5)P₄ (1, 35). We did not recover [³H]Ins(3,4,5,6)P₄; to do so would have required long-term labeling of inositol phospholipids (20, 36). These data are nevertheless consistent with the notion that Ins(3,4,5,6)P₄ is not an immediate intracellular second messenger (24).
use alternative strategies to resolve the controversy about the absolute requirement of Ins(1,3,4,5)P₄ for Ca²⁺ influx (19). Nevertheless, we observed that Ins(1,3,4,5)P₄ could stimulate Ca²⁺ influx when preceded by either Ins(2,4,5)P₃ or Ins(1,4,5)P₃S₂. Thus our data suggest that Ins(1,3,4,5)P₄ could regulate Ca²⁺ influx, even if it was not absolutely required.

Because of the unavailability of nonmetabolizable InsP₄, we cannot rule out the possibility that a metabolite of Ins(1,3,4,5)P₄ is also involved in stimulating Ca²⁺ influx. Ivorra et al. (23) have recently shown that Ins(1,3,4,6)P₄ causes both intracellular Ca²⁺ release and Ca²⁺ influx in Xenopus oocytes. We have recovered Ins(1,3,4,6)P₄ only in cells incubated in the presence of extracellular Ca²⁺. Therefore, the effect of Ca²⁺ influx on the metabolism of Ins(1,4,5)P₃ may promote the formation of more than one compound active in Ca²⁺ homeostasis.

Our data also suggest that Ca²⁺ influx can shunt the metabolism of Ins(1,4,5)P₃ toward the formation of Ins(1,3,4,5)P₄ and away from dephosphorylation to Ins(1,4)P₂. Because we performed our experiments in intact cells, our data suggest that the known Ca²⁺-induced release of the Ins(1,4,5)P₃-kinase may require sustained high levels of InsP₄. Indeed, InsP₄ may remain activated. There may therefore be a positive feedback regulatory mechanism by which Ca²⁺ influx increases the amounts of Ins(1,3,4,5)P₄ produced from Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ stimulates Ca²⁺ influx. However, the physiological contribution of this mechanism to the regulation of Ca²⁺ influx remains to be determined.

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