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A rapid change in phosphorylation on tyrosine accompanies fertilization of sea urchin eggs

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Alterations in protein phosphorylation, particularly phosphorylation on tyrosine, frequently accompany cell change and are important agents in the cascades initiated by extracellular signals. This paper examines whether the activation of the sea urchin egg at fertilization involves an early and rapid phosphotyrosine response. Using an anti-phosphotyrosine antibody and a rapid sampling technique, we find a very early increase in the phosphorylation on tyrosine of two proteins of approximately 91 kDa and 138 kDa. A similar phosphorylation occurs after activation of the eggs by the calcium ionophore, ionomycin, suggesting the stimulation of a Ca^{2+}-sensitive pathway. The timing and Ca^{2+} sensitivity suggest a role in the primary signal transduction events of fertilization.

Tyrosine phosphorylation; Fertilization; Egg; Calcium release

1. INTRODUCTION

It is believed that a rapid and transient increase in intracellular free calcium concentration is the signal responsible for the 'awakening' of sea urchin egg metabolism after fertilization and subsequent entry into the cell cycle [1,2]. Unclear, however, is how the sperm–egg interaction triggers this calcium rise. One possibility is that the sperm binds to a receptor on the plasma membrane which in turn activates a phospholipase C via a G-protein [3]. This involvement of G-proteins has been questioned [4], however, and a second hypothesis is that the sperm might transfer or inject an activating substance into the egg [4–6]. It is intriguing that some of the early responses occurring after fertilization (calcium rise, stimulation of a Na/H exchange, polyphosphoinositide turnover [1,2,7]) are similar to those triggered by activation of receptors with tyrosine kinase activity in somatic cells [8–10]. If similar changes in tyrosine phosphorylation are responsible for the release in Ca_i occurring after fertilization, one might expect to detect such alterations before the initiation of this ionic signal, i.e. during the 'latent period'. This critical but as yet poorly-characterized interval is the time between the first interaction of sperm and egg and the initiation of the calcium rise [4,16]. Detection of any increases in protein tyrosine phosphorylation during this interval is made difficult by the impermeability of the unfertilized egg to $^{32}PO_4$. Indeed, several authors have reported no or little detectable differences in the total phosphoprotein profile between unfertilized and fertilized eggs, even with prolonged incubation in ^{32}P or with loading of eggs with ^{32}P by electroporation [11]. Kinsey and his colleagues have attempted to overcome this limitation by using an antibody generated against a specific tyrosine-phosphate containing peptide, but the tyrosine-phosphate containing proteins detected appeared relatively late after insemination, long after the latent period was over [12,13].

We have re-examined this question using a commercial monoclonal antibody to phosphotyrosine (PY20) as this antibody is commonly used to detect phosphotyrosine changes in growth factor receptors [1]. The data that we report in this paper show that the antibody PY20 immunoprecipitated in both unfertilized and fertilized eggs two proteins of approximately 91 and 138 kDa. As an increase in the phosphorylation on tyrosine of both proteins could be detected during the latent period, we suggest that these two phosphoproteins might play a role in signal transduction following fertilization of sea urchin eggs.

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2. MATERIALS AND METHODS

Gametes were collected from the sea urchin *Strongylocentrotus purpuratus*. Sperm was stored at 4°C and eggs dejellied by several passages through a nylon mesh (90 μm). 5 ml of a 5% suspension were deposited on petri dishes which had been previously covered with protamine sulfate (0.5 mg in distilled water) and then rinsed twice with sea water. At different times after fertilization, the supernatant was removed by aspiration, and eggs were rinsed 2 times with ice-cold glycine medium (1 M glycine, 1 mM EGTA, pH 8.0 adjusted with Tris 1 M). The eggs were then scraped and lysed in ice-cold extraction

buffer (HEPES 50 mM, NaCl 150 mM, glycerol 10%, EDTA 10 mM, $MgCl_2$ 1 mM, Nodinet P-40 1%) containing various phosphatase and protease inhibitors (potassium vanadate 2 mM, $Na_2P_2O_7$ 10 mM, Trasylol 1000 U/ml, bacitracine 1.4 mM, leupeptin 1 mM, NaF 10 mM). The washing procedure takes about 7 s and times indicated in the figures correspond to the time of scraping in lysis buffer. The extracts were centrifuged in an Eppendorf centrifuge for 10 min, and supernatants incubated for 4 h at 4°C with 1 μl of anti-phosphotyrosine antibody (PY20; ICN) coupled to agarose-anti-mouse antibody (Sigma). The immunoprecipitates were rinsed following the procedure described by Whitman et al. [25], twice with the extraction buffer, then with 0.5 M LiCl, 0.1 M Tris, pH 7.4, and finally with 10 mM Tris, 100 mM NaCl, 1 mM EDTA. Immunoprecipitates were fractionated on 10% SDS polyacrylamide gel [26], and the phosphotyrosine-containing proteins revealed by Western immunoblot using the same anti-phosphotyrosine antibody. Bound antibody was detected by incubation with a second antibody linked to alkaline phosphatase (Promega).

The sperm extract was prepared as follows: 500 μl of concentrated sperm was rinsed 2 times with the glycine medium in an Eppendorf centrifuge at 4°C. The pellet was then treated by the same procedure described above as used for eggs.

All immunoprecipitates contained proteins of approximately 28 and 65 kDa, corresponding to the primary antibody.

3. RESULTS

Our initial results, using PY20 antibody to probe egg lysates by a direct Western immunoblot procedure, revealed a slight phosphotyrosine increase within 2 min of fertilization (data not shown). In order to concentrate the proteins phosphorylated on tyrosine and to amplify the signal, we immunoprecipitated the egg proteins before SDS-PAGE and Western blot analysis. We also developed a rapid sampling procedure to detect whether these changes in tyrosine phosphorylation took place in the first seconds following sperm addition. For this, unfertilized eggs were attached to protamine-coated petri dishes and at the indicated times following fertilization, the eggs in the dish were quickly washed and lysed by the addition of a lysis buffer. The proteins were then immunoprecipitated with the anti-phosphotyrosine antibody. The immunoprecipitates were analysed by SDS-PAGE, and the phosphotyrosine content of the proteins visualized with Western blot analysis using the same anti-phosphotyrosine antibody.

Immunoprecipitates from both unfertilized and fertilized eggs contain several phosphotyrosine-containing proteins, detectable by Western immunoblotting (Figs. 1A and C). Fertilization triggered a rapid and major increase in the phosphorylation of two of these proteins (molecular weight of approximately 91 and 138 kDa) (Fig. 1A). This increase was detectable in the first 15 s following sperm addition and the tyrosine phosphorylation content of both proteins further increased during the next 2 min.

The observed increase in tyrosine phosphorylation occurs in egg and not the sperm. Western blot analysis of immunoprecipitates obtained from sperm extracts did not reveal any phosphotyrosine-containing protein (Fig. 1A), nor were there any when sperm was first activated with jelly coat to induce the acrosome reaction (Fig. 1B). The anti-phosphotyrosine antibody was specific. Extracts of eggs obtained before or 2 min after fertilization were immunoprecipitated with the anti-

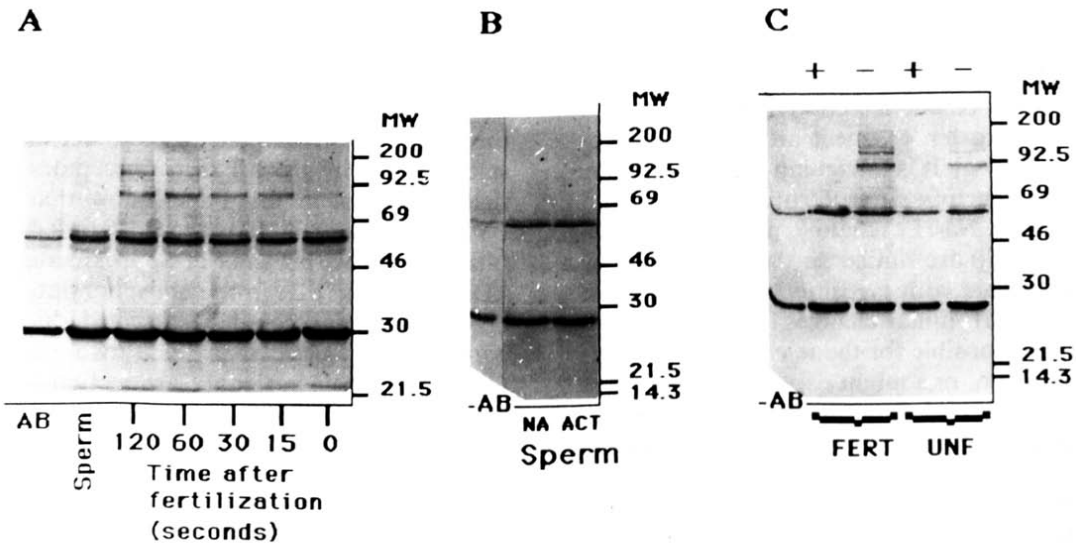

Fig. 1. Western immunoblot of anti-phosphotyrosine immunoprecipitates from eggs and sperm. (A) Changes of the phosphotyrosine content of sperm and of egg proteins at various time points after fertilization. All immunoprecipitates were prepared as described in Materials and Methods. (B) Western blot of anti-phosphotyrosine immunoprecipitates from sperm-activated (ACT) or not activated (NA) by jelly coat. Jelly coat was prepared as in [27]. Activated sperm was rinsed by centrifugation with the glycine medium and treated as described in Materials and Methods. (C) Western blot of anti-phosphotyrosine immunoprecipitates obtained from unfertilized eggs (UNF) or eggs extracted 2 min after fertilization (FERT), and treated in the presence (+) or the absence (−) of phenylphosphate. Samples were prepared as described in Materials and Methods except that the immunoprecipitates marked + were incubated in the presence of 50 mM phenylphosphate. Lanes noted AB were run as control immunoprecipitates (anti-phosphotyrosine coupled to second antibody-agarose), which were treated by the same procedure but in the absence of egg or sperm extract.

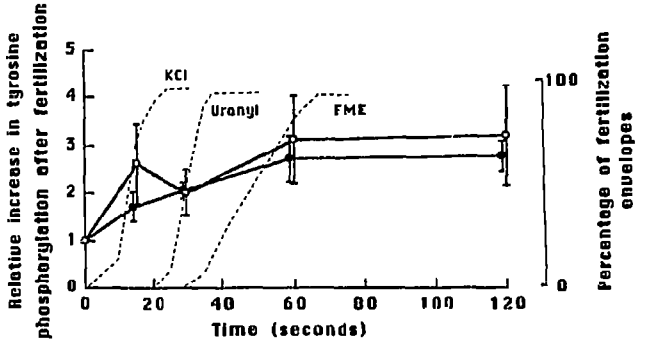

Fig. 2. Time course of tyrosine phosphorylation of the 91 kDa (●) and 138 kDa (○) after fertilization. The relative phosphotyrosine content of the two proteins was determined by densitometric scanning of six Western immunoblots obtained, as described in Materials and Methods, from different batches of eggs. Results are expressed ± SE and relative to that obtained for unfertilized eggs which was arbitrarily taken as 1.0. One dashed curve (FME) indicates the time course of fertilization membrane elevation in an egg population. The two other dashed curves give the percentage of fertilization envelopes in the presence of 0.5 mM KCl or 0.7 mM uranyl acetate, as described in the text.

body in the presence of 50 mM phenylphosphate which competes with the phosphotyrosine for the antibody [15]. Fig. 1C shows that the Western blot of these immunoprecipitates gave no signal. Identical results were obtained by using 1 mM phosphotyrosine instead of phenylphosphate (not shown).

To compare the results from different experiments, we did densitometric scanning of six different Western blots obtained from six different batches of eggs. The results, depicted in Fig. 2, show that the increase in the phosphotyrosine content of both the 91 kDa and 138

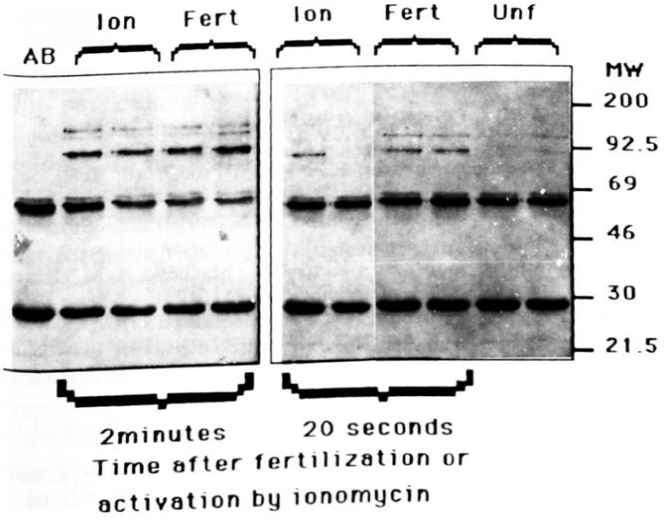

Fig. 3. Comparison of tyrosine phosphorylation triggered by fertilization or activation with ionomycin. Immunoprecipitates were from unfertilized eggs (UNF), 20 s and 2 min after fertilization (FERT) or activation by 7 μM ionomycin (ION), and prepared as described in Materials and Methods. Each experimental condition shows results from two separate batches of eggs.

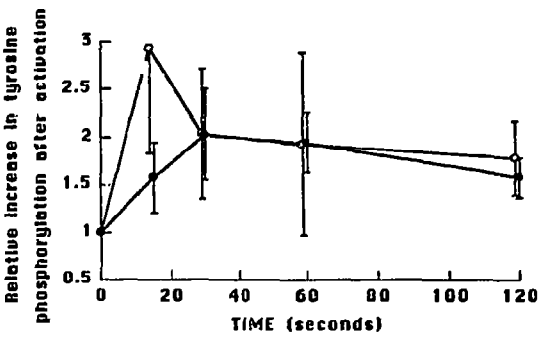

Fig. 4. Time course of tyrosine phosphorylation of the 91 kDa (●) and 138 kDa (○) after activation by 7 μM ionomycin. Densitometric scanning of the Western blots was performed as described in Fig. 2 legend.

kDa proteins triggered by fertilization attains a plateau in 2 min. Most importantly, much of the increase has occurred in the first 15 s following sperm addition.

Fig. 2 shows that the increase in protein phosphotyrosine by 15 s after insemination occurs during the 'latent period'. We determined the latent period in *Strongylocentrotus purpuratus* by adding, at 5-s intervals after sperm addition, KCl-rich sea water or uranyl ions, which respectively act at the beginning or at the end of the latent period [16], and measuring the percentage of activated eggs (Fig. 2). We found the latent period in *S. purpuratus* eggs was 15 s. In these egg populations, fertilization envelopes begin to elevate 30–35 s after sperm addition, and are fully elevated 40 s later [17] (Fig. 2). The kinetics of the Ca^{2+} rise in this species have not been directly determined, but the *Lytechinus pictus* egg exhibits similar temporal characteristics, with a latent period of 12–22 s and with the Ca^{2+} rise initiated at the end of the latent period [4,18]. The Ca^{2+} rise most probably initiates at a similar time (12–22 s) in the *S. purpuratus* zygote. This is also consistent with direct comparisons of the calcium rise with the elevation of the fertilization envelope in *Clypeaster japonicus* eggs. Here the Ca^{2+} rise begins 10 s before fertilization envelope elevation [19]; if one uses this criterion, the Ca^{2+} rise in *S. purpuratus* would then begin at 20–25 s after fertilization.

If the increase in tyrosine phosphorylation occurring during the latent period is part of the pathway leading to the release of intracellular free calcium, one would expect that increasing cytosolic calcium directly, as with a calcium ionophore, would bypass this event. However, when we compared phosphotyrosine formation at fertilization with that induced by activation with 7 μM ionomycin, increased phosphorylation on tyrosine of the two proteins was seen by 20 s after ionophore addition and was similar to that measured after fertilization (Fig. 3). This suggests that the tyrosine phosphorylation can be induced by calcium. After compilation of results from different Western blots analysed by densitometric scanning, we found that the phosphorylation content of

the two proteins, measured 15 s after activation, was similar with activation by ionomycin or by sperm (Fig. 4). However, the subsequent phosphorylation measured after one minute was greater with fertilization (Fig. 2).

4. DISCUSSION

We report in this paper that a very rapid increase in the phosphorylation of two egg proteins occurs after fertilization. The most important finding is that this increase takes place during the latent period. It is then possible that this event might play a role in signal transduction following fertilization.

A major question concerns the identity of these 91 and 138 kDa phosphotyrosine-containing proteins. Phosphorylation of tyrosine can be part of amplification cascades and in some cases the amplification can involve the subsequent activation of polyphosphoinositide hydrolysis, with a resultant increase in cytosolic calcium [10,20,21]. Receptors with tyrosine kinase activity can be linked to phospholipase C and PI kinase activity in other cell types [20,21], and it is intriguing that these two enzymes are also activated after sea urchin fertilization [2,7,22]. The phosphotyrosine changes seen so early after sea urchin fertilization then could be a major component of an amplification system tied to polyphosphoinositide metabolism or the other signaling systems hypothesized to be involved in egg activation [3,40]. In analogy to growth factor action, such as seen with platelet-derived growth factor, the increased tyrosine activity seen after fertilization could result from occupation of a sperm receptor [3] or could result from transfer of an activating factor from the sperm into the egg cytoplasm [4–6]. We observed a stimulation of phosphorylation by ionomycin, which indicates that Ca^{2+} by itself can induce tyrosine phosphorylation. These results also suggest an autocatalytic or positive feedback role for the tyrosine phosphorylation.

A pH increase is also induced by fertilization or calcium ionophore but this increase is probably not involved in the phosphotyrosine change that we report here, since the pH increase does not begin until 45–60 s after insemination, when the described phosphotyrosine change is almost complete. There is, however, a later phosphotyrosine change related to this pH increase; Jiang et al. [12,13] have described a pH-dependent alteration in phosphotyrosine on a 350 kDa egg protein which begins several minutes after fertilization. These data then point to post-fertilization sequences of pCa- and pH-dependent tyrosine kinase and/or phosphatase activities. If sperm induces a local calcium increase, whether by injection of calcium [23], IP_3 [24] or as a consequence of a primary sperm-mediated tyrosine phosphorylation, this calcium increase could then autocatalytically produce more tyrosine phosphorylation. A sperm-mediated phosphorylation, in combination with a calcium-mediated tyrosine phosphorylation, might indeed explain how the local sperm–egg interaction can be amplified into the propagated and global calcium increase which characterizes fertilization.

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