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Microinjection of Inositol 1,2-(Cyclic)-4,5-Trisphosphate, Inositol 1,3,4,5-Tetrakisphosphate, and Inositol 1,4,5-Trisphosphate Into Intact *Xenopus* Oocytes Can Induce Membrane Currents Independent of Extracellular Calcium

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Inositol phosphate action in an intact cell has been investigated by intracellular microinjection of eight inositol phosphate derivatives into *Xenopus laevis* oocytes. These cells have calcium-regulated chloride channels but do not have a calcium-induced calcium release system. Microinjection of inositol 1,3,4,5-tetrakisphosphate (IP_4), inositol 1,2-(cyclic)-4,5-trisphosphate (cIP_3), inositol 1,4,5-trisphosphate (IP_3), or inositol 4,5-bisphosphate [$(4,5)IP_2$], open chloride channels to induce a membrane depolarization. However, inositol 1-phosphate (IP_1), inositol 1,3,4,5,6-pentakisphosphate (IP_5), inositol 1,4-bisphosphate, or inositol 3,4-bisphosphate are unable to induce this depolarization. The depolarization is mimicked by calcium microinjection, inhibited by EGTA coinjection, and is insensitive to removal of extracellular calcium. By means of the depolarization response, the efficacy of various inositol phosphate derivatives are compared. IP_3 and cIP_3 induce similar half-maximal, biphasic depolarization responses at an intracellular concentration of approximately 90 nM, whereas IP_4 induces a mono- or biphasic depolarization at approximately 3400 nM. At concentrations similar to that required for IP_3 and cIP_3, $(4,5)IP_2$ induces a long-term (greater than 40 min) depolarization. The efficacy ($cIP_3 = IP_3 = (4,5)IP_2 \gg IP_4$) and action of the various inositol phosphates in an intact cell and their inability to induce meiotic cell division are discussed.

Key words: inositol phosphates, chloride channel, depolarization, membrane potential, second messengers

The interaction of a hormone with a target cell membrane and the subsequent release of a second messenger to the interior of the cell is a complex and not well-

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understood mechanism. Many hormones (especially those that release intracellular calcium) are thought to activate a phospholipase C which releases inositol 1,4,5-trisphosphate (IP_3), inositol 1,2-(cyclic)-4,5-trisphosphate (cIP_3), and diacylglycerol from the membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2) [1]. This model of hormonal mechanism is referred to as the phosphoinositide (PI) turnover system.

IP_3 has been shown to release intracellular calcium from nonmitochondrial sources [for review, see 2]. Once IP_3 is produced, it is phosphorylated by a 3'kinase to inositol 1,3,4,5-tetrakisphosphate (IP_4) or dephosphorylated by a 5'phosphatase to inositol 1,4-bisphosphate. There is general agreement that inositol 1,4-bisphosphate is unable to release intracellular calcium [2]; however, the function of IP_4 is still a matter of debate. There is a report that IP_4 acts to fill intracellular calcium stores from the extracellular medium [6], and that the IP_4 itself is not effective in releasing intracellular calcium [8]. Unlike IP_3, cIP_3 is not phosphorylated by a kinase but is dephosphorylated by the same 5'phosphatase to inactive cIP_2 [1].

Most studies on inositol phosphate metabolism or action have been conducted in cell homogenates or in permeabilized cells which may not reflect the intracellular milieu of an intact cell [3]. There have been other reports in which inositol phosphates were microinjected into intact *Limulus* photoreceptor [4] or *Xenopus* cells [5,7], but inositol phosphate dose-response relationships have not been reported. These relationships are difficult to assess since the small size of somatic cells precludes accurate measurement of microinjected volumes or, in larger egg cells, the presence of a calcium-induced calcium release system (in which a small amount of calcium is amplified many fold) prohibits measurement of a graded response. The microinjection of IP_3 into *Xenopus laevis* oocytes (which do not have a calcium-induced calcium release system) increases intracellular calcium which, in turn, opens chloride channels to depolarize the plasma membrane [7]. Thus, increases in intracellular calcium can be indirectly followed by measurement of *Xenopus* oocyte membrane potential. This report is the first direct comparison of eight inositol phosphate derivatives in an intact cell, the *Xenopus* oocyte, by recording membrane potential or voltage clamp current in response to intracellular microinjection of these derivatives.

MATERIALS AND METHODS

Cells and Materials

Xenopus laevis oocytes were obtained from toads (Xenopus One, Ann Arbor, MI) that were primed with 35 IU of pregnant mare's serum 3 days before sacrifice. The oocytes were manually dissected from ovarian tissue and placed into a modified Ringer's medium containing 83 mM NaCl, 0.5 mM $CaCl_2$, 1 mM KCl, 1 mM $MgCl_2$, and 10 mM Hepes (pH 7.9) at 22–24°C. For experiments in the calcium-containing medium, oocytes were placed in a 1.5 ml chamber and perfused (1–2 ml/min) with a $CaCl_2$-free Ringer's solution supplemented with a $CaCl_2$-containing Ringer's solution ejected via a syringe pump. Removal of extracellular calcium was accomplished by turning off the calcium syringe pump (Razel, Stanford, CT) and turning on a pump with EGTA (final EGTA concentration in the recording chamber was 1 mM). The use of collagenase to isolate oocytes from the ovarian wall was not used since the enzyme treatment can damage the cell membrane (unpublished results, B. Stith) [12].

Microinjection of *Xenopus* Oocytes

Silanized micropipettes (1.2 or 1.5 mm diameter) were pulled to a tip size of about 1–3 μm, filled with stock solutions (100 μM for inositol phosphates except that 1 mM was used for IP_4) to be microinjected, and connected by tubing to a Picospritzer II (General Valve, Fairfield, NJ). Noncyclic inositol phosphates were obtained from Calbiochem and cIP_3 was a generous gift from Dr. P. Majerus (Washington University, St. Louis, MO). Pressure (90 psi) was applied to the micropipette for 5 ms to 10 s to eject the stock solutions from the micropipette tip. The output of each micropipette was calibrated before and after microinjection into the cell by measuring the diameter of the droplet expressed for various durations of pressure application. This standardization method was proven valid by the microinjection of ^{3}H-sorbitol (New England Nuclear, Wilmington, DE) into *Xenopus* oocytes. For dose-response determinations, intracellular concentrations of inositol phosphates or calcium were calculated from the quantity microinjected, divided by the free intracellular volume (450 nl) [13], with the assumption that the injected material distributed evenly throughout the available cell volume. All numerical results are reported as the mean ± the standard error of the mean (SEM), with the SEM calculated from the number (n) of results obtained from different cells.

Measurement of Oocyte Membrane Potential

To record the response to microinjected inositol phosphates, the membrane potential was recorded by 1.2 or 1.5 mm diameter microelectrodes that were pulled to a tip resistance of 10 to 30 MΩ. They were filled with 2.5 M potassium acetate or potassium methylsulfate and held by a Narishige (Greendale, NY) MP2 manipulator with a David Kopf (Tujuinga, CA) electronic stepping advance system. The electrode signal was fed to an Axoclamp-2 electrometer (Axon Instruments, Burlingame, CA), which was in turn connected to a Tektronix (Beverton, OR) oscilloscope and a Gould (Cleveland, OH) two-channel recorder. In some experiments, membrane voltage was clamped by a standard two-electrode procedure [e.g., 7]: the recording microelectrode measured the clamp voltage, while a second electrode (0.5–1.5 MΩ tip resistance) was used for current passage. Voltage clamping was necessary for the measurement of the reversal potential of the drug response to determine the primary ion channel that opens to induce the depolarization, and to maintain membrane potential in Ca-free medium.

RESULTS

IP_3 (stock 100 μM) was microinjected into oocytes while the membrane potential was continuously recorded via a separate microelectrode. The membrane response was graded and biphasic (Fig. 1) [7]. One to five seconds after microinjection, the membrane depolarized. This first response lasted about 25 seconds and is referred to as the D_1 response. Fifty to one hundred seconds after microinjection, a second depolarization (D_2) began that lasted longer than 100 sec. The D_2 response was characterized by a slower depolarization and repolarization with a series of small, brief depolarizations superimposed. The D_1 response appeared at lower IP_3 doses than the D_2 response (Fig. 1A), but the D_1 magnitude decreased in size (desensitization) during multiple microinjections. The D_2 response was reproducible after multiple injections into a single cell given a 15 min period between injections; thus, the magnitude of the

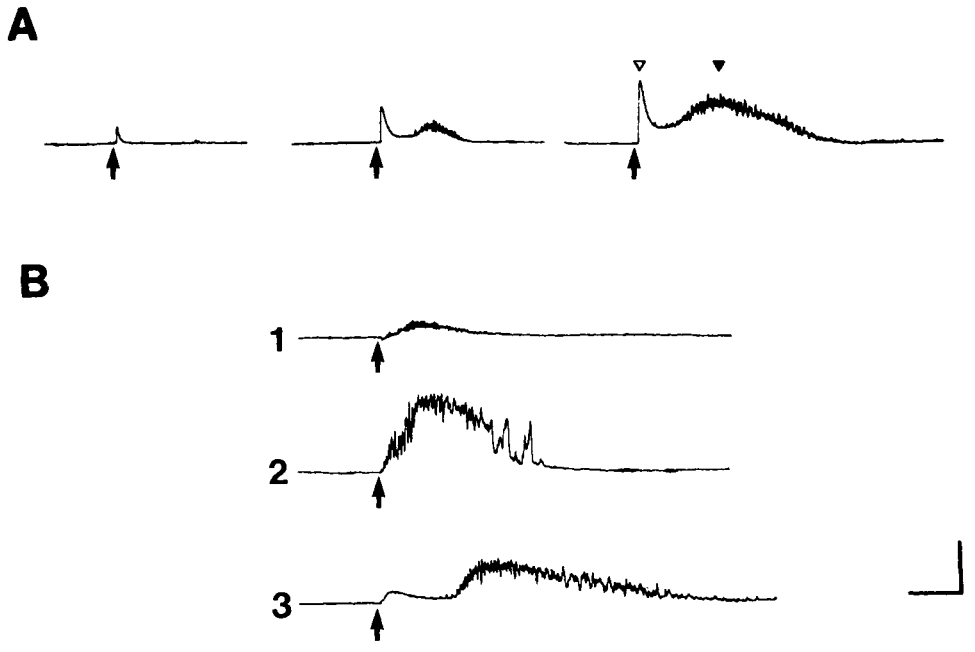

Fig. 1. IP_3 and IP_4 induce a membrane depolarization in *Xenopus* oocytes. **A:** Microinjection of IP_3 induces a biphasic membrane depolarization. The three arrows indicate the times of successive drug microinjection into a single oocyte (approximately 15 min between each injection) and the final intracellular concentrations of IP_3 were 3, 30, and 300 nM (from left to right; see Materials and Methods for dose determination). Note that the biphasic depolarization is referred to as the D_1 (open triangle) and the D_2 (closed triangle) responses and that the D_1 response occurs at lower IP_3 concentrations. The resting membrane potential (rmp) was −40 mV, and the horizontal bar in the lower right corner represents 100 s, whereas the vertical bar depicts 20 mV for both A and B. **B:** The membrane depolarization response to IP_4 was variable. Cell 1, which had an rmp of −38 mV, was microinjected with IP_4 to a final concentration of 1 μM, whereas cell 2, which had a rmp of −48 mV, was injected with IP_4 to 7 μM (injections were at the arrows). Note that the D_1 response usually appears at lower inositol phosphate concentrations (A) than the D_2 response; however, these two cells did not show a D_1 response to IP_4. Cell 3 (rmp of −46 mV) was microinjected with IP_4 to 5 μM and showed a biphasic response.

D_2 peak was used as an accurate measure of the oocyte response to microinjected inositol phosphates.

Although there is evidence for differences between cIP_3 and IP_3 [9], both gave similar biphasic responses that were of the same shape, magnitude, and time course (compare Fig. 1A to Fig. 5). Similarly, IP_4 was able to induce a depolarization, but 4 out of 11 cells tested consistently lacked the D_1 response at all effective IP_4 concentrations; all cells displayed the D_2 depolarization (Fig. 1B). Inositol 1-phosphate (IP), inositol 1,4-bisphosphate [(1,4)IP_2], inositol 3,4-bisphosphate [(3,4)IP_2], and inositol 1,3,4,5,6-pentakisphosphate (IP_5) did not induce a depolarization response at concentrations up to 10 μM (Table I). In an oocyte that did not respond to 5 μM IP_5, subsequent microinjection of 150 nM IP_3 into the same cell induced a full biphasic response.

The maximal D_2 depolarizations at various intracellular IP_3, cIP_3, or IP_4 concentrations produced typical sigmoidal dose-response curves (Fig. 2). For dose-response curves from ten different cells from a total of eight toads, the concentration of IP_3 for a

TABLE I. Intracellular Concentration for Half-Maximal D_2Depolarization Response to Inositol Phosphates*

Derivative	Intracellular concentration for half-maximal depolarization mean ± SEM (n)	Require extracellular Ca^{++}
IP	No effect[a]	N/A
$(1,4)IP_2$	No effect[a]	N/A
$(3,4)IP_2$	No effect[a]	N/A
$(4,5)IP_2$	<100 nM	NT
IP_3	88 ± 16 nM (10)	No
cIP_3	86 ± 29 nM (7)	No
IP_4	3440 ± 1230 nM (7)	No
IP_5	No effect[a]	N/A

*NT = not tested; N/A = not applicable.
[a]No depolarization was noted at intracellular concentrations up to 10 μM.

half-maximal response was 88 ± 16 nM (n = 10; Table I). The concentration of cIP_3 for half-maximal depolarization was 86 ± 29 nM (n = 7; Table I), and the concentration of IP_4 for half-maximal depolarization was 3.44 ± 1.23 μM (n = 7; Table I).

Inositol 4,5-bisphosphate [$(4,5)IP_2$] is capable of inducing a biphasic response (Fig. 3); however, the D_2 response lasted longer than 40 min. Although a dose-response curve from one cell is not feasible, the concentration required for an initial response was similar to that for IP_3 or cIP_3 (1 to 10 nM; 5 cells).

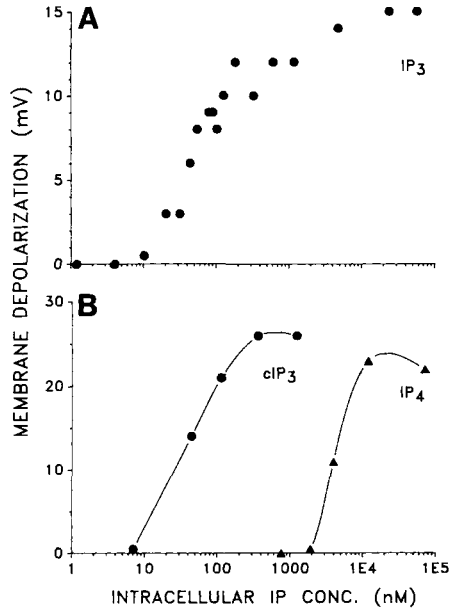

Fig. 2. Typical dose-response relationships between IP_3 (A), cIP_3 and IP_4 (B) and the maximum membrane D_2 response in three different cells. The maximum magnitude of the D_2 response was recorded and plotted against the calculated intracellular concentration for each inositol phosphate.

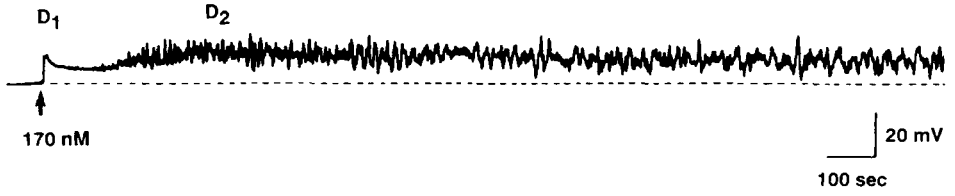

Fig. 3. (4,5)IP_2 induces a prolonged D_2 depolarization. At the arrow, inositol phosphate was microinjected into an oocyte (rmp of −43 mV) to a final intracellular concentration of 170 nM. The electrode was withdrawn after 40 min, at which time the cell was still depolarized and undergoing typical D_2 fluctuations.

To explore the mechanism of action of the inositol phosphates, three active inositol phosphates (IP_3, cIP_3, and IP_4) were each coinjected with 1 mM EGTA, and no depolarization responses were noted (EGTA microinjection alone did not alter membrane potential). To test the effect of an increase in intracellular calcium levels, $CaCl_2$ was microinjected into several oocytes (for a calculated increase in intracellular calcium of 100 μM; see Materials and Methods; Fig. 4). This injection induced an increase in channel activity (the small, transient depolarizations to the left in Fig. 4), but there was no D_1 or D_2 response. When the intracellular calcium was increased by 1 mM, there was an unusually long D_1 response. The D_2 response to this calcium microinjection was more representative of an inositol phosphate response.

It has been found that IP_3 and IP_4 effects are not dependent upon extracellular calcium; thus, they must release calcium from intracellular stores in *Xenopus* oocytes [7,10]. Since removal of extracellular calcium or addition of 1 mM EGTA depolarizes the cell, the cells were voltage clamped at −50 to −70 mV. In the absence of extracellular calcium, with or without EGTA, the microinjection of cIP_3 (as well as IP_3, or IP_4) was still able to induce a biphasic current flow, equivalent to that shown in Figure 5 (top), in six out of six cells.

It has been demonstrated that IP_3 and IP_4 open chloride channels [7,10], but to confirm that the chloride channel is responsible for the cIP_3-induced depolarization,

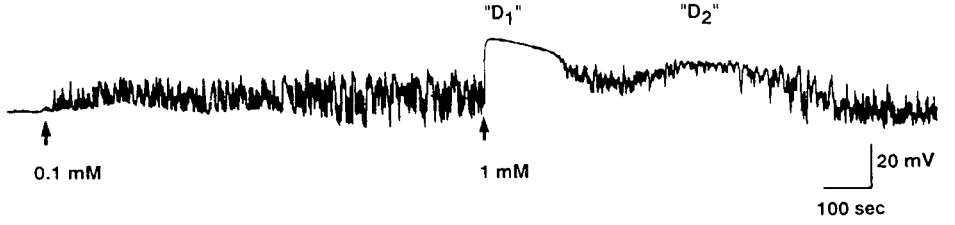

Fig. 4. $CaCl_2$ microinjection induces a two-phase depolarization. At the first arrow, the microinjection resulted in a calculated (see Materials and Methods) increase in total intracellular calcium of 100 μM and produced only an increase in transient depolarizations. The second microinjection (the righthand arrow) increased intracellular calcium by 1 mM and was sufficient to induce a biphasic response (D_1, D_2). The D_1 response has an unusually long duration when compared to the D_1 response to cIP_3, IP_3, IP_4, or (4,5)IP_2 (see Figs. 1, 3, 5).

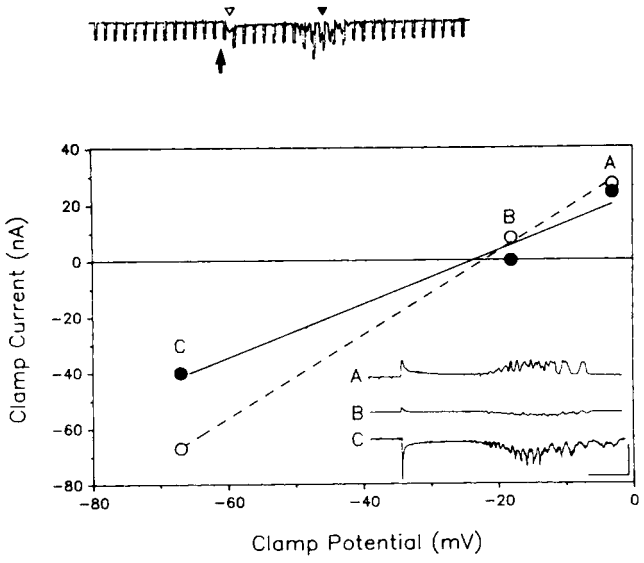

Fig. 5. cIP_3 induces a membrane response in Ca-free medium (**top**) with a reversal potential of about −22 mV (**bottom**). Top: cIP_3 (80 nM) was microinjected (at the arrow) into an oocyte after the cell had been in a medium without $CaCl_2$ and with 1 mM EGTA for 10 min, and the cyclic inositol phosphate-induced D_1 (open triangle) and D_2 (closed triangle) responses. The current response is shown as the cell was voltage clamped at −40 mV (35 nA) to maintain membrane potential at the value in calcium-containing medium. A constant voltage step of 10 mV (hyperpolarizing) was placed on the membrane throughout the experiment. The horizontal bar in the lower right of the figure represents 50 s, and the vertical bar is 80 nA (outward current is downward). Bottom: Reversal potential determination for the cIP_3 response in the presence of extracellular calcium. The oocyte membrane was clamped at −3 (A), −18 (B), and −37 mV (C) and cIP_3 (80 nM) was microinjected into the cell (unclamped rmp of −44 mV). The peak current during the D_1 (open symbols) or D_2 (closed symbols) responses were plotted versus the clamp potential. The resulting reversal potential for both responses is about −22 mV which corresponds to the chloride equilibrium value in *Xenopus* oocytes [7]. The horizontal bar in the lower right insert represents 50 s whereas the vertical bar is 50 nA (outward clamp current is downward).

cells were voltage clamped and the reversal potential determined. Both phases of the cIP_3-induced depolarization were probably due to chloride ion movement since their reversal potentials (−22 mV; Fig. 5, bottom) were similar to that reported in the literature for chloride in *Xenopus* oocytes (−20 to −24 mV) [10].

DISCUSSION

In vitro experiments have shown that the degradative 5′ phosphatase has a 20-fold higher K_m for cIP_3 as compared to the K_m for IP_3 and that cIP_3 is not converted to IP_3 [15]. This suggests that cIP_3 might have a longer half-life than IP_3 in an intact cell [1]. However, the two derivatives may have comparable half-lives in intact cells since our data show that IP_3 and cIP_3 induce a biphasic depolarization response similar in both time course and amplitude. Since similar responses were noted over a wide concentration range of cIP_3 and IP_3, the lack of a difference cannot be attributed to use of high concentrations which would negate the K_m difference.

As noted above, cIP_3 did not induce a prolonged response. However, the oocyte is capable of a prolonged response since $(4,5)IP_2$ was able to depolarize the cell membrane for a period longer than 40 min. The ability of $(4,5)IP_2$ to induce this long-term depolarization could be due to an inability of the 5′ phosphatase to degrade the derivative, that it binds to the calcium release site with higher affinity than other derivatives, or that $(4,5)IP_2$ might act directly on the channel itself. Future studies will examine the metabolism of this inositol phosphate and the duration of the calcium released by this derivative. It is also of interest that 10 μM $(4,5)IP_2$ was unable to induce meiotic cell division in the *Xenopus* oocyte (data not shown). IP_3 was also unable to induce meiosis [14]; however, it may be argued that the brief release of calcium from this derivative is not of sufficient duration. Microinjections of up to 30 μM cIP_3, IP_4, or a combination of 3 μM IP_3, 3 μM cIP_3, and 33 μM IP_4 were also unable to induce meiosis (data not shown). However, 33 μM IP_4 or 10 μM $(4,5)IP_2$ were able to speed insulin-induced meiosis (maturation) in oocytes from four toads in a manner similar to that reported for IP_3 [14]. Control oocytes (microinjected with 1 mM fructose 1,6-phosphate) required 9.5 ± 4.2 ($n = 4$) h for maturation, whereas oocytes preinjected with IP_4 required 6.8 ± 1.9 ($n = 4$) h and cells preinjected with IP_2 required 6.1 ± 1.3 ($n = 4$) h for insulin-induced maturation (significant by paired t-test, $p < 0.05$).

It is important to note that cIP_3 and IP_3 required only about 90 nM concentrations for half-maximal effect, whereas IP_4 required about 40-fold higher concentrations (about 3,400 nM). This is in agreement with a report [10] that finds that the lowest IP_4 concentration for threshold membrane depolarization in four oocytes is some 21-fold higher than that for IP_3. Although earlier reports suggest that IP_4 cannot induce a depolarization response (including the *Xenopus* oocyte) [6,8], there are now two reports (this paper, [10]) that suggest that IP_4 is capable of releasing intracellular calcium to induce a depolarization response. There could be an active contaminant in the IP_4 stock solution that is responsible for the membrane response, but, as recorded by phosphate and two-dimensional NMR analysis, the stock solution does not have other detectable inositol phosphates (Calbiochem). In addition, the lack of a D_1 response to IP_4 in some cells [see also ref. 10 for similar results] suggests that cIP_3 or IP_3 (which always show a D_1 response) are not contaminates.

It is probable that there are two types of calcium-regulated chloride channels corresponding to the two (D_1, D_2) responses since IP_4 induces only the D_2 response in some cells, and because the D_1 response desensitizes faster than the D_2 response after multiple injections. Also, removal of extracellular calcium appears to reduce the magnitude of the D_1 response suggesting that this response may be partially dependent upon extracellular calcium influx. Preliminary data suggest that there might be enzymatic regulation and two different sites of control for the early and late depolarization as the microinjection of protein kinase C delays the D_2, but not the D_1, response (Stith and Proctor, manuscript in preparation).

These data on the effective inositol phosphate concentration are different from many other studies conducted in cell homogenates or permeabilized cells. The effective IP_3 and cIP_3 concentrations reported here for a calcium-dependent depolarization event are significantly lower than those reported to release calcium in various permeabilized cells (an average of 1 μM from twenty different preparations) [2]. The higher concentration of IP_3 for half-maximal effect may be due to the use of permeabilized cells that do not accurately reflect an intact cell. Since the dissociation constant for IP_3 binding is on the order of 5 nM [for review, see ref. 2], the data presented in this paper

may more accurately reflect inositol phosphate efficacy in an intact cell. The approximately 90 nM concentration required for half-maximal effect is, however, much higher than the concentration required for IP_3 induction of the fertilization response in *Xenopus* eggs [5]. In eggs, only about 1 nM IP_3 is required for the large fertilization depolarization (which is also due to calcium-controlled chloride channels) [5]. The lower effective concentration of IP_3 in eggs may be due to the presence of a calcium-induced calcium release system which is absent in oocytes. As shown by the development of extensive plasma membrane-endoplasmic reticulum junctions [11], this calcium release system develops during maturation of the oocyte to the egg and is located near the plasma membrane in the egg cortex. In eggs, microinjection of nanomolar amounts of IP_3 release a large amount of calcium (independent of the IP_3 dose) that triggers subsequent chloride channel opening [5]. It is important to note that the minimum threshold doses required to induce a membrane depolarization (this report, [10]) or the fertilization response [5] are similar (approximately 1 nM).

Busa et al. [5] noted that shallow iontoelectrophoretic microinjections of IP_3 under the *Xenopus* egg membrane resulted in faster fertilization response times than deeper injections. In the present report, attempts at pressure microinjection of IP_3 near the surface or deep into the oocyte did not result in any differences in the response (in both sets of experiments all microinjections were in the animal pole, however, as this is the location of most of the calcium-regulated chloride channels [7]). This difference between the results from eggs and oocytes could also be due to the presence of a calcium-induced calcium release system in the egg cortex but not present in the oocyte.

An interesting question concerns the control of the chloride channel by calcium: why are millimolar levels of microinjected calcium necessary to open chloride channels when IP_3 is effective after it increases calcium by only micromolar levels [9]? This may be due to extensive binding or sequestering of the microinjected calcium or that inositol phosphates release calcium in more optimal locations.

Although it has been shown that IP_3 releases intracellular calcium to open chloride channels and depolarize the *Xenopus* membrane [7], a similar mechanism for IP_4 and cIP_3 has not yet been fully demonstrated. However, since EGTA co-microinjection can block the action of three derivatives that induce a membrane depolarization (IP_3, IP_4, cIP_3), and since calcium microinjection can produce a similar depolarization, there is evidence that they act through an increase in intracellular calcium. Furthermore, these inositol phosphates must release calcium from intracellular stores since lack of extracellular calcium did not inhibit the response to IP_3, cIP_3, or IP_4. These data suggest that IP_4 and cIP_3 act through a calcium-dependent mechanism similar to that used by IP_3.

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