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Inositol 1,4,5-Trisphosphate Mass Changes from Fertilization Through First Cleavage in *Xenopus laevis*

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After fertilization in *Xenopus laevis*, inositol 1,4,5-trisphosphate (IP3) mass increased from 53 to 261 fmol/cell and returned to near basal by 10 min after insemination. IP3 was also elevated over control egg levels during first mitosis and first cleavage. Because IP3 levels and the fertilization calcium wave decline at about the same time and because calcium ionophore or pricking the egg increased IP3, the fertilization calcium wave may be due to calcium-induced IP3 production. In addition, the onset of sperm motility was associated with an increase, whereas the acrosomal reaction was accompanied by a decrease in IP3 mass. Combining our published data with this report, the first chronology of the levels of IP3 from the induction of meiosis (maturation) through fertilization and cleavage in one cellular system is summarized. These data suggest an in vivo dose response for IP3 and calcium release. A small (17 fmol/cell) IP3 change during the induction of meiosis may not be associated with a calcium change. Larger IP3 changes at cleavage (40 fmol/cell) and mitosis (125 fmol/cell) are associated with localized small calcium increases, whereas the largest IP3 change (208 fmol/cell) is associated with the large calcium increase at fertilization.

INTRODUCTION

A sperm induces the events of fertilization due to its ability to increase the cytosolic free calcium ion concentration ($[Ca^{2+}]_i$) in the zygote (Steinhardt and Epel, 1974; for *Xenopus*, see Busa and Nuccitelli, 1985; Kubota *et al.*, 1987; Kline, 1988). After a local, sperm-induced increase in $[Ca^{2+}]_i$, a wave of elevated $[Ca^{2+}]_i$ travels from the sperm binding site to the opposite side of the cell and induces cortical granule breakdown (exocytosis), fertilization envelope elevation, and an increase in intracellular pH and protein synthesis (Gerhart, 1980; Grandin and Charbonneau, 1992). The $[Ca^{2+}]_i$ may increase due to the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol 1,4,5-trisphosphate (IP3) and sn-1,2-diacylglycerol (DAG) (Turner *et al.*, 1984; Whitaker and Irvine, 1984; Kamel *et al.*, 1985; LePeuch *et al.*, 1985).

Evidence for the involvement of PIP2 breakdown at fertilization is twofold. First, microinjection of a PIP2 breakdown product, IP3, can release $[Ca^{2+}]_i$ and induce fertilization events (Whitaker and Irvine, 1984; Busa *et al.*, 1985; Picard *et al.*, 1985). However, many other $[Ca^{2+}]_i$-releasing agents also induce fertilization events (e.g., Clapper *et al.*, 1987), and microinjected IP3 may simply mimic another mechanism used by sperm.

The second line of evidence for PIP2 involvement is that incorporation of label into PIP2, IP3, or DAG has been found to change at fertilization. Typically, turnover studies do not demonstrate the required near-to-equilibrium labeling of precursors (PIP2 for IP3 and PIP2 and other lipids, such as phosphatidylcholine or phosphatidic acid, for DAG), and separation of inactive isomers is not achieved. Possibly due to these difficulties or differences in methodology and species, turnover measurements at fertilization have produced conflicting results. Sea urchin fertilization has been associated with an increase (Turner *et al.*, 1984) or a decrease (Whitaker and Aitchison, 1985) in PIP2 labeling. One report of a large IP3 label increase at sea urchin fertilization (660% by 10 min) (Kamel *et al.*, 1985) does not compare with another report of a smaller biphasic increase (66% IP3 turnover increase at 26 s and a second increase from 2 to 30 min) (Ciapa and Whitaker, 1986). Ciapa and Whi-

[1] Abbreviations used: $[Ca^{2+}]_i$, cytosolic free calcium ion concentration; CICR, calcium-induced calcium release; DAG, sn-1,2-diacylglycerol; DMSO, dimethyl sulfoxide; IP3, inositol 1,4,5-trisphosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; TCA, trichloroacetic acid.

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taker (1986) note that the second IP3 turnover increase is not associated with a $[Ca^{2+}]_i$ increase. Using a method similar to that employed by Turner *et al.* (1984), a report of a 10-fold decrease in PIP2 mass after artificial activation of *Xenopus* eggs (LePeuch *et al.*, 1985) is in apparent opposition to a report of a 2-fold increase (see Busa, 1988). Although phospholipid breakdown at fertilization would suggest that DAG also increases, Alonso *et al.* (1986) reported no change in DAG up to 70 min after sperm addition (at which time there was a 40% decrease). In summary, label turnover measurements do not provide conclusive evidence for PIP2 breakdown at fertilization.

Because IP3 is believed to be produced only from PIP2, one method of demonstrating PIP2 breakdown at fertilization is by measuring IP3 mass. By measuring mass, problems due to prelabeling and inactive isomers are eliminated. This is the first report to find an IP3 mass increase at fertilization and to determine the magnitude and time course of the increase. We also note that IP3 mass declines to near basal level by 10 min after insemination and that IP3 increases during first mitosis and cleavage. Due to two lines of evidence, we provide support for an earlier suggestion that the fertilization $[Ca^{2+}]_i$ wave results from a positive feedback loop involving IP3 and $[Ca^{2+}]_i$. Finally, we note that sperm IP3 mass increases during motility onset and decreases during the acrosomal reaction.

MATERIALS AND METHODS

Fertilization

To obtain eggs from *Xenopus* females (Xenopus One, Ann Arbor, MI), animals were primed by injection of 100 units of pregnant mare's serum gonadotropin (Calbiochem, San Diego, CA) into the lower back region and 4 d later with 850 units of human chorionic gonadotropin (Sigma, St. Louis, MO). The eggs were collected into 100% Barth's medium (88 mM NaCl, 1 mM KCl, 0.8 mM $MgSO_4$, 0.75 mM $CaCl_2$, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, [HEPES] with daily addition of 2.4 mM $NaHCO_3$, pH 7.5) and kept in this medium until just before use. Testes were removed from male frogs and stored in 100% Barth's medium until just before use. To fertilize eggs (~21°C), 50 µl of a solution of sliced testes ([1.88 ± 0.19] × 10^6 sperm [means ± SD]; 3 experiments, 10 determinations total, measured with hemacytometer] was added to 15 eggs (a total volume of 100 µl). Based on earlier work (Wolf and Hedrick, 1971a), we used concentrations of sperm sufficient to ensure high levels of fertilization. At various times after fertilization, groups of 15 zygotes and associated sperm were homogenized in 300 µl of 25% trichloroacetic acid (TCA) with a B pestle in a Dounce homogenizer. The extract was then analyzed for IP3 mass (see DISCUSSION).

For calcium ionophore (1 µM Ionomycin or A23187, Sigma) and pricking experiments, eggs were dejellied in 2% cysteine for ~8 min and washed three times with 100 mM NaCl and 50 mM tris(hydroxymethyl)aminomethane (pH 7). In the ionophore experiments, control groups received an equal amount of the carrier solvent (dimethyl sulfoxide [DMSO]).

In some experiments, zygotes were washed free of excess sperm. After the 50 µl sperm suspension was added to groups of eggs in 250 µl 20% Barth's solution, the dish was flooded with 15 ml of 20% Barth's solution at various times after insemination. After decanting and a second brief wash, the cells were homogenized in 25% TCA (the first step in IP3 mass determination) <45 s after washing began.

For the induction of the acrosomal reaction, egg jelly was manually dissected from one egg and placed into 50 µl of ~1.8 million sperm.

Data Analysis of the IP3 Assay

The displacement of bound, radiolabeled IP3 from the IP3 receptor (calf cerebellum membrane preparation) by IP3 from extracts allowed quantification of IP3 in *Xenopus* cells (Stith *et al.*, 1992) (kit from New England Nuclear, Boston, MA). The cpm obtained in the presence of IP3 from cellular extracts was divided by the cpm obtained without added IP3 and expressed as a percentage. This percentage is directly proportional to the log of the amount of IP3. The assumptions involved in these calculations are discussed in Stith *et al.* (1992). A standard line was performed with each experiment.

To estimate the concentration of IP3 in *Xenopus* eggs, either an intracellular free volume of 0.56 µl (Houle and Wasserman, 1983) or a similar estimation of 52% of total volume (0.598 µl for a 1.3-mm-diameter cell) (Cicirelli *et al.*, 1983) was used.

Results in figures are means ± SE, whereas values reported in text are means ± SD. Tests for significance are with a two-tailed pooled Student's t test. The n values represent the number of experiments, and in each experiment there were four to six determinations per treatment.

RESULTS

Sperm IP3 Mass Increases at the Onset of Motility

Because fertilization is optimal in a dilute saline medium (Wolf and Hedrick, 1971a), sperm were transferred from 100 to 20% Barth's solution. Upon transfer to this dilute medium, sperm became motile, and there was a 1.44 ± 0.05-fold (from 5.3 to 7.7 attomol/sperm; $n = 4$; paired t test; $\alpha < 0.0004$) increase in IP3. Sperm IP3 measurements from within 10 s to 2 min after dilution were similar and the values combined.

Summarizing these and other experiments, a motile sperm in dilute (20%) Barth's solution had 12.33 ± 7.64 amol of IP3 ($n = 6$).

Sperm IP3 Mass Decreases During the Induction of the Acrosomal Reaction

In the dilute solution, the sperm acrosomal reaction can be induced by addition of egg jelly. By observation (100× magnification), the acrosomal reaction appeared complete by ~3 min after the egg jelly addition. During the acrosomal reaction, IP3 levels declined from 16 ± 2 to 1.11 ± 0.87 amol/sperm (data from 22 individual determinations of sperm with acrosomal projections; Figure 1). Egg jelly alone contained no IP3 ($n = 3$).

Although we believe the above method is more accurate, we determined IP3 in "acrosomal" sperm by two other methods that depend on the difference between two large numbers. First, fertilization was induced with increasing amounts of sperm, and IP3 mass in the egg-sperm mixture was measured at 1 min after insemination. Fertilization with 0.18, 1.8, and 18 million sperm resulted in IP3 values of 13.2 ± 1.8, 17.5 ± 2.0, and 32 ± 16 pmol (4 determinations at each sperm concentra-

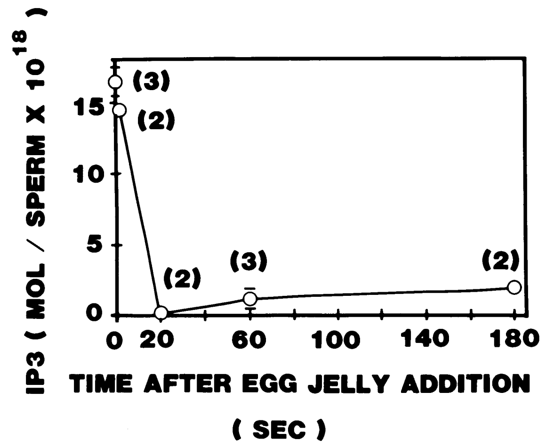

Figure 1. Sperm IP3 mass decreased during the acrosomal reaction. Sperm (1.8 million) were added to one egg jelly in a total volume of ~50 μl. The numbers in parentheses represent the number of experiments (with, as usual, 4–6 determinations per experiment).

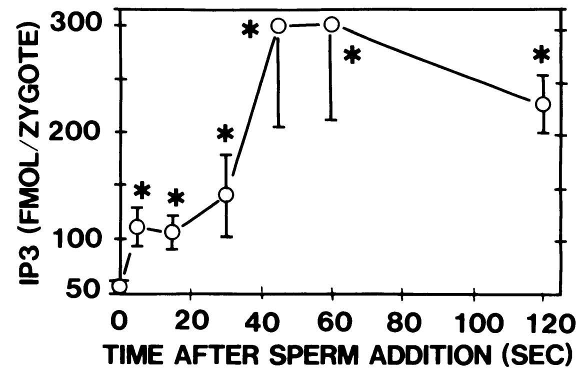

Figure 2. IP3 mass increased at fertilization from basal values to 304 ± 148 fmol/zygote (average of points from 45 s to 5 min after insemination; see Figure 4 for 5-min point). IP3 values from 5 to 30 s were greater than control value ($p < 0.008$) but less than the average of DAG values from 45 s to 5 min ($p < 0.0007$). After correction for sperm, the maximal IP3 value was 234 fmol/zygote (see RESULTS). The first IP3 increase (from 5 to 30 s; 0.89 ± 0.40 pmol/15 zygotes plus 50 μl of sperm, n = 8) is probably due to sperm as IP3 in an equivalent amount of sperm measured during some of these experiments (0.55 ± 0.12 pmol/50 μl of sperm, n = 4) was not significantly different. Asterisks denote significance at $p < 0.02$.

tion). By dividing the change in IP3 (pmol) by the difference in the number of sperm, one can estimate the amount of IP3 per sperm (which, at this point, have undergone the acrosomal reaction). The first difference produced 2.7 amol and the second produced 0.9 amol IP3 per sperm. Another method of estimation of sperm IP3 is from the IP3 increase at 5 and 15 s after sperm addition to eggs (Figure 2). This estimate assumes that the IP3 increase at these times is due to sperm alone, that sperm have interacted with the egg jelly, and that sperm–egg membrane contact has not taken place (see DISCUSSION). The increase in IP3 at 5 and 15 s divided by the number of sperm results in an estimate of 0.5 amol/sperm. Averaging the data from these two other methods for determining IP3 mass in "acrosomal" sperm, the results (1.4 ± 1.2 amol/sperm) are similar to that obtained with egg jelly addition (1.1 amol/sperm).

Basal Level of IP3 in a Xenopus Egg

The basal amount of IP3 in a *Xenopus* egg was 53.18 ± 9.68 fmol (n = 12; i.e., 12 experiments with 4–6 determinations per experiment). This IP3 level is comparable with those of the 8-, 16-, and 32-cell *Xenopus* embryo (~75 fmol/embryo) (Maslanski *et al.*, 1992).

IP3 Changes at Fertilization

There was an immediate increase in IP3 at 5 and 15 s after addition of sperm to egg that was due to sperm alone (Figure 2). Note that time values will be given as "time after insemination." There was a second IP3 increase at ~45 s after sperm addition, and IP3 remained at a relatively constant high level through 5 min (data from 45 s to 5 min were not significantly different; see also Figure 4). We suggest that sperm–egg interaction begins at about 45 s because there is an IP3 increase at this time. This suggestion was supported by "washing experiments" to estimate sperm–egg contact. After insemination, the washing of zygotes to remove nonfertilizing sperm should inhibit development if the wash is before sperm–egg contact has taken place. Under our fertilization conditions, we noted that washing sperm away before ~1 min after insemination inhibited sub-

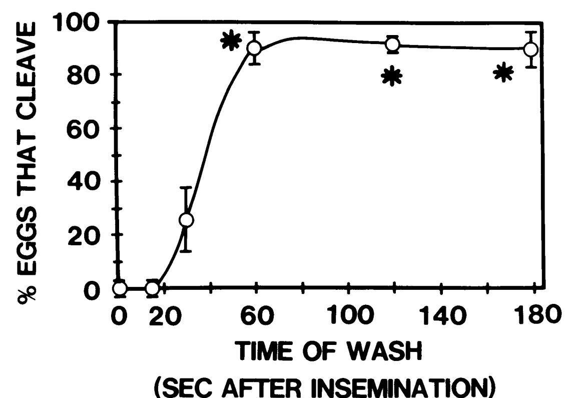

Figure 3. Estimation of sperm–egg contact by washing sperm from eggs. The time that eggs were washed free of "excess" sperm was expressed relative to the time of insemination (which was time zero). Because washing zygotes at 1, 15, and 30 s after sperm addition inhibited development, we estimated that sperm–egg contact begins at ~30 s. Development was defined by the percentage of eggs that were fertilized and go through cleavage after washing. Results are from three experiments and asterisks denote significance at $p < 0.01$.

sequent cleavage (Figure 3). This suggested that sperm–egg contact begins at ~30 s and is complete by ~1 min.

Because sperm that do not fertilize contribute IP3, we needed an estimate of sperm IP3 to subtract from the maximal IP3 value obtained from both zygote and sperm. The peak zygote IP3 mass value was determined by two methods of correction for sperm IP3. First, we used the sperm–egg jelly data to correct the peak IP3 value from Figures 2 and 4. Because the average "acrosomal" sperm IP3 value was 1.1 amol/sperm and because 1.8 million sperm were used to fertilize, the excess sperm correction value would be 1.99 pmol. Combining the IP3 values from 45 s to 5 min (Figures 2 and 4), we report a maximal IP3 value of 5.50 ± 1.99 pmol per 15 zygotes plus excess sperm (n = 11) for this period. After subtracting the sperm correction factor from 5.5 pmol and dividing by 15 (number of zygotes), the peak IP3 value would be 234 ± 85 fmol/zygote.

As a second method, we washed away excess sperm at 5 min after fertilization. Compared with egg levels (57 ± 10 fmol/egg; 5 determinations), washing the zygote at 5 min after insemination produced an IP3 value that was significantly higher (288 ± 43 fmol/zygote; 6 determinations). This value was not significantly different from the zygote IP3 mass estimate obtained by the sperm–egg jelly correction factor (234 fmol/zygote).

Thus, two different procedures for sperm IP3 correction produced similar estimates of maximal IP3 mass in the zygote. In summary, IP3 increased at fertilization from a basal level of 53 fmol/egg to an average IP3 peak value of 261 fmol/zygote.

Artificial Activation Induces a Small Increase in IP3

Addition of a calcium ionophore or pricking of the egg increased IP3 from ~60 to ~100 fmol/egg (Table 1). These increases were smaller than that induced by addition of sperm. Pricking a *Xenopus* oocyte (which is not believed to induce a large $[Ca^{2+}]_i$ wave) did not induce a measurable increase in IP3.

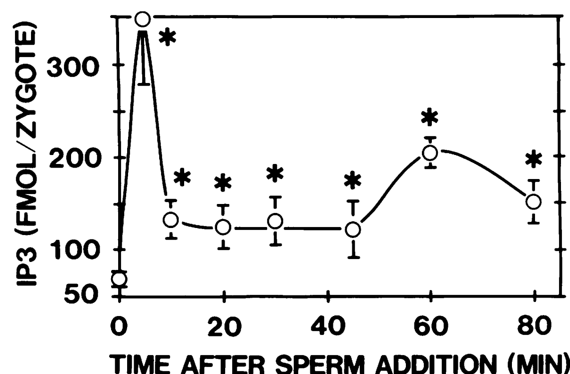

Figure 4. IP3 mass declined by 10 min after insemination but remained above control levels. Peak IP3 mass declined from ~350 fmol/zygote at 5 min to ~126 fmol/zygote from 10–45 min. During the time of first mitosis (60–80 min), IP3 increased again to ~178 fmol/zygote. Asterisks denote $p < 0.04$ (n = 5).

Table 1. Artificial activation and IP3 changes in dejellied *Xenopus* eggs

	Control	45 s	2 min
Calcium ionophore (n = 5)[a]	61.7 ± 2.7	110 ± 7.3[b]	72.3 ± 4.8[b]
Prick activation (n = 3)	59.8 ± 1.4	93.7 ± 8.3[b]	68.0 ± 6.1[b]

Values are fmol per egg ± SD. Control group for ionophore experiments received an equivalent amount of carrier DMSO. As noted in MATERIALS AND METHODS, each experiment had four to five determinations per sample time.

[a] Results of three experiments with ionomycin and two with A23187 (there was no significant difference between the results of the two treatments, so the data were combined).

[b] Significant difference from controls ($\alpha < 0.05$).

Late IP3 Changes

IP3 levels declined after 5 min (Figure 4) but were slightly elevated from 10 through 45 min (from 65 to 126 fmol/zygote). The excess sperm contribution was not significant at these times because washing away excess sperm at 20 min resulted in similar elevated IP3 levels in the zygote. At the time of first mitosis (60–80 min), IP3 was again elevated to ~178 fmol/zygote.

Under our conditions for fertilization, cleavage began at ~103 ± 26 min (*n* = 7). Due to this variability, we grouped zygotes and zygotes in cleavage before IP3 analysis. A group of zygotes referred to as "BEF" was analyzed about 10 min before cleavage (with cleavage time determined by a group that was fertilized earlier). Zygotes in the BEF group had IP3 levels that were similar to control eggs (53 fmol/zygote) (Figure 5). From the appearance of the cleavage dimple through cleavage furrow formation, IP3 was ~1.8-fold of control values. Thus, during cleavage, IP3 was elevated to ~93 fmol/zygote (an average from early, mid, and late groups; n = 4).

DISCUSSION

Sperm IP3 Changes

We have examined sperm IP3 mass under two conditions. First, placement of sperm into dilute medium induced sperm motility and an increase in IP3 mass. Changes induced by the dilute media (membrane potential changes? volume changes? intracellular pH changes?) may increase sperm IP3. As high levels of extracellular calcium induce motility even in concentrated solutions (see Table 1 in Wolf and Hedrick,

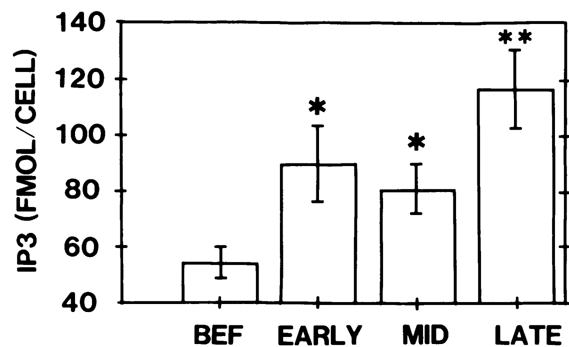

Figure 5. IP3 levels increase through first cleavage. Cleavage results in two cells, but we record the amount of IP3 on a per cell basis. As cleavage occurs at different times in zygotes from different animals, we grouped zygotes by the following characteristics. BEF is ~10–15 min before a cleavage dimple appears (as denoted by a group that was fertilized earlier), EARLY is when a dimple appears, MID is when the dimple expands to a cleavage furrow that was halfway around the oocyte (~10 min after dimple appearance), and LATE is when the cleavage furrow was around the whole circumference of the oocyte (~15–20 min after dimple appearance). One asterisk denotes significance at $p < 0.01$, whereas two denote significance at $p < 0.002$ ($n = 4$).

1971b), elevated IP3 may be involved in the induction of sperm motility.

The second condition was induction of the sperm acrosome reaction by egg jelly. Our report of an IP3 decrease during the acrosomal reaction is surprising but not in disagreement with a requirement for extracellular calcium and a calcium influx (Wolf, 1974). The calcium influx associated with the acrosomal reaction may induce the breakdown of IP3. In opposition to this possibility, we found that elevated egg $[Ca^{2+}]_i$ induced an increase in IP3. Our IP3 determination in sperm that have undergone an acrosomal reaction is based on three methods that produce similar values (~1.1 amol/sperm).

IP3 and Egg Activation

There has been much speculation that a soluble agent diffuses from the sperm to activate the egg (e.g., Swann *et al.*, 1992). If IP3 is the soluble agent, an estimate of the amount of IP3 required to activate the egg is needed. With iontophoresis, ~0.2–0.7 fmol IP3 were needed to activate the *Xenopus* egg (Busa *et al.*, 1985). Because the amount of IP3 in "acrosomal" sperm is 180-fold lower than this estimated value, one sperm's IP3 (*Xenopus* eggs are typically monospermic) (Grey *et al.*, 1982) may not be sufficient to activate the egg. However, the estimate of required IP3 is from studies that utilized IP3 injection deep into the egg, and less IP3 may be needed if it is released into a localized area (i.e., sperm binding site) of the cortex. For example, Whitaker and Irvine (1984) have suggested that only about 2.5 amol of IP3 are required to activate sea urchin eggs, and this value is similar to our estimate of sperm IP3 (1.1 amol). Because direct measurements of IP3 diffusion into the egg have not been made, the question of whether sperm IP3 is responsible for activating the egg is still unanswered (see Rakow and Shen, 1990).

Alternate methods of sperm activation of the egg involve a membrane receptor (for *Xenopus*, see Kline *et al.*, 1991) or diffusion from the sperm of an agent other than IP3 (see summary in Nuccitelli, 1991). The measurement of a "latent period" between sperm binding and IP3 production would help decide which method sperm use to activate the egg (Whitaker *et al.*, 1989; Nuccitelli, 1991). Our estimation of a latent period between sperm addition and the IP3 increase in the zygote is about 45 s, and this agrees with wash experiments that may estimate sperm–egg contact (Figure 3). However, lag period estimation varies due to differences in sperm concentration, solution composition, procedures, and even the eggs themselves (e.g., noted for sea urchin) (Allen and Griffin, 1958). Sperm–egg binding can be as early as 20–55 s (Figures 1 and 4 in Webb and Nuccitelli, 1985), 1.5–3 min (Grey *et al.*, 1982), or 6 ± 4 min (eggs matured in vitro) (Kline *et al.*, 1991). One important factor is the time eggs and sperm spend in dilute medium. If eggs are incubated in low tonicity medium for 90 min before insemination, fertilization (as noted by the fertilization potential) can require up to 30 min (Grey *et al.*, 1982).

Our determination of the changes in IP3 may be compared with those of $[Ca^{2+}]_i$ at fertilization. IP3 mass increases about fivefold (from 53 fmol/egg to 261 fmol/zygote), whereas $[Ca^{2+}]_i$ increases about threefold (Busa and Nuccitelli, 1985). Both IP3 mass and $[Ca^{2+}]_i$ decrease after 5 min, with $[Ca^{2+}]_i$ falling to basal values by ~12 min (Busa and Nuccitelli, 1985).

Nanomolar amounts of IP3 increase $[Ca^{2+}]_i$ in sea urchin eggs (Whitaker and Irvine, 1984; Turner *et al.*, 1986) and *Xenopus* oocytes (Stith and Proctor, 1989) and eggs (Busa *et al.*, 1985). If the fertilization IP3 mass increase is uniformly distributed throughout the cell, IP3 concentration would increase from ~95 to 466 nM. Larabell and Nuccitelli (1992) have suggested that IP3 production takes place largely near the surface of the egg. If the IP3 increase takes place only in the outer 200 or 5 μm (the latter being an estimate of egg cortex) of the zygote, IP3 peak concentrations in these locations would be ~1.15 or 15.85 μM, respectively.

Our estimate of the IP3 increase does not compare well with a prior estimate based on PIP2 breakdown. LePeuch *et al.* (1985) estimate that 1100 nmol of IP3 would be produced by 9 min after pricking the *Xenopus* egg (PIP2 mass decreased from 1200 to 100 nmol/egg). Our IP3 mass increase due to pricking (34 fmol/egg) was ~10^7-fold smaller than this estimate from PIP2 mass. Han *et al.* (1992) did not measure PIP2 but esti-

mated that the *Xenopus* egg has 10 pmol. Both of these estimates (1.2 μmol and 10 pmol PIP2 per egg) may be too high as two careful estimates of PIP2 in sea urchin eggs found much lower values. Although the sea urchin egg has a surface area that is ~170-fold smaller than that of the *Xenopus* egg, sea urchin PIP2 has been found to be ~3 fmol/egg (3 fmol/egg, Turner *et al.*, 1984; 2.8 fmol/egg assuming a 100 μm diameter, Swann *et al.*, 1987).

Early Fertilization Events Including the $[Ca^{2+}]_i$ *Wave*

Various events can be compared with the time course of the early IP3 increase. After sperm addition, a $[Ca^{2+}]_i$ wave originates at the sperm binding site and travels across the *Xenopus* zygote from ~2 to 7 min (Busa and Nuccitelli, 1985; Kubota *et al.*, 1987). This wave induces cortical granule breakdown, cortical contraction, and an intracellular pH increase (Webb and Nuccitelli, 1981; Grandin and Charbonneau, 1992).

There has been much debate about whether the fertilization $[Ca^{2+}]_i$ wave is due to calcium-induced calcium release (CICR) or an IP3–$[Ca^{2+}]_i$ positive feedback loop (i.e., elevated IP3 releases $[Ca^{2+}]_i$ and elevated $[Ca^{2+}]_i$ then stimulates IP3 production). In the sea urchin $[Ca^{2+}]_i$ wave, there is evidence of a role for IP3 (Whitaker and Irvine, 1984; Whitaker and Aitchison, 1985; Swann and Whitaker, 1986) and CICR (Clapper *et al.*, 1987; Rakow and Shen, 1990; Galione *et al.*, 1991; McPherson *et al.*, 1992). In contrast to these studies with sea urchins, the $[Ca^{2+}]_i$ wave at hamster fertilization may be solely due to IP3. Monoclonal antibody to the IP3 receptor completely blocked both the initial localized $[Ca^{2+}]_i$ increase at the sperm binding site and the subsequent $[Ca^{2+}]_i$ wave in hamster zygotes (Miyazaki *et al.*, 1992).

In *Xenopus* eggs, most evidence suggests that IP3 is involved in the $[Ca^{2+}]_i$ wave. Injection of IP3 induces a $[Ca^{2+}]_i$ wave (Busa *et al.*, 1985) that is not inhibited by procaine or mimicked by caffeine (CICR regulators) (Busa, 1988). An antibody to PIP2 reduces wave $[Ca^{2+}]_i$ near the plasma membrane (Larabell and Nuccitelli, 1992). Heparin inhibits IP3 binding to the *Xenopus* IP3 receptor (Parys *et al.*, 1992) and inhibits the $[Ca^{2+}]_i$ wave in oocytes (DeLisle and Welsh, 1992). Finally, because no ryanodine receptors have been found in the *Xenopus* egg (Parys *et al.*, 1992), CICR may not play a role in the fertilization $[Ca^{2+}]_i$ wave. In opposition, IP3 may not be responsible for the $[Ca^{2+}]_i$ wave due to experiments demonstrating two pools of $[Ca^{2+}]_i$ in eggs (Busa *et al.*, 1985). In oocytes, $[Ca^{2+}]_i$ regulation of IP3 action (Parker and Ivorra, 1990; DeLisle and Welsh, 1992; Lechleiter and Clapham, 1992) and not increased IP3 may be involved in wave propagation. Our evidence supports a role for an IP3/$[Ca^{2+}]_i$ feedback loop in the $[Ca^{2+}]_i$ wave of the *Xenopus* egg: IP3 levels and the $[Ca^{2+}]_i$ wave decline at about the same time and two methods that artificially increase intracellular $[Ca^{2+}]_i$ (pricking and calcium ionophore) increase IP3.

Because artificial elevation of $[Ca^{2+}]_i$ in an oocyte does not induce a $[Ca^{2+}]_i$ wave, it has been suggested that elevated $[Ca^{2+}]_i$ does not stimulate IP3 production (DeLisle and Welsh, 1992). Our preliminary report of the inability of pricking to increase oocyte IP3 supports this notion. However, there are differences between the oocyte and the egg (e.g., the oocyte lacks a cortical reticulum) (see summary in Larabell and Nuccitelli, 1992). As opposed to the oocyte, artificial elevation of egg $[Ca^{2+}]_i$ can induce a wave in the egg (Kubota *et al.*, 1987) and increase IP3 mass.

Pricking is one method of artificial elevation of $[Ca^{2+}]_i$, and, as noted, it induces a $[Ca^{2+}]_i$ wave (Kubota *et al.*, 1987) and a small increase in IP3. At 2 min after pricking, a well-defined $[Ca^{2+}]_i$ wave is present, yet IP3 levels are only 8 fmol above basal (Table 2). Using a torus to represent the area of the $[Ca^{2+}]_i$ wave at 2 min after pricking, an estimate of the $[Ca^{2+}]_i$ wave free volume would be 0.056 μl (with free volume 52% of total volume; a 1.3-mm-diameter egg; a $[Ca^{2+}]_i$ wave diameter of 0.2 mm per Kubota *et al.*, 1987; and torus volume $2\pi^2Rr^2$, where $R = 0.055$ cm, $r = 0.01$ cm). If the increase in IP3 at 2 min after pricking (8 fmol; Table 1) occurs largely in the $[Ca^{2+}]_i$ wave, IP3 concentration would increase from control levels (in these experiments 107 nM) to ~250 nM. Thus, a small but localized increase in IP3 mass may be sufficient to raise IP3 concentration to levels that release $[Ca^{2+}]_i$.

Late Events After Fertilization

The time (10–30 and 60–80 min; Figure 4) of elevated IP3 can be related to the time of various late events of fertilization (unless noted, Gerhart, 1980; Ubbels *et al.*, 1983). Completion of meiosis II (when the second polar body appears) occurs at ~15–20 min and sperm aster formation and postfertilization waves at ~16–45 min. Other events and their times include onset of DNA syn-

Table 2. Inositol 1,4,5-trisphosphate increases during four developmental periods

Period	IP3 (fmol/cell) Change	Magnitude
Induction of meiosis (oocyte to egg)	From 29 to 46	17
Fertilization (egg to zygote)	From 53 to ~261	208
First mitosis (60–80 min after fertilization)	Increase to ~178	125
First cleavage (before to late cleavage)	Increase to ~93	40

thesis (27–40 min), cortical rotation (30–45 min), pronuclear migration (25–50 min) followed by fusion (~70 min), an increase in maturation promoting factor activity (60–80 min) (Gerhart *et al.*, 1984), mitosis (75–90 min), and surface contraction waves (70–90 min). Due to the overlapping nature of these events, direct correlation between IP3 elevation and a single event is not feasible.

Although IP3 was elevated at 10–30 min and during mitosis, no elevation of $[Ca^{2+}]_i$ has been found during these periods in *Xenopus* (Rink *et al.*, 1980; Busa and Nuccitelli, 1985). In sea urchin cells, $[Ca^{2+}]_i$ increases have been found during pronuclear migration, possibly at nuclear envelope breakdown, at anaphase, and at cleavage (summaries in Whitaker and Patel, 1990; Han *et al.*, 1992). There is also evidence that IP3 production may be required for these events (Forer and Sillers, 1987; Petzelt *et al.*, 1989). The inability to describe a $[Ca^{2+}]_i$ increase in *Xenopus* during these periods may be a technical problem, because Kline (1988) has noted that prevention of a $[Ca^{2+}]_i$ increase prevents chromatin decondensation, pronuclei and aster formation, and migration of sperm nuclei in *Xenopus* zygotes. Our report of elevated IP3 during these periods suggests that more sensitive techniques may demonstrate small localized $[Ca^{2+}]_i$ increases from 20 min through mitosis in the *Xenopus* zygote.

IP3 Mass Increase During Cleavage

Our demonstration of an increase in IP3 during cytokinesis is not surprising in light of the small $[Ca^{2+}]_i$ increase during *Xenopus* cleavage (Baker and Warner, 1972; Grandin and Charbonneau, 1991). A $[Ca^{2+}]_i$ increase during sea urchin cleavage (Whitaker and Patel, 1990) and at the cleavage furrow of medaka zygotes also has been described (Fluck *et al.*, 1991). Further evidence of an IP3–$[Ca^{2+}]_i$ requirement during this period is that calcium chelators (Baker and Warner, 1972; Han *et al.*, 1992), an antibody to PIP2 (which reduces calcium release), or heparin (which blocks IP3 release of $[Ca^{2+}]_i$) (Han *et al.*, 1992) inhibit cleavage in *Xenopus* embryos.

In Vivo IP3-$[Ca^{2+}]_i$ Dose-Response Relationship

By including earlier data (Stith *et al.*, 1992), IP3 changes during the induction of meiosis in the oocyte, fertilization, first mitosis, and cleavage of the zygote can be compared (Table 2). The smallest IP3 change is associated with the induction of meiosis (17 fmol/oocyte), and most but not all studies are unable to show any $[Ca^{2+}]_i$ change during this period (for summary, see Stith *et al.*, 1992). During fertilization, there is a 12-fold larger IP3 increase and a well-described large increase in $[Ca^{2+}]_i$. Intermediate IP3 increases were noted during mitosis (125 fmol/zygote) and cleavage (40 fmol). A small $[Ca^{2+}]_i$ increase has been found during cleavage (Grandin and Charbonneau, 1991) but, possibly due to a localized $[Ca^{2+}]_i$ increase deep within the cell (at the nuclear envelope and mitotic spindle), no $[Ca^{2+}]_i$ changes have been noted during mitosis in *Xenopus* zygotes. Thus, for large *Xenopus* cells, intermediate IP3 changes on the order of 40–125 fmol/cell may produce only localized $[Ca^{2+}]_i$ increases that are difficult to detect.

In light of the above relationship, comments about the distribution of IP3 in an unstimulated egg may be made. If basal IP3 is distributed throughout the free volume of the *Xenopus* egg (~0.56 μl; see MATERIALS AND METHODS), basal IP3 concentration would be ~95 nM. If the IP3 is concentrated in the outer 5 (0.0132 μl) or 200 μm (0.198 μl) of the egg (with 52% free volume), basal concentration would be 4.0 μM and 269 nM, respectively. Although IP3 could be sequestered, the last two estimates suggest that IP3 is not concentrated at the plasma membrane of the unstimulated cell.

In conclusion, we find support for the idea that fertilization events are due to IP3 production. We find that IP3 mass increases ~45 s after sperm addition and reaches a maximal value of about fivefold over control levels. Because artificial elevation of $[Ca^{2+}]_i$ increases IP3 and because IP3 declines at about the same time as the fertilization $[Ca^{2+}]_i$ wave, support for the presence of a positive feedback loop of $[Ca^{2+}]_i$ and IP3 is described. Elevation of IP3 mass during the completion of meiosis, first mitosis, and cleavage is noted. Finally, onset of sperm motility is associated with an increase in IP3 mass, whereas the acrosomal reaction is accompanied by a decrease. A future article will report diacylglycerol mass changes during these same time periods.

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