Fertilization Results in Increased Tyrosine Phosphorylation of Egg Proteins*

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The sea urchin egg contains one or more tyrosine-specific protein kinases which are active during the response of the egg to sperm fusion. Fertilization results in an 8-fold increase in the relative incorporation of [32P]orthophosphate into phosphotyrosine as compared to phosphoserine and phosphothreonine. Under defined in vitro conditions, plasma membranes from fertilized eggs incorporated 5-10-fold more phosphate into tyrosine than plasma membranes from unfertilized eggs. Analysis of the phosphorylated plasma membrane proteins by polyacrylamide gel electrophoresis demonstrated that at least four proteins were more actively phosphorylated in plasma membranes from fertilized eggs. Of these, a closely spaced doublet of approximately 120 kDa was found to contain phosphotyrosine.

The properties of the egg tyrosine-specific kinase were studied using an artificial peptide substrate. The enzyme is membrane-bound and is enriched 8-fold in the egg plasma membrane. Enzyme activity in egg homogenates and plasma membranes increased 2- and 4-fold, respectively, as early as 20 min, post-insemination. These results suggest that the fertilization-dependent increase in tyrosine-specific protein kinase activity may play a role in the onset of embryonic development.

Tyrosine-specific protein kinases have recently been discovered in a number of vertebrate cells such as avian and mammalian tumor cells (1-4), cultured fibroblasts (5), liver cells (6), lymphocytes (7), and human red blood cells (8). Phosphotyrosine is a minor cellular component, normally accounting for between 0.02 and 0.05% of the phosphoamino acids (2, 9). However, cells stimulated to divide by certain tumorigenic RNA viruses or by binding of epidermal growth factor to its receptor may accumulate levels of phosphotyrosine which are 5-10-fold higher than that of the resting cell (9-11). This has led to the concept that tyrosine-specific protein kinases may play a role in regulating cell growth. Studies with cells transformed by a temperature sensitive mutant of Rous sarcoma virus showed that the virally encoded tyrosine-specific protein kinase p60<sup>src</sup> plays an essential role in cell transformation (2). Similarly, the epidermal growth factor receptor has been shown to have tyrosine-specific protein kinase activity which is stimulated by the binding of epidermal growth factor (12). These findings suggest that control of cell division by mechanisms as diverse as viral infection and receptor-ligand interaction may involve a common pathway in which tyrosine phosphorylation is an early step.

Fertilization is another example in which a resting cell (the egg) begins to divide in response to a stimulus (sperm fusion). A recent study (13) has shown that the tyrosine-specific kinase activity associated with the sea urchin egg membranes increased dramatically during embryonic development. As part of our investigation of the fertilization-dependent changes in the egg plasma membrane, we have studied the effect of fertilization on plasma membrane tyrosine-specific kinase activity. In vitro labeling studies showed that fertilization results in an 8-fold increase in the relative incorporation of [32P]orthophosphate into tyrosine residues of egg proteins. In vitro studies demonstrated the existence of a tyrosine-specific kinase associated with the egg plasma membrane. The plasma membrane-associated activity increased an average of 4-fold within 20 min of insemination. In vitro, the kinase can phosphorylate a synthetic, tyrosine-containing peptide as well as a 120-kDa protein which is present in plasma membrane preparations. We suggest that this tyrosine-specific kinase may play some important role during fertilization, possibly involving the control of cell growth.

EXPERIMENTAL PROCEDURES

Materials—Sea urchins (Lytechinus variegatus) were collected in the Miami area, and gametes were obtained and processed as described (14). The following chemicals were obtained from the suppliers indicated: [γ-32P]ATP (1-3 Ci/mmol) and L-[3,4,2-3H] valine (65 Ci/mmol), New England Nuclear; (32P)orthophosphate (carrier-free), Amersham Corp.; Dowex AG 50W-X8, Bio-Rad; and phosphoserine, phosphothreonine, ATP, emetine, and HEPES, Sigma. The synthetic peptide RR-SRC (15) was a generous gift from Dr. J. Caneville of E. G. Krebs' laboratory (University of Washington, Seattle, WA) or was obtained from Peninsula Laboratories. The following solutions were used: artificial seawater (16); calcium- and magnesium-free sea water, 0.5 M NaCl, 10 mM KCl, 25 mM EGTA, 2.5 mM NaH<sub>2</sub>CO<sub>3</sub>, pH 8; and Buffer A (12.5 mM HEPES, 10 mM MgCl<sub>2</sub>, 5 mM 2-mercaptoethanol, pH 7.4 (13)).

Membrane Preparation—Plasma membranes were prepared from eggs before and 20 min after fertilization by methods described earlier (14, 17). The ouabain inhibitable Na<sub>a</sub>-K<sub>a</sub>-ATPase activity was determined as described earlier (14).

To prepare a particulate fraction, eggs (200 mg of protein) were fertilized and the excess sperm were washed out. Twenty min post-insemination, the eggs were washed by centrifugation in 10 volumes of calcium- and magnesium-free seawater, then resuspended in 35 ml of this solution and homogenized by hand in a glass homogenizer with a Teflon pestle. The membranes were pelleted in a DuPont AH627 rotor at 25,000 rpm for 1 h, resuspended in 35 ml of Buffer A and centrifuged again. The pellet was suspended in Buffer A except

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that the 2-mercaptoethanol was not added until after samples were taken for protein determination by the method of Lowry (18) as modified (19).

**In Vivo Labeling**—Fertilized eggs were washed several times in artificial seawater to remove excess sperm and suspended in 3 volumes of artificial seawater with continuous stirring. At 15 min post-insemination, [32P]orthophosphate was added to 50 μCi/ml and the suspension was stirred for 30 min at 25 °C. At 45 min post-insemination, the reaction was stopped by adjusting the suspension to 10% (w/v) CCl₄/COOH. Unfertilized eggs were incubated at 0.5 mCi/ml to make up for their low rate of phosphate intake (20).

**In Vitro Phosphorylation**—Phosphorylation of plasma membranes was carried out in Buffer A containing 0.2-0.75 μg of membrane protein/ml. The reaction was started by addition of [γ-32P]ATP to a concentration of 0.75 μCi/ml, and the membranes were incubated at 25 °C for 15 s. The reaction was stopped by heating to 90 °C for 5 min, after which membrane lipids were extracted with 20 volumes of chloroform/methanol (2:1) and the protein residue dried under N₂. Phosphorylation of the RR-SRC peptide (15) was carried out in 50 μl of Buffer A containing 0.5% (v/v) Nonidot P-40, 2.5 mM RR-SRC, and 10-25 μg of membrane protein. The reaction was started by adding [γ-32P]ATP (6.25 μCi/μmol) to a final concentration of 80 μM and the mixture was incubated at 25 °C for 1 min, then stopped as above. The reaction products were analyzed by electrophoresis on Whatman 3MM paper in pyridine (0.5% v/v), acetic acid (5% v/v), pH 3.5, at 2000 V for 1 h. The phosphopeptide was quantitated by cutting the paper into 1-cm strips which were placed in vials containing Liquiscint (National Diagnostics) and counted in a scintillation counter. Where indicated, the reaction products were analyzed by reverse phase high performance liquid chromatography as described previously (15).

**Phosphoamino Acid Analysis**—Samples from acrylamide gels were electroeluted (21) and freeze-dried. Membrane and total egg samples were precipitated in ice-cold 10% (w/v) CCl₄/COOH and washed five times in 100 volumes of 10% CCl₄/COOH. The pellets were then extracted five times in 100 volumes of ethyl ether and dried under N₂. Seventy-five μg each of phosphoserine, phosphothreonine, and phosphotyrosine were added, and the samples were hydrolyzed in 6 N HCl at 110 °C for 2 h under vacuum. The reaction mixture was filtered, then dried under vacuum and redissolved in 1 ml of 0.1 M formic acid. This was loaded on a column (0.7 × 6 cm) of Dowex AG 50W-X8 (hydrogen form) equilibrated in 0.1 M formic acid and eluted with 50 ml of 0.1 M formic acid. The eluate was then freeze-dried. In separate experiments, the recovery of phosphotyrosine from this column was determined by absorbance at 270 nm to be greater than 90%. Phosphoamino acids were analyzed by two-dimensional high voltage paper electrophoresis (22), and the standards were localized by spraying with ninhydrin/cadmium acetate.

### RESULTS

**Phosphorylation of Endogenous Egg Proteins**—To determine whether the rate of tyrosine phosphorylation changed significantly as a consequence of fertilization, we labeled unfertilized and fertilized eggs with [32P]orthophosphate and measured the incorporation into phosphotyrosine. The fertilized eggs were labeled for a 30-min period beginning 15 min post-insemination. The first cell division occurs 60 min post-insemination, and other studies have shown that the rate of protein and DNA synthesis begins to increase about 20 min post-insemination (16). Since unfertilized eggs take up phosphate very slowly, we were not able to establish steady state labeling conditions. Therefore, the values in Table I represent the relative incorporation of label into these phosphoamino acids and not their relative amounts. As seen in Table I, prior to fertilization the phosphorylation of tyrosine residues represents a very small proportion of the total incorporation detected in phosphoamino acids (less than 0.05%). However, after fertilization, phosphotyrosine accounted for 0.466% of the label in phosphoamino acids. This suggests either that the activity of a tyrosine-specific protein kinase increases or that the activity of a phosphotyrosine protein phosphatase decreases at fertilization.

Similar results were obtained when plasma membranes were incubated with [32P]orthophosphate (2.5 μCi/μmol) for the indicated time, and the incorporation of label into these phosphoamino acids was determined by electrophoresis on Whatman 3MM paper in pyridine (0.5% v/v), acetic acid (5% v/v), pH 3.5, at 2000 V for 1 h. The phosphopeptide was quantitated by cutting the paper into 1-cm strips which were placed in vials containing Liquiscint (National Diagnostics) and counted in a scintillation counter. Where indicated, the reaction products were analyzed by reverse phase high performance liquid chromatography as described previously (15).

**Table I**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Phosphotyrosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfertilized</td>
<td>5</td>
</tr>
<tr>
<td>Fertilized</td>
<td>5</td>
</tr>
</tbody>
</table>

**Table II**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Phosphotyrosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfertilized</td>
<td>12</td>
</tr>
<tr>
<td>Fertilized</td>
<td>12</td>
</tr>
</tbody>
</table>

**In Vivo Phosphorylation of Tyrosine Residues in Plasma Membranes**

The labeling of plasma membranes was carried out under conditions similar to those described for unfertilized eggs. Unfertilized and fertilized eggs (800 ng of protein) were incubated for 30 min with [32P]orthophosphate, and the phosphorylated proteins were separated as described under "Experimental Procedures." The ninhydrin-staining spots were cut out, bleached in 0.1% H₂O₂, and counted in a scintillation counter. The radioactivity in each phosphoamino acid is expressed as a percentage of the total in the three identified phosphoamino acids. The results are the average of five separate experiments ± S.D. The total radioactivity in phosphoamino acids from unfertilized eggs ranged from 175,000 to 350,000 cpm and in fertilized eggs was as high as 2,527,000 cpm.

**Table I**

<table>
<thead>
<tr>
<th>Radioactivity</th>
<th>Phosphoserine</th>
<th>Phosphothreonine</th>
<th>Phosphotyrosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unfertilized</td>
<td>76,806 ± 5,098</td>
<td>23,178 ± 5,085</td>
<td>&lt;0.05 ± 0.024</td>
</tr>
<tr>
<td>Fertilized</td>
<td>73,177 ± 3,396</td>
<td>26,388 ± 3,236</td>
<td>0.466 ± 0.364</td>
</tr>
</tbody>
</table>

**In Vitro Phosphorylation of Tyrosine Residues**

Unfertilized and fertilized eggs were incubated in vitro with [γ-32P]ATP. The plasma membranes from fertilized eggs incorporated at least five times as much phosphate into tyrosine residues as plasma membranes from unfertilized eggs (Table II). The phosphorylation of serine and threonine residues increased 5- and 2-fold, respectively, indicating that a variety of other plasma membrane-associated kinases may be activated at fertilization.

**Analysis of the Membrane Proteins Phosphorylated in Vitro**—In order to determine which plasma membrane proteins were phosphorylated under these in vitro conditions, plasma membranes from unfertilized and fertilized eggs were incubated with [γ-32P]ATP and analyzed by polyacrylamide gel electrophoresis. As seen in Fig. 1, fertilization results in increased phosphorylation of four proteins (molecular mass species) of about 120, 76, 42, and 38 kDa. Analysis of the plasma membranes by two-dimensional gel electrophoresis and radioautography indicated that the 38- and 42-kDa phosphoproteins were probably not related to actin.

To evaluate the possibility that the differences in labeling patterns seen in Fig. 1 resulted from the action of proteases or phosphatases rather than protein kinases, we did a mixing experiment in which equal amounts of fertilized and unfertilized plasma membranes were incubated with [γ-32P]ATP. The resulting phosphoproteins were analyzed by electrophoresis and radioautography as in Fig. 1. The densitometric scan presented in Fig. 2 reveals that the 76- and 38-kDa bands were not labeled in the mixture of fertilized and unfertilized plasma membranes. This suggests that some component in the unfertilized plasma membrane reduced the phosphorylation of the 76- and 38-kDa proteins (kinase in-
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FIG. 1. Analysis of plasma membrane proteins phosphorylated in vitro. Plasma membranes (200 µg of protein) from fertilized eggs (top) and unfertilized eggs (bottom) were incubated with [γ-32P]ATP at a concentration of 0.25 µM (0.75 mCi/ml) for 15 s. The phosphoproteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% gel, and the Coomassie blue-stained gel is shown at the top of each panel (origin at left). The gel was then dried and analyzed by autoradiography. The x-ray film was then scanned with an LKB densitometric scanner, and the resultant profiles are presented beneath each gel. The apparent molecular mass of selected bands is indicated in kilodaltons.

FIG. 2. Phosphorylation of a mixture of unfertilized and fertilized plasma membranes in vitro. A mixture containing plasma membranes from unfertilized eggs (100 µg) and plasma membranes from fertilized eggs (100 µg) was phosphorylated in vitro as in Fig. 1 and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography as above. A densitometric scan of the x-ray film is presented with the origin at the left and apparent molecular mass of selected bands is indicated in kilodaltons.

FIG. 3. Phosphoamino acid analysis of fertilized plasma membrane proteins phosphorylated in vitro. The phosphorylated bands and intervening sections of gel from Fig. 1 were electroeluted, hydrolyzed, and analyzed by two-dimensional paper electrophoresis and autoradiography (6 days). The autoradiographs are presented with the first dimension, pH 3.5, from bottom to top and the second dimension, pH 1.9, from left to right. The position of each phosphoamino acid was determined by ninhydrin staining of authentic standards. The samples presented are: top of gel to 130 kDa (A), doublet at 120 kDa (B), 76 kDa (C), 75 to 45 kDa (D), 42 kDa (E), and 38 kDa (F).

FIG. 4. Phosphorylation of the RR-SRC peptide by fertilized egg plasma membranes. The RR-SRC peptide, phosphorylated as described under "Experimental Procedures," was analyzed by paper electrophoresis at pH 3.5. The strip of paper was then cut into 1-cm pieces and counted in a scintillation counter. ●, 2.5 mM RR-SRC; ○, no peptide.

Phosphorylation in Vitro—The RR-SRC peptide, synthesized by Dr. J. Casnellie, has been used as a substrate for the tyrosine-specific kinase associated with the epidermal growth factor receptor (15). Since the peptide contains tyrosine as the only phosphate acceptor, it is a specific probe for tyrosine-specific kinases. The sea urchin egg kinase also phosphorylates this peptide (Fig. 4). The reaction is linearly dependent on enzyme (membrane protein) concentration (Fig.
Tyrosine Phosphorylation at Fertilization

**FIG. 5.** Dependence of peptide phosphorylation on plasma membrane protein. Fertilized egg plasma membranes were suspended in Buffer A containing 0.5% Nonidet P-40, and increasing amounts of the solubilized membranes were used to phosphorylate the RR-SRC peptide. The specific activity of the $[\gamma-^{32}P]ATP$ was 25 mCi/µmol.

**FIG. 6.** Effect of divalent cations on peptide phosphorylation by fertilized egg plasma membranes. The RR-SRC peptide was phosphorylated by plasma membranes (15 µg of protein) as described under “Experimental Procedures” except that the concentration of $Mg^{2+}$ (O) or $Mn^{2+}$ (C) was varied.

5), and half-maximal reaction rate required 35 µM ATP. The kinase requires $Mg^{2+}$ or $Mn^{2+}$ for (Fig. 6) activity. The sea urchin enzyme is similar to other tyrosine-specific kinases in that the activity in the presence of 5 mM $Mn^{2+}$ is over 3-fold higher than that obtained with $Mg^{2+}$ (4, 5, 15). $Mn^{2+}$ (10 mM) also stimulated phosphorylation of the 120-kDa doublet approximately 2-fold when fertilized plasma membranes were phosphorylated in vitro, although the labeling of other proteins was not affected (data not shown). We chose to use 10 mM $Mg^{2+}$ in our assays since this more closely approximates the intracellular environment.

Using this quantitative assay, we tested different subcellular fractions for tyrosine-specific kinase activity. As seen in Table III, the activity is tightly membrane-bound and is enriched about 8-fold in the plasma membrane. The recovery of tyrosine-specific kinase activity in the plasma membrane fraction was somewhat lower than that of the plasma membrane marker Na/K-ATPase. The kinase activity in plasma membranes prepared from eggs 20 min post-insemination was about four times higher than that in plasma membranes from unfertilized eggs (Table IV). This suggested that fertilization results in an increase in total cellular kinase activity or an

**TABLE III**

Membrane localization of tyrosine-specific protein kinase activity

Fertilized eggs were homogenized in 10 volumes of calcium- and magnesium-free seawater and divided into two aliquots. One aliquot was centrifuged at 100,000 × g for 1 h to yield soluble and particulate fractions. The second aliquot was used to prepare plasma membranes as described previously (17). The kinase activity in each fraction was measured, and the specific activity is expressed as picomoles of $^{32}P$ incorporated into RR-SRC peptide/mg of protein/min. The recovery of ouabain-inhibitable Na/K-ATPase activity (a plasma membrane marker enzyme) in the plasma membrane fraction was 25%.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Specific activity</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>1.24 ± 0.08</td>
<td>100</td>
</tr>
<tr>
<td>Particulate</td>
<td>5.27 ± 0.04</td>
<td>107</td>
</tr>
<tr>
<td>Soluble</td>
<td>0.03 ± 0.00</td>
<td>1.5</td>
</tr>
<tr>
<td>Plasma membranes</td>
<td>0.02 ± 0.01</td>
<td>200</td>
</tr>
</tbody>
</table>

**TABLE IV**

Tyrosine-specific kinase activity in plasma membranes and homogenates from unfertilized and fertilized eggs

Homogenates and plasma membranes were prepared from eggs before and 20 min after fertilization. Kinase activity was then measured using the RR-SRC peptide as a substrate. In some experiments, equal amounts of unfertilized and fertilized egg homogenates were mixed, and the mixture was assayed for kinase activity (mixed homogenate). Specific activity is expressed as picomoles of peptide phosphorylated per mg of protein/min + S.D. $n$ represents the number of different preparations from which the data were obtained.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Specific activity</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma membrane</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unfertilized</td>
<td>2.32 ± 0.74</td>
<td>4</td>
</tr>
<tr>
<td>Fertilized</td>
<td>8.68 ± 1.95</td>
<td>7</td>
</tr>
<tr>
<td>Homogenate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unfertilized</td>
<td>0.87 ± 0.25</td>
<td>8</td>
</tr>
<tr>
<td>Fertilized</td>
<td>1.69 ± 0.48</td>
<td>8</td>
</tr>
<tr>
<td>Mixed</td>
<td>1.43 ± 0.40</td>
<td>4</td>
</tr>
</tbody>
</table>

**FIG. 7.** Tyrosine-specific kinase activity during early development. Eggs were suspended (1% v/v) in filtered seawater, fertilized, and quickly washed by centrifugation, then allowed to develop. Homogenates were prepared before and at various times after fertilization and immediately assayed for kinase activity using the RR-SRC peptide as a substrate. The reaction products were analyzed by reverse phase high pressure liquid chromatography as described under “Experimental Procedures.” Each fraction was assayed in duplicate, and the range is indicated by the vertical bars.
TABLE V

Effect of inhibition of protein synthesis on kinase activity

Unfertilized eggs were preincubated with emetine (50 µg/ml) for 30 min, then fertilized and cultured in the presence of emetine for 2 h. At various times, eggs were collected, washed by centrifugation, homogenized, and assayed for kinase activity using the RR-SRC peptide as a substrate. Control eggs were fertilized and cultured as above but were not exposed to emetine. To evaluate the effectiveness of the emetine treatment, we measured its inhibition of incorporation of [3H]valine into protein. To avoid possible effects on amino acid transport, we prelabeled the eggs for 1 hr with [3,4-3H]valine (5 µCi/ml) (36). The eggs were then washed and treated with emetine or sea water (control) and fertilized as above. The incorporation of tritiated valine into protein was measured by trichloroacetic acid precipitation in every case, protein synthesis was inhibited by greater than 99% by emetine treatment.

<table>
<thead>
<tr>
<th>Time post-insemination (min)</th>
<th>Kinase activity (pmol/mg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Unfertilized</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.81</td>
</tr>
<tr>
<td>30</td>
<td>1.26</td>
</tr>
<tr>
<td>120</td>
<td>1.46</td>
</tr>
</tbody>
</table>

In vivo labeling studies showed that fertilization results in increased phosphorylation of tyrosine relative to serine and threonine residues of egg proteins. Prior to fertilization, the relative incorporation of [32P]orthophosphate into tyrosine was below the limit of detection (0.05%), a value comparable to that in the resting mammalian or avian cell. However, between 15 and 45 min post-insemination, the relative incorporation rose to 0.47%, a value comparable to rapidly dividing transformed tumor cells (9–11). Because the rate of phosphate uptake by unfertilized eggs is so low, we were unable to establish equilibrium labeling conditions without reducing viability. Therefore, an increase in the relative incorporation of 32PO4 into phosphotyrosine as compared to phosphoserine and phosphothreonine could reflect an increase in total phosphotyrosine or an increase in the relative rate of turnover of PO4 into phosphotyrosine. The fertilization process resembles cell transformation in that a quiescent cell is changed into a rapidly dividing cell. The observation that both virally transformed cells and fertilized eggs exhibit an unusually high rate of tyrosine phosphorylation suggests that these two systems may make use of a common pathway in the as yet unknown series of events involved in growth control.

Earlier studies demonstrated that fertilization resulted in increased activity of endogenous protein kinases toward several proteins when these membranes were phosphorylated in vitro (13, 24). We have done similar experiments using purified plasma membranes prepared from eggs before and 20 min after fertilization. Analysis of the plasma membrane proteins labeled in vitro showed that proteins of 120, 76, 42, and 38 kDa were phosphorylated more extensively in plasma membranes from fertilized eggs. This undoubtedly reflects increased activity of several types of protein kinases, tyrosine-specific and other. Mixing experiments demonstrated that the unfertilized plasma membranes contained something that reduced phosphorylation of the 76- and 38-kDa proteins, suggesting that a complex system of control mechanisms may exist. Phosphoamino acid analysis of the plasma membranes labeled in vitro showed that the incorporation of [32P]orthophosphate into phosphotyrosine of total plasma membrane protein was 5–10-fold higher in the membranes from fertilized eggs. When the individual proteins were separated by gel electrophoresis and analyzed for incorporation into phosphotyrosine, a closely spaced doublet of approximately 120 kDa was found to contain several phosphorylated tyrosine proteins. This is in contrast to the results of an earlier study using a membrane fraction from Strongylocentrotus purpuratus embryos (13). Dasgupta and Garbers (13) reported the phosphorylation of tyrosine residues in nine molecular mass classes including one of 120 kDa. This suggests that additional substrates exist in other subcellular fractions.

In order to quantitate the activity of the tyrosine kinase and study its properties, we made use of a synthetic peptide substrate (RR-SRC) which contains tyrosine as the only phosphate acceptor (15). Using this assay, we were able to demonstrate that the kinase is membrane-bound and is enriched about 8-fold in the plasma membrane. In this respect, the egg enzyme resembles pp60c-src and epidermal growth factor receptor kinases which have been localized to the plasma membrane (25–28). As with tyrosine kinases from other sources, the egg enzyme is more active in the presence of Mn++ than Mg++. As suggested by our in vivo labeling experiments, the tyrosine-specific kinase activity was higher in homogenates and plasma membranes from fertilized eggs. This supports the finding of Dasgupta and Garbers (13) that tyrosine-specific kinase activity increased 2–3-fold by 1 h after fertilization.

DISCUSSION

In this report, we have studied the role of a tyrosine-specific protein kinase in the response of the sea urchin egg to fertilization. In every case, protein synthesis was inhibited by greater than 99% by emetine treatment. This demonstrates that the components of the unfertilized homogenate had no inhibitory effect on the tyrosine-specific kinase activity in the fertilized homogenate.

To more accurately establish the time course of the increase in enzyme activity, we prepared homogenates at various times after fertilization and immediately assayed kinase activity using the peptide substrate. The results (Fig. 7) show that the kinase activity remains constant for 15 min post-insemination, then begins to increase at 30 min and continues to increase through the four-cell stage (2 h). Since the above time course closely corresponds to the onset of protein synthesis (16), it seemed likely that the increase in kinase activity may reflect the synthesis of additional enzyme molecules. To test this possibility, we used emetine, a potent inhibitor of protein synthesis in sea urchins (23), to prevent enzyme biosynthesis and measured the effect of fertilization on enzyme activity. Eggs treated with emetine underwent the cortical reaction and nuclear centration, but no protein synthesis was detectable and the eggs did not divide. In spite of this, the initial increase in kinase activity occurred on schedule at 30 min (Table V); however, further increase in kinase activity was partially inhibited. This suggests that the initial increase in kinase activity does not require protein synthesis.

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Our results demonstrate that the enzyme activity begins to increase at 20–30 min post-fertilization or about 40 min prior to the first cell division. The initial increase in kinase activity (30 min post-insemination) does not require protein synthesis. However, further work will be needed to determine whether or not the continued increase in kinase activity beyond the two-cell stage is a direct result of enzyme biosynthesis.

In separate experiments, we have detected tyrosine-specific kinase activity in sperm using endogenous sperm proteins and the RR-SRC peptide as substrates. However, the activity/sperm cell is one ten-thousandth of that in the fertilized egg. Thus, the tyrosine-specific kinase activity delivered to the egg by the sperm cannot directly account for the fertilization-dependent increase in kinase activity.

It is clear that fertilization results in increased activity of several protein kinases (25, 30) of which the tyrosine-specific activity is a relatively small part. This raised the possibility that the tyrosine phosphorylation observed in our work and that of Dasgupta and Garbers (13) represented a lack of specificity of one or more kinases rather than an enzyme such as pp60^c-src which is entirely specific for tyrosine residues. Using partially purified plasma membranes (prepared on a Ca^{2+}-containing sucrose gradient (17)) as starting material, we have solubilized the tyrosine-specific kinase activity in 0.5% Nonidet P-40 and purified it by chromatography on hydroxyapatite as described for the pp60^c-src enzyme (37). When this partially purified material is incubated with [γ-32P]ATP, phosphorytrosine accounted for 52% of the radioactivity in identified phosphoprotein amino acids. While not conclusive, the enrichment of tyrosine phosphorylating activity suggests that a tyrosine-specific kinase does exist in the egg plasma membrane.

The response of the egg to fertilization includes a series of pre-programmed events which convert the resting egg into a rapidly dividing embryo. These include cytoplasmic events such as activation of NAD kinase (29), phosphorylation of the ribosomal S6 protein (30), as well as increased protein and DNA synthesis (16, 31). In addition, several important events involve the plasma membrane such as the electrical block to polyspermy (32), calcium influx (33), increased transport of small molecules (20, 34), and cortical vesicle exocytosis (35).

The results of our study suggest that the activity of a plasma membrane-bound tyrosine-specific protein kinase also increases as a consequence of fertilization. The significance of this phenomenon is at present unknown, but the analogy with the pp60^c-src enzyme and the epidermal growth factor and insulin receptors (36) suggests that it may play a role in growth control. The mechanism by which this egg kinase in controlled and the function of its protein substrate remain questions for future research.

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REFERENCES