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

SYLVAIN DELISLE, DIDIER PITTET, BARRY V. I. POTTER, P. DANIEL LEW, AND MICHAEL J. WELSH
Howard Hughes Medical Institute, Departments of Internal Medicine and Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, Iowa 52242, Infectious Diseases Division, University Hospital, CH-1211 Geneva 4, Switzerland; and School of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, United Kingdom

Delisle, Sylvain, Didier Pittet, Barry V. I. 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-trisphosphate [Ins(1,4,5)P₃] 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.

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-trisphosphate [Ins(1,4,5)P₃] 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.

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 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 (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 (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 back-filled with Ins(1,3,4,5)P₃ (Calbiochem, San Diego, CA), Ins(1,4,5)P₃ (Amersham, Arlington Heights, IL), and 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 1,3,4,5-triphosphorothioate [Ins(1,3,4,5)P₃S₃] (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.
**INFLUX OF EXTRACELLULAR Ca\(^{2+}\) IN XENOPUS OOCYTES**

**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 had 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 nonobscuratory 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 6 \(\mu\)Ci of labeled Ins(1,4,5)P\(_3\) (spec act 20–60 Ci/mmol) mixed with 10 \(\mu\)l 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 \(\sim 0.11\) mM. Given an injection volume of 10 nl and an oocyte volume of 1 \(\mu\)l, the calculated intracellular Ins(1,4,5)P\(_3\) concentration was \(\sim 1.1\) \(\mu\)M, 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 (14\(^\circ\)C, 800 g) for 10 min, and the supernatant was transferred into a glass tube. Three successive dialy whole extractions (5.1, vol/vol) were performed to remove lipids and phospholipids, and the aqueous phase was frozen at \(-70^\circ\)C. When feasible, i.e., during the steady-state current that followed the [Ca\(^{2+}\)]\(_i\) oscillations (Fig. 1C), 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\(_3\) 3\(_S\) (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\(_3\) 3\(_S\) (estimated intracellular concentration of 10 \(\mu\)M) caused prolonged [Ca\(^{2+}\)]\(_i\) oscillations (30/30 cells) (Fig. 1D). 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). This result indicates that Ins(1,4,5)P\(_3\) 3\(_S\) 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\(_3\) \(S_3\) (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\(_3\) 3\(_S\), 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. 1E). These results suggest that the combination of

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**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 \(\mu\)M) into Xenopus oocytes caused a biphasic increase in [Ca\(^{2+}\)]\(_i\) (Fig. 1A). 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. 1A). 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 \(\mu\)M). Ins(1,3,4,5)P\(_4\) caused [Ca\(^{2+}\)]\(_i\) oscillations (12/12 cells) (Fig. 1B and C). In previous experiments, we (11, 36) and others (28, 29) had 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. 1B with Fig. 1C). 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. 1A), we also added Mn\(^{2+}\) during the steady-state current that followed the [Ca\(^{2+}\)]\(_i\) oscillations (Fig. 1C). 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).
Ins(1,4,5)P$_3$S$_3$ 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$_3$S$_3$/Ins(1,3,4,5)P$_4$-induced Ca$^{2+}$ influx varied considerably from cell to cell [191 ± 46 (SF) nA, range = 57–440 nA]. There was no relationship between the amplitude of Ins(1,4,5)P$_3$ induced Ca$^{2+}$ influx and the time interval between Ins(1,4,5)P$_3$S$_3$ 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 P, 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$_3$S$_3$ (3 cells). This failed to cause Ca$^{2+}$ influx. Second, we injected Ins(1,4,5)P$_3$S$_3$ (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$_3$ 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$^{2+}$], oscillations (12/13 cells) when injected alone and did not cause Ca$^{2+}$ influx when injected after Ins(1,4,5)P$_3$S$_3$ (5/5 cells). These results suggest that the ability to cause Ca$^{2+}$ influx following an injection of Ins(1,4,5)P$_3$S$_3$ 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$_3$S$_3$, releases Ca$^{2+}$ from intracellular stores (11, 13, 36). In contrast to Ins(1,4,5)P$_3$S$_3$, 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$_3$S$_3$, 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

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**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$^{2+}$]). 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$^{2+}$], followed by a more sustained increase in [Ca$^{2+}$], Mn$^{2+}$ (bar) blocked sustained increase in [Ca$^{2+}$]. 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$^{2+}$], oscillations more clearly, we have expanded time scale of boxed area (insert in C). Addition of Mn$^{2+}$ (4 mM) did not change the average Cl$^-$ current. D: injection of D-myo-inositol 1,4,5-trisphosphorothioate [Ins(1,4,5)P$_3$S$_3$] alone did not cause Ca$^{2+}$ influx. E: injection of Ins(1,4,5)P$_3$S$_3$ (10 pmol) caused prolonged [Ca$^{2+}$], 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$_3$S$_3$] alone did not cause Ca$^{2+}$ influx. F: injection of Ins(1,4,5)P$_3$S$_3$ (10 pmol) caused prolonged [Ca$^{2+}$], 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$_3$S$_3$] alone did not cause Ca$^{2+}$ influx. E: injection of Ins(1,4,5)P$_3$S$_3$ (10 pmol) followed by Ins(1,3,4,5)P$_4$ (10 pmol). In this case, a nonoscillatory current developed after [Ca$^{2+}$], 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$_3$.**
**INFLUX OF EXTRACELLULAR Ca²⁺ IN XENOPUS OOCYTES**

**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 Ca²⁺ influx. Under these conditions, 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

**Fig. 3.** Injection of \(\text{Ins(2,4,5)P}_3\) (10 pmol) followed by five sequential injections of \(\text{Ins(1,4,5)P}_4\) (doses of 0.3, 1.8, 3, 30, and 150 pmol at closed arrows) (top trace). First \(\text{Ins(1,3,4,5)P}_4\) injection inhibited \([\text{Ca}^{2+}]_i\) oscillations. Magnitude of \(\text{Ins(1,4,5)P}_3\)-induced extracellular \(\text{Ca}^{2+}\) influx increased with amount of \(\text{Ins(1,3,4,5)P}_4\) injected. Note that \(\text{Mn}^{2+}\) caused a recurrence of \([\text{Ca}^{2+}]_i\) oscillations before blocking current. Area enclosed by box is enlarged in bottom trace.

**Fig. 4.** Injection of \(\text{CaCl}_2\) (5 \(\mu\)M) followed by an injection of \(\text{Ins(1,3,4,5)P}_4\). Absence of \(\text{Mn}^{2+}\)-inhibitable current indicates that an increase in \([\text{Ca}^{2+}]_i\) was not sufficient to confer \(\text{Ins(1,3,4,5)P}_4\) with ability to stimulate \(\text{Ca}^{2+}\) influx. To test this possibility further, we injected \(\text{CaCl}_2\) in doses expected to increase the average \([\text{Ca}^{2+}]_i\) to 25 \(\mu\)M in Xenopus oocytes. \(\text{Ins(1,4,5)P}_3\) stimulation increases \([\text{Ca}^{2+}]_i\) to 0.25-1 \(\mu\)M (6, 17). Subsequent injection of \(\text{Ins(1,3,4,5)P}_4\) caused \([\text{Ca}^{2+}]_i\) oscillations but failed to cause \(\text{Ca}^{2+}\) influx (8 cells) (Fig. 4). Thus an increase in \([\text{Ca}^{2+}]_i\) is not sufficient to induce \(\text{Ins(1,3,4,5)P}_4\)-dependent \(\text{Ca}^{2+}\) influx. The presence of \(\text{InsP}_3\) appears to be required.

**Effect of \(\text{Ca}^{2+}\) influx on \(\text{Ins(1,3,4,5)P}_4\) generation.** Once formed following membrane-receptor activation, \(\text{Ins(1,4,5)P}_3\) follows two main metabolic pathways. In one pathway, it is dephosphorylated to \(\text{Ins(1,4)P}_2\) by the \(\text{Ins(1,4,5)P}_3\) 5-phosphatase (12). In a second pathway, it is phosphorylated to \(\text{Ins(1,3,4,5)P}_4\) by the \(\text{Ca}^{2+}\)/calmodulin-dependent \(\text{Ins(1,4,5)P}_3\) 3-kinase (4, 8, 39). The second pathway might be favored by the cell, in part, because the \(\text{Ins(1,4,5)P}_3\)-induced increase in \([\text{Ca}^{2+}]_i\) would activate the 3-kinase and not the 5-phosphatase (3, 32). Because \(\text{Ca}^{2+}\) influx increases \([\text{Ca}^{2+}]_i\), we hypothesized that it might shunt the metabolism of \(\text{Ins(1,4,5)P}_3\) toward the formation of \(\text{Ins(1,3,4,5)P}_4\). To examine the effect of \(\text{Ca}^{2+}\) influx on the metabolism of \(\text{Ins(1,4,5)P}_3\), we injected \([\text{H}]\text{Ins(1,4,5)P}_3\) into single Xenopus oocytes (2-20 \(\times\) 10\(^3\) counts \(\text{min}^{-1}\) cell\(^{-1}\)) incubated in the presence or absence of extracellular \(\text{Ca}^{2+}\). We then stopped \(\text{InsP}\) metabolism at predetermined time points. In cells with time points \(\geq 1\) min \((n = 56)\), we recorded the electrophysiological response and found typical changes in \([\text{Ca}^{2+}]_i\) (Fig. 1A). In cells incubated in the absence of extracellular \(\text{Ca}^{2+}\), the sustained increase in \([\text{Ca}^{2+}]_i\) shown in Fig. 1A was not present. HPLC separation of the \([\text{H}]\text{InsP}\) was performed for each individual cell \((n = 72)\).

By 40 min after injection, \([\text{H}]\text{Ins(1,4,5)P}_3\) was almost completely metabolized (Fig. 5A). The rate of \(\text{Ins(1,4,5)P}_3\) metabolism was comparable to that reported by Irvine et al. (20) and was not significantly influenced by extracellular \(\text{Ca}^{2+}\) (Fig. 5A).

The predominant \(\text{InsP}_4\) isomer recovered was \(\text{Ins(1,3,4,5)P}_4\). In the presence of extracellular \(\text{Ca}^{2+}\), the maximal accumulation of \([\text{H}]\text{Ins(1,3,4,5)P}_4\) counts [11.9% of total disintegrations/min (dpm) injected] occurred 3 min after \([\text{H}]\text{Ins(1,4,5)P}_3\) injection (Fig. 5B, open circles). In the absence of extracellular \(\text{Ca}^{2+}\) (Fig. 5B, closed circles), the maximum increase in
The product of the Ins(1,4,5)P₃ 5'-phosphatase, Ins(1,4)P₂, was the only InsP₂ isomer recovered. The effect of extracellular Ca²⁺ on [³H]Ins(1,4)P₂ 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)P₂ counts began to rise at 3 min and gradually increased to a maximum (3.3% of initial dpm) throughout the 40-min time course (Fig. 5C, open circles). In the absence of extracellular Ca²⁺, [³H]Ins(1,4)P₂ counts reached their maximum (5.3% of initial dpm) 5 min after injection and then gradually decreased (Fig. 5C, closed circles). These results suggest that less Ins(1,4)P₂ 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)P₂ 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)P₂ counts 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 InsP₃s 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)P₃S₃ 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)P₃S₃ 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

![Graph](http://example.com/graph.png)

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

<table>
<thead>
<tr>
<th>Time, min</th>
<th>[Ca²⁺]o</th>
<th>InsP₃/InsP₂ ratios</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>nominal</td>
<td>0.4±0.1*</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>0 mM</td>
<td>4.8±0.1*</td>
<td>4</td>
</tr>
<tr>
<td>10</td>
<td>5 mM</td>
<td>0.2±0.0*</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>5 mM</td>
<td>2.1±0.6</td>
<td>3</td>
</tr>
</tbody>
</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)P₂ was calculated for each Xenopus oocyte. Oocytes were bathed with high (6 mM) or low (nominal) extracellular Ca²⁺ concentration ([Ca²⁺]o). Values are expressed as means ± SE. * Value at 0 mM [Ca²⁺]o, statistically different (P < 0.05) from value at nominal [Ca²⁺]o.
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_2S_2. 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 unavailability 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(1,3,4,5)P_4 toward the formation of Ins(1,3,4,5)P_4 and away from dephosphorylation to Inos(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-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(1,3,4,5)P_3-kinase may require sustained high levels of [Ca^{2+}], 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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Address for reprint requests: M. J. Welsh, Howard Hughes Medical Institute, Departments of Internal Medicine and Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, Iowa 52242.

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