Activation of sea urchin eggs by inositol phosphates is independent of external calcium

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We investigated the contribution of external calcium ions to inositol phosphate-induced exocytosis in sea urchin eggs. We show that: (a) inositol phosphates activate eggs of the sea urchin species *Lytechinus pictus* and *Lytechinus variegatus* independently of external calcium ions; (b) the magnitude and duration of the inositol phosphate induced calcium changes are independent of external calcium; (c) in calcium-free seawater, increasing the volume of inositol trisphosphate solution injected decreased the extent of egg activation; (d) eggs in calcium-free sea water are more easily damaged by microinjection; microinjection of larger volumes increased leakage from eggs pre-loaded with fluorescent dye. We conclude that inositol phosphates do not require external calcium ions to activate sea urchin eggs. This is entirely consistent with their role as internal messengers at fertilization. The increased damage caused to eggs in calcium-free seawater injected with large volumes may allow the EGTA present in the seawater to enter the egg and chelate any calcium released by the inositol phosphates. This may explain the discrepancy between this and earlier reports.

INTRODUCTION

The transient increase in the cytosolic free calcium ion concentration (Ca^{2+}_i) that occurs at fertilization in sea urchin eggs (Steinhardt *et al.*, 1977) is the trigger that initiates development (Whitaker & Steinhardt, 1985). The most obvious sign of egg activation is the elevation of a fertilization envelope. The fertilization envelope rises as a consequence of the exocytosis of cortical secretory granules caused by the increase in Ca^{2+}_i (Baker & Whitaker, 1978). Fertilization, parthenogenetic activation and the elevation of the fertilization envelope can occur in calcium-free sea water, which suggests that the increase in Ca^{2+}_i is due to release of calcium ions from internal stores (Steinhardt & Epel, 1974; Schmidt *et al.*, 1982). It has been suggested that the fertilization calcium transient is the result of the liberation of the calcium-releasing second messenger inositol 1,4,5-trisphosphate [$\text{Ins}(1,4,5)\text{P}_3$]. This idea rests on the demonstration of an increased turnover of polyphosphoinositide phospholipid (Turner *et al.*, 1984) and the production of inositol trisphosphate at fertilization (Ciapa & Whitaker, 1986). Microinjecting $\text{Ins}(1,4,5)\text{P}_3$ into sea urchin eggs causes an increase in Ca^{2+}_i and the elevation of a fertilization envelope (Whitaker & Irvine, 1984; Swann & Whitaker, 1986). Sea urchin egg homogenates contain an ATP-driven, membrane-bound calcium-sequestering system that is sensitive to $\text{Ins}(1,4,5)\text{P}_3$ (Clapper & Lee, 1985).

It has recently been reported that microinjection of $\text{Ins}(1,4,5)\text{P}_3$ causes activation of sea urchin eggs (judged by fertilization envelope elevation) only if calcium ions are present externally (Slack *et al.*, 1986; Irvine & Moor, 1986). These findings have been taken as an indication that inositol polyphosphates regulate plasma membrane calcium fluxes (Irvine & Moor, 1986). Yet fertilization

and development in sea urchins can occur in sea water containing less than 10^{-8} M- Ca^{2+} (Chambers, 1980), that is, under conditions in which calcium influx cannot occur (Poenie *et al.*, 1985). If $\text{Ins}(1,4,5)\text{P}_3$ activates eggs only in calcium-containing sea water then it cannot be the crucial internal messenger at fertilization.

We undertook this study to determine more precisely the contribution of external calcium ions during InsP_3 -induced egg activation. We used microinjection techniques to assess the effects of a number of inositol phosphates on cortical granule exocytosis both in the presence and absence of external calcium. We have also assessed the contribution that calcium influx makes to the inositol phosphate-induced calcium transient by measuring Ca^{2+}_i using the calcium sensitive fluorescent dye, fura2. We find that all the inositol phosphates tested cause cortical granule exocytosis independently of external calcium. Measurements of Ca^{2+}_i indicate that calcium influx makes no significant contribution to the inositol phosphate-induced calcium transient. Our data support the idea that $\text{Ins}(1,4,5)\text{P}_3$ is the internal messenger that causes the increase in Ca^{2+}_i at fertilization and demonstrate that inositol phosphates do not cause a significant influx of calcium when they activate sea urchin eggs.

MATERIALS AND METHODS

Obtaining gametes

Gametes were obtained by the intracoelomic injection of 0.5 M-KCl. Eggs of the sea urchin species *Lytechinus pictus* (Pacific Biomarine Laboratories Inc., Venice, CA, U.S.A.) and *Lytechinus variegatus* (collected off Key Biscayne, FL, U.S.A.) were collected in artificial seawater (ASW; for composition, see below). The egg jelly was

Abbreviations used: InsP_3 and InsP_4 , inositol trisphosphate and tetrakisphosphate respectively; locants are given in parentheses, e.g. $\text{Ins}(1,2\text{cyc}4,5)\text{P}_3$ is inositol 1,2-cyclic 4,5-trisphosphate; ASW, artificial seawater.

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removed by triple passage through 100 μm Nitex mesh and the eggs were washed twice with, then resuspended in, either ASW or calcium-free ASW. For microinjection the eggs were attached to coverslips pretreated with poly(L-lysine) (0.02 mg/ml). Sperm was collected in a minimal volume of ASW and kept at 4 °C until use.

The viability of eggs from each female was assessed at the end of a series of experiments on eggs from that female. A sample of eggs was fertilized and development was observed. For all data reported here > 90 % of the sample elevated fertilization envelopes and > 80 % developed to at least the four-cell stage.

All experiments were conducted at 16 °C.

Composition of artificial seawater

ASW: 435 mM-NaCl, 40 mM-MgCl₂, 15 mM-MgSO₄, 11 mM-CaCl₂, 10 mM-KCl, 2.5 mM-NaHCO₃, 1 mM-EDTA, pH 8.0.

Calcium-free ASW: 445 mM-NaCl, 50 mM-MgCl₂, 10 mM-KCl, 2.5 mM-NaHCO₃, 2 mM-EGTA, 1 mM-EDTA, pH 8.0.

Calcium-free ASW was calculated to have a free calcium ion concentration of less than 100 nM by using the binding constants of Martell & Smith (1974). This was confirmed by using a calcium-sensitive electrode (World Precision Instruments, New Haven, CT, USA).

Microinjection techniques

Micropipettes were pulled from glass capillary tube (1.5 mm i.d.; Clarke Electromedical, Pangbourne, U.K.) using a Palmer Bioscience Microelectrode Puller (Sheerness, U.K.). The tip resistance was typically 5–10 Mohm when filled with 3 M-KCl and the diameter was such that a 10–50 ms pulse of 450 kPa pressure ejected a volume of 1 pl (Swann & Whitaker, 1986). Pulses were delivered to the pipette at a frequency of 1 Hz during periods of immersion in sea water to prevent contamination of the pipette contents. The micropipette was held in a micromanipulator (Prior Instruments, Cambridge, U.K.) mounted onto the stage of a Leitz Diavert microscope.

Microinjections were performed by using brightfield microscopy with a 40 $\times$, 0.65 NA achromat objective. The injected volume was estimated by measuring the cytoplasmic displacement of the injected fluid with an eyepiece graticule. For experiments to determine the damage done to eggs by microinjection a low pressure injection procedure based on that described by Turner *et al.* (1986) was used.

Observation of the egg cortical granules

We used cortical granule exocytosis as a morphological marker to indicate that an elevation of Ca²⁺_i had occurred. However, as well as looking at fertilization envelope elevation as an indirect assay of exocytosis, we observed the cortical granules themselves. The cortical granules were observed by using differential interference contrast microscopy with a Leitz 100 $\times$, 1.2 NA oil-immersion objective.

Fluorescence measurements of Ca²⁺_i and of dye efflux

Ca²⁺_i was measured by using the calcium-sensitive fluorescent dye fura2. The fura2 (pentapotassium salt; Molecular Probes, Junction City, OR, U.S.A.; 10 mM in 0.5 M-KCl/20 mM-Pipes, pH 6.7) was introduced into the eggs by microinjection to give a cytoplasmic concentra-

tion of about 100 μM . The fluorescence from single eggs was measured by the methods described by Swann & Whitaker (1986). Ca²⁺_i was calculated by using the methods and binding constants of Poenie *et al.* (1985). On occasion, eggs were loaded with the fluorescent dye fluorescein (20 μM -fluorescein diacetate for 30 min) and the leakage of the dye following injection was measured using a Nikon Optiphot epifluorescence microscope (excitation 450–490 nm, emission 510 nm) and a SIT camera attached to a video recorder and video analyser (Colorado Video Inc., Boulder, CO, U.S.A.).

Inositol phosphates

The inositol phosphates were a generous gift from Dr. R. F. Irvine of the Institute of Animal Physiology, Babraham, U.K. They were dissolved in an injection vehicle containing 500 mM-KCl, 20 mM-Pipes and 0.1 mM-EGTA, pH 6.7.

RESULTS

Inositol phosphates and cortical granule exocytosis

We microinjected Ins(1,4,5)P₃, inositol 2,4,5-trisphosphate [Ins(2,4,5)P₃], inositol 1,2-cyclic 4,5-trisphosphate [Ins(1,2cyc4,5)P₃], inositol 1,3,4-trisphosphate [Ins(1,3,4)P₃] and inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P₄] to test their effects on cortical granule exocytosis. Ins(1,4,5)P₃ induced the exocytosis of cortical granules and the resultant elevation of a fertilization envelope. Fig. 1 shows two eggs, one microinjected in artificial seawater (ASW) and one in calcium-free ASW. Both eggs have elevated a complete fertilization envelope. However, the fertilization envelope in calcium-free ASW is much less refractile and more difficult to see. For this reason, we decided to observe the cortical granules directly when determining the effects of inositol phosphates.

In experiments to determine the sensitivity of eggs to inositol phosphates we injected a constant volume of inositol phosphate (1.5 pl, 0.3 % egg volume) and varied the pipette concentration. The reasons for this were twofold. First, the sensitivity of the eggs to inositol phosphates may be a function of the concentration injected as well as the total dose. Second, we were concerned about possible damage to eggs caused by microinjecting larger volumes, particularly in calcium-free ASW (see below). The results of microinjecting various inositol phosphates into *Lytechinus pictus* eggs are shown in Fig. 2. In ASW the most active inositol phosphate tested was Ins(1,4,5)P₃: half-maximal activation occurs at an Ins(1,4,5)P₃ concentration of 2.5 μM , similar to the concentration determined in earlier work (Whitaker & Irvine, 1984; Turner *et al.*, 1986). Other inositol phosphates also caused cortical granule exocytosis. Ins(1,2cyc4,5)P₃, Ins(2,4,5)P₃ and Ins(1,3,4,5)P₄ activated eggs at half-maximal concentrations of 19, 56 and 390 μM respectively. We also tested Ins(1,3,4)P₃. It showed little activity: a pipette concentration of 1 mM caused only 20 % of the eggs injected to undergo complete cortical granule exocytosis. Microinjection of vehicle alone caused no sign of exocytosis ($n = 28$).

Fig. 2 also shows that removing the external calcium did not affect the sensitivity of cortical granule exocytosis to any of the inositol phosphates tested. This result was very different from the previous reports (Slack *et al.*, 1986; Irvine & Moor, 1986) of an external calcium

Fig. 1. The $\text{Ins}P_3$ -induced elevation of a fertilization envelope in normal and calcium-free ASW

Two eggs microinjected with $\text{Ins}(1,4,5)P_3$ (1.5 pl, 10 μM pipette concentration). On the left, an egg in ordinary ASW; on the right, an egg in calcium-free ASW. Both have elevated fertilization envelopes, but the envelope of the egg in calcium-free ASW is much less distinct

Fig. 2. Inositol phosphate-induced cortical granule exocytosis

Eggs were microinjected with a constant volume of inositol phosphate (1.5 pl) and the pipette concentration was varied. The eggs were scored for cortical granule exocytosis by direct observation of the cortical granules by using differential interference contrast microscopy. Closed symbols refer to injections performed in normal ASW. Open symbols refer to injections performed in calcium-free ASW. Each point on the graph represents the injection of between 10 and 15 eggs.

requirement for $\text{Ins}(1,4,5)P_3$ -induced exocytosis. To determine whether a species difference might account for this discrepancy, we repeated our experiments using eggs from *L. variegatus*, the species used in the fuller of the two reports. We obtained very similar results in *L. variegatus* and *L. pictus* (Table 1).

We also attempted to reproduce the reported synergism between $\text{Ins}(1,3,4,5)P_4$ and $\text{Ins}(2,4,5)P_3$ (Irvine & Moor, 1986). We co-injected submaximal doses: 10 μM -

$\text{Ins}(1,3,4,5)P_4$ with either 10 μM - $\text{Ins}(2,4,5)P_3$ or 1 μM - $\text{Ins}(1,4,5)P_3$. We did not observe any synergistic effects.

Dye leakage from microinjected eggs

Our observations were so clearly different from those reported by Irvine & Moor (1986) that we made experiments in which we attempted to reproduce their methods as closely as we could. For these experiments we attempted to use their low-pressure microinjection

Table 1. Inositol phosphate-induced cortical granule exocytosis in eggs of the sea urchin species *L. variegatus*

Only complete fertilization envelope elevation was scored. N.D., not determined.

	Volume (pl)	Pipette concentration	Score	
			Normal ASW	Calcium-free ASW
Ins(1,4,5) P_3	0.5	10 μ M	20/20	20/20
Ins(1,3,4,5) P_4	0.5	100 μ M	0/10	N.D.
	0.5	1 mM	9/11	12/19
Ins(2,4,5) P_3	0.5	10 μ M	4/10	N.D.
	5	10 μ M	10/10	N.D.
Ins(1,2cyc4,5) P_3	0.5	10 μ M	0/10	N.D.
	0.5	100 μ M	10/11	N.D.

method and their larger injection volumes (4% egg volume). We found that larger injection volumes (> 2% egg volume) caused more visible damage to eggs, particularly in calcium-free seawater. We did not find low pressure injection to be a satisfactory method for introducing large volumes of solution into the egg. To test the idea that large injection volumes may damage eggs we use high pressure to microinject eggs in calcium-free ASW with either 1.5 pl or 20 pl of 10 μ M-Ins(1,4,5) P_3 . In these experiments, the injection of 1.5 pl (0.3% egg volume) of 10 μ M-Ins(1,4,5) P_3 caused 85% ($n = 33$) eggs to undergo complete cortical granule exocytosis in calcium-free ASW, while the injection of 20 pl (4% egg volume) of 10 μ M-Ins(1,4,5) P_3 caused 38.6% ($n = 38$) of the eggs injected to undergo complete cortical granule exocytosis. Thus microinjecting larger volumes of the same concentration of Ins(1,4,5) P_3 caused significantly less activation ($P < 0.005$).

We made an estimate of the leakiness of eggs after microinjection by pre-loading the eggs with a fluorescent dye and measuring the rate of leakage of the dye from the eggs after large and small volume microinjections using high pressure pulses (see the Materials and methods section). We found that dye leakage was not detectable after microinjection in normal ASW with injected volumes of up to 12 pl (2.4% egg volume). In calcium-free ASW, large volume injections (12 pl, 2.4% egg volume) caused more rapid dye leakage than small volume injections (1.5 pl, 0.3% egg volume): the former caused a loss of $14 \pm 1.3\%$ (mean $\pm$ S.E.M., $n = 3$) of the dye 2 min after injection, the latter only $2.7 \pm 2.66\%$ ($n = 3$). This difference is significant ($P < 0.02$, one tailed t -test).

Measurement of Ca^{2+}_i after microinjection of inositol phosphates

Our observations that inositol phosphate-induced cortical granule exocytosis is independent of external calcium ions did not exclude the possibility that inositol phosphates cause a calcium influx that affects the duration and magnitude of the inositol phosphate-induced increase in Ca^{2+}_i , since exocytosis is not a strictly quantitative measure of Ca^{2+}_i . We therefore measured changes in Ca^{2+}_i following the injection of Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 by using the calcium-sensitive fluorescent dye fura2. The results of these experiments are shown in Fig. 3(a). We found that the injection of Ins(1,4,5) P_3 (1.5 pl of 10 μ M) in ASW caused a transient

increase in Ca^{2+}_i with a peak of 2 μ M and a duration of 3 min. Removing the external calcium ions affected neither the magnitude nor duration of the calcium response. We obtained similar results when Ins(1,3,4,5) P_4 (1 pl of 1 mM) was microinjected (Fig. 3(b)).

DISCUSSION AND CONCLUSION

We have investigated the parthenogenetic activation of sea urchin eggs by inositol phosphates and the requirements for external calcium. Ins(1,4,5) P_3 was the most active inositol phosphate tested, causing complete cortical granule exocytosis at a half-maximal concentration of 2.5 μ M, a value in good agreement with earlier findings (Whitaker & Irvine, 1984; Turner *et al.*, 1986). We have confirmed Irvine & Moor's (1986) observation that Ins(1,3,4,5) P_4 has little calcium-releasing activity in sea urchin eggs. The effects we observed may be accounted for by the contamination of our sample of Ins(1,3,4,5) P_4 with Ins(1,4,5) P_3 . In contrast we found that Ins(2,4,5) P_3 had an activity two orders of magnitude greater than that previously reported (Irvine & Moor, 1986). Our observations point to a similar sensitivity for Ins(2,4,5) P_3 in sea urchin eggs as in other tissues (Irvine *et al.*, 1984). Our finding that the two main metabolites of Ins(1,4,5) P_3 , Ins(1,3,4,5) P_4 and Ins(1,3,4) P_3 (Berridge, 1987), have little calcium-mobilizing activity in unfertilized sea urchin eggs argues against their having a significant role as internal messengers controlling Ca^{2+}_i at fertilization.

We find that inositol phosphate-induced cortical granule exocytosis occurs independently of extracellular calcium ions. Moreover, we find that the inositol phosphate-induced calcium transient is not measurably affected by the removal of external calcium. These data indicate that inositol phosphates do not increase calcium influx in sea urchin eggs in a way that significantly affects Ca^{2+}_i . Our findings are entirely consistent with the idea that Ins(1,4,5) P_3 is the crucial internal messenger at fertilization, where it brings about release of calcium from an internal store (Whitaker & Irvine, 1984; Clapper & Lee, 1985; Swann & Whitaker, 1986). These results and, indeed, previous work (Chambers, 1980; Schmidt *et al.*, 1982) make schemes in which calcium influx plays a significant part at fertilization (Irvine & Moor, 1986; Michell, 1986; Houslay, 1987) highly unlikely. Our observations clearly differ from earlier findings (Slack *et al.*, 1986; Irvine & Moor, 1986). One possible explanation

Fig. 3. Changes in Ca^{2+}_i induced by microinjection of $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$

(a) Ca^{2+}_i was measured with the calcium-sensitive dye fura2 following the microinjection of $Ins(1,4,5)P_3$ at $t = 0$ (1.5 pl, 10 μM pipette concentration). Closed symbols ($\blacktriangle$, $\blacksquare$, $\bullet$) represent injections in ordinary ASW and open symbols ($\triangle$, $\square$, $\circ$) represent injections performed in calcium-free ASW. Three experiments were performed in each case and each injection caused the elevation of a complete fertilization envelope. There is no obvious difference in the extent and duration of the $InsP_3$ -induced calcium transient in normal and calcium-free ASW. (b) Ca^{2+}_i following the microinjection of $Ins(1,3,4,5)P_4$ at $t = 0$ (1.5 pl, 1 mM pipette concentration). As in (a), closed symbols represent injections performed in ordinary ASW and open symbols represent injections performed in calcium-free ASW. Three experiments were performed in each case and each injection caused the elevation of a complete fertilization envelope.

for this difference may lie in the damage that the microinjection of large volumes causes to eggs in calcium-free ASW. It is possible that the leakiness that we measured using fluorescent dyes is sufficient to allow the EGTA present in the calcium-free ASW to enter the eggs during microinjection and chelate any calcium ions

released by the inositol phosphates. We chose to microinject small volumes to avoid this problem.

We have no explanation for our failure to observe the synergism between $Ins(1,3,4,5)P_4$ and $Ins(2,4,5)P_3$ previously reported (Irvine & Moor, 1986), though it appears that others have had similar difficulties (Irvine & Moor,

1987; Irvine *et al.*, 1988). Irvine & Moor (1987) have suggested that their inability to reproduce this crucial observation stems from a seasonal variation in the response of *L. variegatus* eggs to inositol phosphates. We find no such variation in the responses of *L. pictus* eggs. It has been suggested that whether or not a cell requires external as well as internal calcium for a response depends crucially on the size of the internal store relative to the cytosolic free calcium concentration change required to produce the response. This postulate implies that the synergism between $\text{Ins}(2,4,5)P_3$ and $\text{Ins}(1,3,4,5)P_4$ might be observed only in eggs with a depleted intracellular calcium store. This does not seem to us a satisfactory explanation, since eggs that manifested the synergism had a normal cellular response to $\text{Ins}P_3$. Well-behaved *L. variegatus* eggs have a robust intracellular store that cannot be depleted by long incubations in calcium-free sea water (Azarnia & Chambers, 1976): the postulate is therefore difficult to test.

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