Polyphosphoinositide metabolism during the fertilization wave in sea urchin eggs

BRIGITTE CIAPA¹, BÉATRICE BORG¹ and MICHAEL WHITAKER²*

¹Laboratoire de Physiologie Cellulaire et Comparée, Faculté des Sciences, Parc Valrose, 06034 Nice Cedex, France
²Department of Physiology, University College London, Gower Street, London WC1E 6BT, UK

*Author for correspondence

Summary

A transient increase in intracellular free calcium is believed to be the signal responsible for the stimulation of the egg metabolism at fertilization and the resumption of the cell cycle. We have studied how the polyphosphoinositides (PPI) turn over at fertilization in sea urchin eggs, in order to determine the relationship between the metabolism of these lipids and the calcium signal. We compare the patterns of PPI turnover that occur during the first minute following fertilization in eggs in which PPI are labelled to steady state with [³H]inositol or [³H]arachidonate with that in which PPI are labelled for a shorter period with [³H]inositol. When eggs are labelled to apparent isotopic equilibrium with either [³H]inositol or [³H]arachidonate, no early increase in [³H]PtdInsP₂ occurs while PtdIns decreases slightly. On the contrary, when not labelled to isotopic equilibrium, all [³H]PPI increase during the first 15 seconds following fertilization. We find that, within seconds, fertilization triggers a 600-fold increase in the turnover of PPI, producing an amount of InsP₃ apparently sufficient to trigger calcium release. We suggest that phosphoinositidase C and PtdIns₃ kinase, responsible respectively for the hydrolysis and synthesis of PtdInsP₂, are both stimulated to a comparable degree in the first 30 seconds following fertilization and that net changes in the amount of PtdInsP₂ at fertilization are very sensitive to the relative levels of activation of the two enzymes. Activating the eggs with the calcium ionophore A23187 showed that both these enzymes are sensitive to calcium, suggesting that calcium-dependent InsP₃ production might play a role in the initiation and/or the propagation of the fertilization calcium wave. A comparison of the rates of PPI turnover in fertilized and A23187-activated eggs confirms that the fertilizing sperm stimulates an early and possibly calcium-dependent increase in PtdInsP₃ kinase activity.

Key words: calcium, fertilization, sea urchin egg, phosphoinositide, phosphoinositidase C, PI kinase

Introduction

It is well accepted that the activation of most deuterostome eggs at fertilization is initiated by a transient increase in intracellular free calcium (Ca) which is due to the release of calcium from intracellular stores (reviewed by Jaffe, 1983, Whitaker and Steinhardt, 1985 and Gillot et al., 1990). This ionic signal appears in the form of a wave of calcium that propagates in the egg from the point of sperm-egg interaction (reviewed by Whitaker et al., 1989; Mohri and Hamaguchi, 1991). It is, then, important to understand how the calcium wave is initiated and how it propagates.

Calcium waves are also a feature of calcium signalling in somatic cells (Berridge and Galione, 1988). In most cells where it has been studied, the calcium signal emanates from the hydrolysis by phosphoinositidase C of the plasma-membrane polyphosphoinositide lipids (PPI) (reviewed by Berridge and Irvine, 1989; Downes and McPhee, 1990 and Bansal and Majerus, 1990). There is good evidence to support the idea that the polyphosphoinositide messengers, inositol trisphosphate (InsP₃) and diacylglycerol (DAG), play a crucial role in sea urchin egg activation (reviewed by Whitaker, 1989). It appears that InsP₃ can release calcium from intracellular stores in the egg both in vivo (Whitaker and Irvine, 1984) and in vitro (Clapper and Lee, 1983). In addition, activating protein kinase C with phorbol esters, which mimic the effects of DAG (reviewed by Nishizuka, 1986), leads to the stimulation of several metabolic processes in the egg normally induced at fertilization, the most important being the stimulation of the Na/H+ exchange (reviewed by Shen, 1989 and Ciapa et al., 1991). The best arguments that the activating pathway set up by the fertilizing sperm involves PPI turnover can be made from experiments where changes have been measured in vivo. An increase in InsP₃ (or related inositol phosphates) and
DAG production occurs after fertilization (Ciapa and Whitaker, 1986; Swann et al., 1987). It has also been reported that PtdInsP$_2$ and PtdInsP$_{2'}$ levels increase (Turner et al., 1984) while that of PtdIns decreases after fertilization (Kamel et al., 1985). Incorporation of $[^3H]$inositol into PtdInsP$_2$ and PtdInsP$_{2'}$ also rises substantially at fertilization (Turner et al., 1984; Kamel et al., 1985). These reported increases are perhaps surprising, given that the two messengers, InsP$_3$ and DAG, are produced by the hydrolysis of PtdInsP$_2$, implying a decrease in the levels of PtdInsP$_2$. We undertook this study in order to determine the relationship between the metabolism of PPI and the production of DAG and InsP$_3$ that we have described previously. Our data indicate that fertilization massively stimulates both the production and hydrolysis of PtdInsP$_2$ and that the net increase in PtdInsP$_2$ at fertilization represents only a fraction of the PtdInsP$_2$ turned over during the fertilization calcium wave.

**Materials and methods**

**Handling of gametes**

Experiments were performed with gametes of *Paracentrotus lividus*, collected from the bay of Villefanche-sur-Mer, or of *Lytechinus pictus*, obtained from Pacific Biomarine Inc. Gametes of *Paracentrotus lividus* were obtained by dissecting and shaking the gonads in artificial sea water (for all experiments performed with *Paracentrotus lividus*: ASWp is from Mannemix Wiegant GMBH, pH 8.0). Gametes of *Lytechinus pictus* were obtained by intracoelomic injection of 0.5 M KCl and shed into artificial sea water (for all experiments performed with *Lytechinus pictus*: ASW is 450 mM NaCl, 50 mM MgCl$_2$, 11 mM CaCl$_2$, 10 mM KCl, 2.5 mM NaHCO$_3$, 1 mM EDTA, pH 8.0). For all eggs, egg jelly was removed by passage through a 90 μm nylon mesh. Eggs were fertilized in a 5% v/v suspension in ASW at a sperm density of 10$^6$/ml at 13°C.

**Labelling polyphosphoinositides**

A 20% suspension of unfertilized eggs was incubated at 13°C for 18 to 36 hours (according to experiments) in ASW containing an antibiotic (0.1% sulfadiazine) and *myo-2* $[^3H]$inositol (10 μCi/ml) or $[^3H]$arachidonic acid (10 μCi/ml) (Amersham Int. Inc.). Eggs were rinsed 4 times by decantation in cold ASW and then dejellied before all experiments.

**Extracting and separating polyphosphoinositides**

Samples were taken at intervals into ice-cold trichloracetic acid (TCA: 10% w/v final concentration). After centrifugation, the TCA pellet was washed with distilled water and extracted with chloroform/methanol/11 N HCl (100/200/5 v/v) overnight at 4°C. $[^3H]$inositol-labelled polyphosphoinositides were separated by chromatography on oxalate-treated silica gel 60 thin-layer chromatography plates (T-6395 Sigma) in chloroform/methanol/aceton/acetate acid/water (40/35/5/12.5/12.8) (Allan and Cockroft, 1983). Two-dimensional chromatography was used to distinguish PtdIns from phosphatidylcholine labelled with $[^3H]$arachidonic acid, in which case the first dimension was chloroform/methanol/7 M ammonium hydroxide (60/35/5; Kirchner, 1967). $[^3H]$arachidonic-DAG was separated by developing thin layer plates in light petroleum/diethyl ether/acetic acid (97.5/2.5/1.5; Homa et al., 1980). TLC plates were sprayed with a scintillation mixture (0.4% PPO in β-methyl naphthalene dissolved in toluene) and developed by autoradiography. An autoradiogram of each plate (films X-OMAT Kodak) was used to identify radioactive lipids. Lipids were recovered from the substrate and measured by liquid scintillation counting. All lipids were identified using comigration with commercial standards (Sigma) as the criterion.

Protein content of the extracted pellets was determined using a Bradford assay (Bradford, 1976) for *Lytechinus pictus* or according to the method described by Lowry et al. (1951) for *Paracentrotus lividus*.

**Statistical significance of data**

Although the general pattern of alterations in PPI metabolites under the various experimental conditions we report here was consistently observed, the precise kinetics of the changes varied between one egg batch and the next. This was not due to variations in fertilization rate between egg batches, nor to variable recovery of PPI metabolites: both of these possibilities were checked in control experiments. The consequence of this kinetic variability is that pooled data at each time point from experiments on different egg batches shows considerable dispersion. We have indicated this dispersion in some figures by using error bars. In other figures, we have shown representative, individual experiments. We believe (see Discussion) that the kinetic variation is the result of small differences in the relative rates of production and destruction of PPI metabolites between egg batches, though we cannot formally exclude other reasons for the variation than the two we have investigated. Others find similar variation (Turner et al., 1984).

**Results**

**Incorporation of $[^3H]$arachidonate and $[^3H]$inositol into polyphosphoinositides of unfertilized eggs**

In order to compare the turnover of PPI in unfertilized and fertilized eggs, we first measured the rate of incorporation of two radio-labelled precursors ($[^3H]$arachidonate and $[^3H]$inositol) into PPI of unfertilized eggs of two species, *Lytechinus pictus* and *Paracentrotus lividus*.

Fig. 1A shows the time course of accumulation of $[^3H]$inositol into unfertilized eggs and into eggs lipids in these two species. It appears that the aqueous cytoplasmic phase equilibrates relatively slowly and contributes therefore to the slow rate of incorporation of $[^3H]$inositol into PPI. For both species, steady state was reached only after 30 hours of incubation with the precursor.

Fig. 1B shows the time course of incorporation of $[^3H]$inositol into PtdIns, PtdInsP and PtdInsP$_2$ of *Lytechinus pictus*. The kinetics of incorporation in all of the PPI were sigmoidal, most probably due to the slow increase in $[^3H]$inositol intracellular specific radioactivity. In this experiment, apparent isotopic equilibrium was reached after 30 hours of incubation. The initial rates of incorporation of $[^3H]$inositol in the three PPI indicates that the precursor appeared first in PtdIns, then in PtdInsP and later in PtdInsP$_2$. This reflects the fact that inositol enters the PPI cycle during PtdIns synthesis. Similar results were found when experiments were performed with *Paracentrotus lividus* (not shown).
Polyphosphoinositide turnover at fertilization

The rates shown in Table 2A, measured from the time course of incorporation of $[^{3}H]$inositol into each PPI of *Lytechinus pictus*, indicate that sea urchin egg PPI turns over at around 10%/hour, and that the rate of incorporation of this precursor into PtdIns is similar to the rate of phosphorylation of PtdIns to PtdInsP and PtdInsP to PtdInsP$_2$. This inference is supported by the observation that the PtdInsP and PtdInsP$_2$ of eggs incubated with $^{32}$P reach isotopic steady state after 20-25 hours (not shown).

We have shown that $[^{3}H]$arachidonate-labelled DAG is generated during the calcium transient at fertilization (Ciapa and Whitaker, 1986). To investigate the relation between the production of this messenger and the metabolism of PPI, we followed the incorporation of this precursor in eggs and lipids of *Lytechinus pictus*. Arachidonate is found at the 2-position in PPI (Baker and Thompson, 1972). We found that $[^{3}H]$arachidonate was incorporated rapidly into PPI in unfertilized eggs, and that PPI were labelled to constant specific activity within 3 hours (results not shown). This presumably reflects the relative ease with which phospholipid acyl side chains are exchanged (Irvine, 1986). After three hours incubation with $[^{3}H]$arachidonate (steady state), 50% of the radioactivity was incorporated into endogenous lipids and of that, 70% was found in PPI, 0.6% in phosphatidylethanolamine (PE) and 3% in phosphatidylcholine (PC). The remaining extractable counts were found in neutral lipids (4%), phosphatidic acid (0.2%) and in unidentified arachidonate hydrolysis products (22%).

All these experiments allowed us to determine the proportion of PtdIns, PtdInsP and PtdInsP$_2$ when steady state was reached, i.e. after 3 hours labelling with $[^{3}H]$arachidonate, or after 30 hours labelling with $[^{3}H]$inositol. The proportion of label found in each lipid as indicated in Table 1 was similar in both *Lytechinus pictus* and *Paracentrotus lividus*, and comparable for $[^{3}H]$inositol and $[^{3}H]$arachidonate: around 1-3% in PtdInsP$_2$, 3-12% in PtdInsP and 84-96% in PtdIns. These relative proportions are not dissimilar to their relative abundance after steady state labelling in the developing embryo (Schmell and Lennarz, 1974), but there is a large excess of PtdIns when compared with its abundance in other cell types (Michell, 1975).

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**Table 1. Distribution of $[^{3}H]$inositol and $[^{3}H]$arachidonate label in polyphosphoinositides of unfertilized eggs**

<table>
<thead>
<tr>
<th></th>
<th>PtdInsP$_2$</th>
<th>PtdInsP</th>
<th>PtdIns</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>$[^{3}H]$inositol</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Lytechinus pictus</em></td>
<td>1.3±0.26</td>
<td>10.5±1.9</td>
<td>88.2±2.13</td>
</tr>
<tr>
<td>(n=5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Paracentrotus lividus</em></td>
<td>2.9±0.2</td>
<td>12.2±0.3</td>
<td>84.9±0.4</td>
</tr>
<tr>
<td>(n=12)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>$[^{3}H]$arachidonate</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Lytechinus pictus</em></td>
<td>1.0</td>
<td>2.9</td>
<td>96.1</td>
</tr>
<tr>
<td>Expt. 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expt. 2</td>
<td>1.0</td>
<td>5.1</td>
<td>93.6</td>
</tr>
</tbody>
</table>

Mean and s.e.m are shown.
Alterations in $[^3H]$inositol and $[^3H]$arachidonate incorporation into PPI at fertilization

The alterations in PPI determined by labelling to isotopic steady state should reflect alterations in the chemical amounts of PPI, if we assume that the entire pool of PPI is exchangeable. The results obtained after steady state labelling are shown in Fig. 2A. We did not observe any significant change during the period of the fertilization calcium transient (10 to 40 seconds) and production of DAG and InsP$_3$ (Ciapa and Whitaker, 1986). Subsequent to this, PtdInsP$_2$ and PtdInsP increase while PtdIns decreases, though the precise timing of these changes varied from experiment to experiment: when the data are pooled, this variation is apparent in the error bars for each time point and the later changes do not appear to be statistically significant from the pooled data, due to the temporal variation. Nonetheless, we saw the same pattern in each individual experiment. We verified the steady state alterations in PPI using $[^3H]$arachidonate label. The alterations in $[^3H]$inositol PPI are very similar to those measured using $[^3H]$arachidonate as a label (Fig. 3: here a single representative experiment is shown); in particular, $[^3H]$arachidonate-labelled PtdInsP$_2$ did not change during the first 20 seconds (relative change in the $[^3H]$arachidonate incorporation of 0.96±0.19, n=5, when compared to the unfertilized level). Given that the production of both InsP$_3$ and DAG occurs within 20 seconds of fertilization, coincident with the calcium wave (Ciapa and Whitaker, 1986), our steady state labelling experiments suggest either that only a small proportion of the PtdInsP$_2$ participates in the activation process at fertilization, or that rates of synthesis and hydrolysis and synthesis of PtdInsP$_2$ increase in concert during the fertilization wave.

Despite the similarities in the pattern of PPI

![Graph showing alterations in PPI](image-url)
Polyphosphoinositide turnover at fertilization

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Table 2. Modification after fertilization in the rate of incorporation of [3H]inositol into polyphosphoinositides of Lytechinus pictus

<table>
<thead>
<tr>
<th></th>
<th>PtdInsP_2</th>
<th>PtdInsP</th>
<th>PtdIns</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Unfertilized eggs</td>
<td>0.14</td>
<td>0.12</td>
<td>0.10</td>
</tr>
<tr>
<td>B. Fertilized eggs (n=5)</td>
<td>84±21</td>
<td>39±30</td>
<td>84±24</td>
</tr>
</tbody>
</table>

Incorporation rates (A and B) were calculated as a percentage increase per minute.

The bottom line gives the relative rate, the ratio B/A

Mean and s e m. are shown

Fig. 3. Relative changes in [3H]arachidonate-labelled PPI after fertilization of eggs of Lytechinus pictus. A single experiment in which PPI were separated by two-dimensional chromatography is shown. Results are expressed relative to the amount of [3H]arachidonate that was incorporated into each lipid at the end of the incubation period and arbitrarily taken as 1.0. Initial disints/minute in PtdInsP_2, 2240; PtdInsP, 2900 and PtdIns, 541437. The experiment shown is representative of five experiments with [3H]arachidonate labelling.

Alterations in [3H]inositol incorporation into PtdInsP_2 after artificial activation

Table 2 shows that the rate of incorporation of [3H]inositol increases approximately 600-fold into PtdInsP_2, 300-fold into PtdInsP, and about 800-fold into PtdIns in the 10 seconds following insemination. The subsequent increase in PtdInsP_2 and in PtdInsP were then slower than after steady state labelling. A precise, quantitative interpretation of the large increases of [3H]inositol into PPI that we observe is difficult because the increase is rapid and massive, but it is very clear that PPI turnover is increased several hundred fold at fertilization.

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metabolism after steady state labelling with either [3H]inositol or [3H]arachidonate, we noted that the longer-term labelling experiments with [3H]inositol resulted in a slower rate of fertilization, giving a slightly delayed population calcium transient peak that occurred between 40 and 60 seconds after insemination. To estimate the interconversion rates of PPI at fertilization, we used unfertilized eggs whose PPI were not labelled to steady state with [3H]inositol.

Eggs incubated for 18 hours at 16°C in ASW with [3H]inositol show comparable rates of fertilization to control eggs (a population calcium transient centred at 30 seconds with a half-width of 20 seconds; Ciapa and Whitaker, 1986). After this time of incubation, we estimate that the label in PtdInsP_2, PtdInsP and PtdIns has reached only 30-50%, 50-60% and 70% respectively of its steady state value (Fig. 1B). The alterations in [3H]PPI under these conditions are shown in Fig. 2B. [3H]PtdInsP_2, [3H]PtdInsP and [3H]PtdIns all increase transiently during the time at which Ca_2 increases after fertilization, rather than remaining relatively constant as they do after steady state labelling with [3H]inositol (Fig. 2A) or [3H]arachidonate (Fig. 3). Table 2 shows that the rate of incorporation of [3H]inositol increases approximately 600-fold into PtdInsP_2, 300-fold into PtdInsP, and about 800-fold into PtdIns in the 10 seconds following insemination. The subsequent increase in PtdInsP_2 and in PtdInsP were then slower than after steady state labelling. A precise, quantitative interpretation of the large increases of [3H]inositol into PPI that we observe is difficult because the increase is rapid and massive, but it is very clear that PPI turnover is increased several hundred fold at fertilization.

Two major inferences can be made from the data described above: no net hydrolysis of PtdInsP_2 appears to occur during the calcium transient; and accumulation of this lipid is markedly accelerated as Ca_2 decreases. In order to investigate the relationship between alterations in intracellular calcium concentrations and PtdInsP_2 turnover, we studied how PtdInsP_2 was metabolised when the eggs are activated with the calcium ionophore A23187. The calcium ionophore stimulates a Ca_2 transient with very similar properties to the fertilization Ca_2 transient (Zucker et al., 1978).

Experiments performed with Lytechinus pictus show that 50 μM A23187 provoked a 15-20% decrease in the amounts of [3H]inositol in PtdInsP_2 in the first 10 seconds following addition (Fig. 4). This was then followed by a marked increase in the incorporation of the isotope, at a rate similar to that measured one minute after fertilization. We also observed an increase in the incorporation of [3H]arachidonate into DAG during the first minute following addition of the calcium ionophore, very similar to that measured after fertilization (Ciapa and Whitaker, 1986).

Comparable results (Fig. 5) were obtained with Paracentrotus lividus. We found in this species that
incorporation of $[^{3}H]$inositol into PtdInsP$_{2}$ measured in the first 30 seconds was always, for each batch of eggs, greater after fertilization than after activation with A23187. Again, the rate of incorporation of $[^{3}H]$inositol into PtdInsP$_{2}$ measured between 30 and 60 seconds after activation was increased and similar to that measured after fertilization, as is the case in *Lytechinus pictus*. The changes in $[^{3}H]$inositol relative to the unfertilized egg were from 1.47±0.24 at 30 seconds to 1.60±0.27 at 60 seconds after fertilization and from 1.04±0.15 at 30 seconds to 1.31±0.21 at 60 seconds after activation with A23187 (n=4 for both data sets). Direct comparison of the kinetics of PtdInsP$_{2}$ turnover after fertilization or activation with A23187 in individual experiments (*Paracentrotus lividus*: Fig. 5; *Lytechinus pictus*: not shown) indicates that turnover is always smaller in A23187-activated eggs, implying that hydrolysis of PtdInsP$_{2}$ is favoured by treatment with A23187; indeed, in two of the three cases illustrated in Fig. 5 hydrolysis predominates during the first 30 seconds.

Because, in both *Lytechinus pictus* and *Paracentrotus lividus*, A23187 triggers an early decrease in $[^{3}H]$PtdInsP$_{2}$, followed by an increase in the accumulation of $[^{3}H]$inositol into PtdInsP$_{2}$, we can infer that both the phosphodiesterase and the kinase involved in the hydrolysis and synthesis of PtdInsP$_{2}$ were activated and are, therefore, calcium-sensitive. It appears that the stimulation of PtdInsP$_{2}$ hydrolysis is transient, with a time course that corresponds to the transient increase in Ca$_{i}$, while the stimulation of the kinase persists after Ca$_{i}$ has returned to resting levels.

Discussion

We have labelled PPI of unfertilized eggs with $[^{3}H]$arachidonate or $[^{3}H]$inositol to determine the alterations in the metabolism of these lipids during the transient increase in Ca$_{i}$ that occurs shortly after fertilization. The first point to be considered is whether the alterations in $[^{3}H]$-labelled metabolites that we measured in the first seconds following insemination can be correlated with this increase in Ca$_{i}$. The second point that we will discuss is whether the alterations in PPI metabolism form part of the mechanism of the calcium wave that propagates across the egg from the point of sperm-egg interaction.

**PPI metabolism in the unfertilized egg**

We observed that $[^{3}H]$arachidonate labels PPI to steady state within 3 hours and observed that 95% of the $[^{3}H]$arachidonate incorporated into phospholipids was found in PPI, reflecting the generally high proportion of
arachidonate in PPI and indicating that PPI are most probably the source of the arachidonate-labelled DAG which is produced during the first 30 seconds following insemination (Ciapa and Whitaker, 1986) or activation with the calcium ionophore (this paper). On the contrary, steady-state labelling of PPI by [3H]inositol is only achieved after 30 hours incubation of unfertilized eggs. The rate of incorporation of inositol into PPI under these conditions (10%/hour) is some 50 times slower than the rate of incorporation into PPI of hepatocytes and 10 times slower than incorporation into platelets (Abdel- Latif, 1986, Vickers and Mustard, 1986), a rate that presumably reflects the lower temperature, relative metabolic quiescence and relative impermeability to inositol of sea urchin eggs. For the two species that we examined (Lytechinus pictus and Paracentrotus lividus), the relative proportion of label that we measured at steady state labelling with either [3H]inositol or [3H]arachidonate in PtdInsP2, PtdInsP and PtdIns are close to those found in other species of sea urchin egg by other authors (Turner et al., 1984; Schmell and Lennarz, 1974); it is therefore probable that these proportions of label reflect chemical concentrations of these phospholipids.

**PPI turnover at fertilization**

After 18 hours incubation with [3H]inositol, PtdIns and PtdInsP2 were labelled respectively to 70% and 30% of their steady state value. We measured an increase of around 30% in [3H]PtdIns and 50% [3H]PtdInsP2 during the first 30 seconds after insemination. This indicates that fertilization triggers a rapid increase in the rate of phosphorylation of PtdIns and PtdInsP into PtdInsP and PtdInsP2 respectively, augmenting the [3H]inositol incorporation into the whole pool of PPI by around 20% during this 30 second period. The early increase that we measure in Lytechinus pictus is of a similar magnitude to that observed in Strongylocentrotus purpuratus (Turner et al., 1984). The chemical amount of PtdInsP2 in Strongylocentrotus purpuratus is 8 μmoles/l packed eggs (Turner et al., 1984). If InsP3 were the trigger of calcium release at fertilization, it should reach a cytoplasmic concentration of 5-10 μM (Whitaker and Irvine, 1984; Swann and Whitaker, 1986), implying that most of the egg PtdInsP2 should be turned over during the first 60 seconds after fertilization. This conclusion is in agreement with our minimum estimate of a 600-fold increase (from 0.1 to 80% per minute: Table 2) in turnover of PPI immediately after fertilization.

It is important to consider whether the acceleration of the turnover of the PPI cycle, illustrated by the increased incorporation of the radioactive precursor, leads to a de novo synthesis of PPI. This seems indeed the case, as an increase in chemical concentrations of PtdInsP2 and PtdInsP (Turner et al., 1984; Kamel et al., 1985) together with a decrease in the concentration of PtdIns (Kamel et al., 1985) has been observed. Similar results should also be obtained when measuring incorporation of a radioactive precursor to steady state. Steady state labelling of PPI with [3H]arachidonate is relatively straightforward since [3H]arachidonate labels PPI to steady state within 3 hours. However, the alterations in [3H]inositol-PPI metabolites that we measured after fertilizing eggs incubated for 36 hours in inositol may be an underestimate, because of the asynchrony in the population of the individual calcium transients of eggs that had been kept for 36 hours during [3H]inositol labelling (Ciapa and Whitaker, 1986). Nonetheless, the pattern of alterations of [3H]PPI after fertilization of eggs labelled to steady state with [3H]inositol indicates a similar sequence of events to those of [3H]arachidonate-labelled eggs: an early phase during which PPI do not change substantially and a later phase during which PtdInsP2 increases at the expense of PtdIns: each set of data confirms the other.

There is a slight discrepancy with results reported by other authors, who found a detectable, though variable, early increase in the chemical concentrations of both PtdInsP2 and PtdInsP (Turner et al., 1984; Kamel et al., 1985). How might we explain this? The alterations after fertilization in the incorporation of a radioactive tracer at equilibrium or in the chemical amount of PtdInsP2 represent a net change in both the synthesis and the hydrolysis of this lipid. A rapid increase in the rate of PtdInsP2 hydrolysis may therefore be more or less hidden by an enhanced phosphorylation of this lipid, and vice versa. Small variations in the relative rates of activation of the phosphoinositidase C and the PtdInsP kinase could lead to small quantitative differences between sea urchin species and even between batches of eggs of same species. This is indeed the case in Strongylocentrotus purpuratus where 32P incorporation into PPI shows considerable variation from experiment to experiment (Turner et al., 1984), and in Paracentrotus lividus, where the increase in the incorporation of [3H]inositol occurring after fertilization or activation of these eggs (shown in Fig. 5) varies considerably in the different batches of eggs. Moreover, a similar phenomenon to the one we report here for inositol turnover is evident in 32P-labelled eggs: a 30 minutes incubation with 32P phosphate leads to a 50% increase in incorporation of 32P into PtdInsP2 30 seconds after fertilization (Kamel et al., 1985), while a 2 hour incubation with 32P phosphate results in a 30% increase under similar conditions. Both these experiments were performed far from equilibrium, since we have found that 32P phosphate equilibrates in PPI only after 24 hours of incubation (not shown).

The differences that we see between steady state and non-steady state labelling might also be explained if the PPI that participate at fertilization were confined to a small, metabolically active pool. Distinct pools of PPI lipids have been described in platelets (Vickers et al., 1986) and human erythrocytes (Gascard et al., 1989). On this hypothesis, changes in the smaller pool would be masked at steady-state labelling by the large, but quiescent pool. On this interpretation, the amounts of InsP3 generated would be similar, since the smaller quantity of participating PtdInsP2 would be balanced by a greater turnover rate. We think this explanation of our data less likely, because it is hard to reconcile a
small, metabolically active pool of PtdInsP₂ with the substantial increases in chemical amounts of PPI that can occur 60 seconds or so after fertilization (Turner et al., 1984). It appears that the net changes in PPI at fertilization in sea urchin eggs are best understood as small differences between two large numbers: the rates of synthesis and hydrolysis. This idea also explains why production of the second messengers InsP₃ and DAG can be accompanied by net increases in their precursor PtdInsP₂.

**PPI metabolism and the calcium wave**

Activating the eggs of *Lytechinus pictus* with the calcium ionophore showed a clear decrease followed by an increase in the incorporation of [³H]inositol into PtdInsP₂. These results indicate that both the phosphoinositidase C and the PtdInsP-kinase are sensitive to calcium, which is in agreement with the calcium-dependence of these two enzymes observed with plasma membrane preparations in vitro (Whitaker and Aitchison, 1985; Oberdorf et al., 1989). Since we have found that the stimulation of PPI turnover after fertilization occurs during the calcium transient (Ciapa and Whitaker, 1986 and this paper), it seems very likely that the enzymes involved in the hydrolysis and regeneration of PtdInsP₂ are influenced by the increase in cytoplasmic calcium concentration at fertilization.

Given ours and others' data (Turner et al., 1984; Kamel et al., 1985; Oberdorf et al., 1989), there is no doubt that both classes of enzyme involved in the PPI metabolism (phosphodiesterases and kinases) are stimulated during, if not before, the calcium transient. The important question is whether this activation in PPI turnover plays a role in the generation and propagation of the calcium wave. Very recent data indicate that the wave of calcium is not restricted to the cortex, but propagates also throughout the egg cytoplasm (McCulloh, 1991; Mohri and Hamaguchi, 1991). The velocity of the calcium wave is not uniform, being rapid in the vicinity of the sperm entry site (close to the plasma membrane) and slower in the central region of the egg (Mohri and Hamaguchi, 1991). We might therefore envisage the presence of more than one mechanism to explain how the calcium wave propagates. The two obvious mechanisms that could participate in the initiation and the propagation of the wave are a calcium-induced calcium release (CICR) and the phosphoinositide machinery (Whitaker and Irvine, 1984; reviewed by Exton, 1988; Jacob, 1990; Meyer, 1991). Our results confirm the suggestion that a regenerative mechanism in addition to CICR, involving InsP₃-induced calcium release and calcium-stimulated InsP₃ production (Swann and Whitaker, 1986), acts to propagate the calcium wave in the sea urchin egg. Such a model involving a sequential diffusion and amplification of InsP₃ has subsequently been proposed to explain the calcium waves in somatic cells (Meyer and Stryer, 1991). A mechanism in which the PPI kinases are stimulated by Ca, is also evident in our data; this would provide a further positive feedback mechanism during the fertilization calcium wave.

Finally, our data show that PtdInsP₂ accumulation is always more rapid after fertilization than after activation of the eggs with the calcium ionophore. This suggests that sperm may trigger other pathways in addition to the alteration in intracellular calcium concentration that affects the activities of the PtdIns kinase and phosphoinositidase C. One candidate pathway involves phosphorylation on tyrosine, by analogy with receptors with tyrosine kinase activity (Ullrich and Schlessinger, 1990) and indeed, a rapid and early increase in tyrosine phosphorylation has recently been described (Ciapa et al., 1991). Another candidate pathway involves phosphorylation on histidine (Jaffe, 1989). Nonetheless, though it is evident that a massive increase in PPI metabolism is one of the earliest detectable events at fertilization, how this comes about remains uncertain (Crossley et al., 1991).

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