Relationship between release of surface proteins and metabolic activation of sea urchin eggs at fertilization

(cell surface/cell transformation/protein synthesis/glycoproteins)

JAMES D. JOHNSON AND DAVID EPEL

Marine Biology Research Division, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, Calif. 92038

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ABSTRACT Macromolecular components are released from sea urchin eggs when their metabolism is activated at fertilization or by incubation in ammonia. When the released material is dialyzed, concentrated, and added back to partially activated eggs the rate of protein synthesis is suppressed to the level of the unactivated egg. The surface proteins of the unfertilized eggs can be labeled with $^{125}\text{I}$ by a lactoperoxidase procedure. When fertilized or activated with various parthenogenetic agents, 15–25% of the total labeled protein is released; most of the label is associated with a 150,000-dalton glycoprotein. The extent of metabolic activation, as assessed by measuring increased protein synthesis, is correlated with the amount of surface label released. Several other proteins are released during activation but are not labeled by the lactoperoxidase procedure in the intact cell. We have not yet identified which of these components is responsible for suppressing protein synthesis, nor do we know if any of the other metabolic changes of fertilization such as K$^+$ conductance and DNA synthesis are also suppressed. We suggest that these released components are surface molecules involved in maintaining the low metabolic state occurring at the end of oogenesis and that removal of these components during fertilization results in the release of the suppression of the egg.

Fertilization of sea urchin eggs results in a cascade of events occurring in a defined temporal sequence. As part of the ensuing metabolic transformation, there occurs a massive reorganization of the plasma membrane resulting from the fusion of approximately 15,000 secretory granules (cortical granules) with the egg plasma membrane (1). This exocytosis occurs about 30 sec after fertilization, but is not a prerequisite for the array of metabolic events that follow, since incubating eggs in 1–10 mM NH$_4$Cl initiates a number of the metabolic changes such as K$^+$ conductance of the plasma membrane and protein and DNA synthesis (2–4), but does not initiate the cortical granule exocytosis (2). Also, development of eggs is activated when fertilized in the presence of inhibitors of the cortical exocytosis (5, 6).

Using a lactoperoxidase-$^{125}\text{I}$ technique for labeling the egg surface, we had previously observed that fertilization and several different types of parthenogenetic activation resulted in the release of a labeled, large molecular weight surface protein (7). We here show that release of labeled protein and nonlabeled proteins occurs when the eggs are activated with a resultant cortical granule exocytosis or are treated with agents such as ammonia, nicotine, or procaine, which only activate cytoplasmic events such as protein and DNA synthesis. This finding suggested a relationship between the release of protein and the subsequent metabolic activation of the egg. This correlation is directly supported by experiments showing that adding released surface components to activated eggs suppresses the protein synthetic capacity of the cell back to the level of the unfertilized egg.

Loss of surface protein accompanying a metabolic activation has also been reported for vertebrate somatic cells upon viral, proteolytic (8), or chemical (9) transformation and suggests that metabolic repression by loosely held surface (peripheral) proteins is a common feature of cells. We suggest that in oocytes the metabolic stasis occurring at the end of oogenesis results from addition of proteins or other materials to the oocyte surface and that the removal of these surface components at fertilization results in the metabolic derepression of the oocytes.

MATERIALS AND METHODS

Handling of Gametes. Ovulation of Strongylocentrotus purpuratus eggs was induced by intracoelomic injection 0.5 M KCl. Egg jelly was removed by exposure to pH 5.0 seawater with swirling for 2 min. The pH was then titrated back to 8.0 with 1.0 M Tris-HCl (pH 8.0) in seawater, and the dejellied eggs were then washed several times in filtered seawater. Temperature in all experiments was 16°.

Activation of Eggs with Ionophore. Ionophore A23187 was obtained from R. L. Hamill, Eli Lilly Co., Indianapolis, Ind. Eggs were activated as described by Steinhardt and Epel (10).

Labeling of Surface Proteins with $^{125}\text{I}$. The procedure of Huber and Morrison (11) was followed, modified as described below. To 1 ml of a seawater-lactoperoxidase (0.3 mg/ml, Calbiochem) solution in a 10-ml beaker was added 10 $\mu$l of Na$^{125}$I (2 mCi/ml in 0.01 M NaOH, New England Nuclear). Sodium sulfite (10 $\mu$m) was added to reduce any I$_2$ to I$^-$. After thorough mixing, 1 ml of a 10% suspension of dejellied eggs was added. Ten microliters of a 0.06% solution of H$_2$O$_2$ in filtered seawater was added at zero time, and further 10-$\mu$l additions were made at 2-min intervals until 8 min had elapsed. The eggs were kept in suspension by swirling during the entire procedure. After a total time of 10 min, the eggs were poured into 40 ml of filtered seawater in a conical centrifuge tube and washed four times by gentle hand centrifugation and resuspension in filtered seawater. Eggs iodinated by this procedure exhibited normal development upon fertilization. Approximately 5% of the total $^{125}\text{I}$ added was bound. The radioactivity of the $^{125}\text{I}$-labeled material was determined by placing a portion of the protein solution or egg suspension in 10 ml of Aquasol (New England Nuclear) and counting in a Beckman LS-230 Scintillation counter.

Removal of the Vitelline Layer. The vitelline layer was removed by the dithiothreitol method of Epel et al. (12) or by a new procedure developed by Carroll et al.* In the latter technique a partially purified protease fraction from the cortical granules of S. purpuratus is used to detach the vitelline layer from the plasma membrane; a subsequent incubation would be necessary.
tion of the eggs in the 10 mM dithiothreitol in seawater at pH 9.1 completely removes the vitelline layer. This procedure is more reliable than the original dithiothreitol method (12), which variably removed 20–40% of the 125I from eggs labeled as above. It is more specific than proteolytic procedures utilizing trypsin or Pronase, which alter many surface proteins; the cortical granule proteases carry out a very limited proteolysis of the vitelline layer.

**Measurement of Protein Synthesis.** The extent of metabolic activation of a 0.2% suspension of eggs or embryos was determined by measuring the rate of incorporation of L-[3H]valine into trichloroacetic acid-insoluble protein after a 5-min pulse, as described by Epel et al. (3). Radioactivity in both the trichloroacetic acid-soluble and insoluble fractions was measured (3), and total dpm in each fraction was calculated by standard procedures. Incorporation into protein is expressed as percent incorporation, using the ratio (dpm in acid-insoluble)/(dpm in acid-soluble and acid-insoluble). Radioactivity in the trichloroacetic acid-soluble fraction was determined as above; radioactivity of the protein pellet was measured by liquid scintillation counting after solution in NCS and counting in a toluene-based cocktail. Counting efficiency was determined by addition of internal standards.

**Preparation of Released Proteins for Bioassay or Gel Electrophoresis.** A 10% suspension of eggs was incubated for 15 min in 10 mM NH4Cl, pH 8.0, the eggs were removed by hand centrifugation, and the supernatant fluid was then centrifuged at 48,000 × g for 30 min. For bioassays, the resulting supernatant was dialyzed against several changes of filtered seawater at 4°C. For electrophoresis, samples were dialyzed against distilled water, frozen, and lyophilized. The resulting residue was weighed and solubilized in sodium dodecyl sulfate buffer (14) to a concentration of approximately 1–2 mg/ml.

**Polyacrylamide Gel Electrophoresis with Sodium Dodecyl Sulfate.** Electrophoresis on polyacrylamide slab gels was described by Studier (13) with 7.5% acrylamide in the running gel and 4% acrylamide in the stacking gel. All gels were run at a constant 45 V. Gels were stained for protein with Coomassie blue according to Laemmli (14) and for carbohydrate with the periodic-acid Schiff stain as described by Segrest and Jackson (15). For molecular weight determination and for quantifying the radioactivity of 125I-labeled protein, 7.5% sodium dodecyl sulfate disc gels were run by the procedure of Laemmli (14). Standards for molecular weight included *Escherichia coli* β-galactosidase (130,000), rabbit muscle glycogen phosphorylase (94,000), bovine serum albumin (68,000), rabbit muscle aldolase (40,000), and bovine pancreas carboxypeptidase A (34,000). For determining radioactivity, gels were sliced into 0.20-cm slices, placed in scintillation vials with 0.5 ml of NCS tissue solubilizer, and heated for 4 hr at 55°C. After 12 hr at room temperature, 2,5-diphenyloxazole (PPO)-1,4-bis(2,5-phenyloxaz-dyl)benzene(POPOP)-toluene scintillation fluid was added and radioactivity determined in a LS-230 Beckman liquid scintillation system.

**RESULTS**

The iodination procedure as described had no adverse effects on development through the pluteus stage. Incubation of labeled eggs in 1 mg/ml of Pronase for 1 hr removed 88–92% of the label, indicating the bulk of iodination was at the cell surface and available to these enzymes. Similar results were earlier obtained with eggs from another species (Arbacia punctulata), and here surface labeling was further confirmed by autoradiography (16).

When eggs were fertilized with sperm or activated with ionophore A23187, approximately 25% of the 125I label was released into the seawater in a nondialyzable form (Fig. 1). These released counts were covalently bound to protein, as indicated by precipitation in 10% trichloroacetic acid and migration as a single Coomassie blue staining band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

This release could result from the membrane vesiculation accompanying exocytosis of the cortical granules or be related to some aspect of the metabolic activation accompanying fertilization. These two alternatives were distinguished by incubating eggs in 10 mM NH4Cl, pH 8.0, which activates metabolism but does not initiate cortical exocytosis. As shown in Fig. 1, incubation in ammonia also releases 125I label at a rate slower than that accompanying ionophore activation or fertilization. This suggests that the release is related to activation. Metabolic activation in ammonia is slower than that induced by ionophore or normal fertilization (3).

**Correlation of protein release and metabolic activation**

If the release of protein is related to metabolic activation, then there should be a correlation between the activation of metabolic processes and the release of protein by agents other than ammonia. Carroll (personal communication) found that 1 mM nicotine would activate chromosome condensation, but not the cortical reactions. Vacquier and Brandiff (6) have reported similar observations for procaine. We compared release of protein and metabolic activation by these agents, assessing activation by measuring percent incorporation of amino acid into protein. As shown (Fig. 2), there is strong correlation between increased protein synthesis and release of iodinated surface protein. This correlation also applies to incubation in ammonia at pH 6.0, which neither activates protein synthesis nor results in release of surface label.
Characterization of proteins released during incubation of eggs in ammonia

The major component released during incubation of eggs in ammonia is a 150,000-dalton glycoprotein, as judged by positive staining with the Coomassie blue and periodic acid-Schiff stain. This same molecular weight was observed over a 5–10% range of polyacrylamide gel concentrations. A detectable amount of this protein is released after only 3 min of incubation in ammonia; other lower molecular weight proteins become apparent after 20 min of incubation in ammonia (Fig. 3), and two of these (A and B) stain positively with the periodic acid-Schiff stain (Fig. 3). However, only the 150,000-dalton protein is labeled with $^{125}$I (Fig. 3); either this protein is the only one containing exposed tyrosine residues or is the only protein released during the ammonia treatment that is sufficiently exposed on the egg surface to be iodinated.

Site of the $^{125}$I-labeled protein released by ammonia

The following experiments indicate that the iodinatable protein released by ammonia is most likely on the plasma membrane itself and is not a component of the vitelline layer. This latter layer contains major surface proteins of the unfertilized egg and elevates at fertilization as the template of the fertilization membrane. The vitelline layer of iodinated eggs was removed with either the dithiothreitol (12) or cortical granule protease* procedure (see Materials and Methods). Eggs denuded by either of these procedures were not metabolically activated, as judged by measurement of rate of protein synthesis. The eggs were then activated by incubation in ammonia; labeled surface protein was released that was identical in amount and in molecular weight to that released by normal eggs.

Results of experiments in which eggs were incubated in trypsin or Pronase suggest that only a small portion of the 150,000-dalton protein is susceptible to proteolysis by exogenous proteases. Eggs were treated in 1 mg/ml of trypsin for 1 hr and incubated in ammonia. The proteins released into the seawater were collected and electrophoresed on sodium dodecyl sulfate gels as described. A major protein of approximately 150,000 daltons was released from these eggs after incubation in ammonia. Eggs treated with 1 mg/ml of trypsin or Pronase for 30 min were not metabolically activated, as judged by measurement of protein synthesis.

Biological activity of nondialyzable components released upon activation

The above results indicate that release of labeled surface protein is closely correlated to metabolic activation. The following experiment asks if the activation is reversible, i.e., can the nondialyzable material released by eggs upon activation be added back and suppress the metabolism of already activated eggs? Three samples of eggs (0.2% suspension) were activated by incubation in 10 mM NH$_4$Cl, pH 8.0. At 30 min the percent incorporation of $[^3]$Hvaline into protein was measured. The samples were then washed twice in filtered seawater and resuspended in ammonia solution, filtered seawater, or the solution of nondialyzable material from activated eggs (10–30 µg/ml of protein). This represents a 50-fold concentration of material (see Materials and Methods). The rate of protein synthesis of each group was then measured at 60 and 90 min. In five experiments, the rate of protein synthesis of eggs incubated in the nondialyzable material was significantly suppressed when compared to the eggs resuspended in seawater only or resuspended in 10 mM NH$_4$Cl in filtered seawater (Fig. 4). Ammonia (10 mM, pH 8.0) reversed the suppressive effect. When ammonia-activated eggs were incubated with 50 µg/ml of bovine serum albumin or ovalbumin, no suppression of protein synthesis was observed. Heating the solution of nondialyzable material in a boiling-water bath for 15 min did not inactivate the activity. If protein is responsible for the suppressor activity, then the heat stability of the molecule would be consistent with the characteristics of glycoproteins (17). We have not yet directly established, however, that the iodinatable glycoprotein is rebound to the surface of already activated eggs.

**DISCUSSION**

Fertilization normally initiates a programmed sequence of changes, which can be temporally divided into two distinct phases (18). The initial or “early” changes occur in the first...
FIG. 4. The suppression of protein synthesis by nondialyzable components released by eggs. A 0.2% suspension of eggs was incubated in 10 mM ammonia for 30 min, at which time a 2-ml sample of eggs was pulsed with [3H]valine for 5 min. Eggs were then washed twice in filtered seawater, and separate groups were incubated in ammonia (X), filtered seawater (○), or components released by eggs (■). Controls remained in filtered seawater throughout (△). Samples (2 ml) from each group were pulsed for 5 min with [3H]valine beginning at 60 and 90 min. Error bars are standard deviation of five experiments.

60 sec after sperm-egg interaction and appear to involve the release of Ca^{2+} from intracellular stores (10) and the resultant exocytosis of cortical granules (19). The “late” changes, which begin at 5 min after insemination, normally follow the early changes as a part of the programmed sequence of fertilization. The early changes, however, are not a prerequisite for the late changes, since they can be bypassed by incubation of eggs in agents such as ammonia (2–4), nicotine (this paper), and procaine (6). Also, these late changes act independently of each other, as opposed to the early changes which are all dependent upon one another (3). On these grounds one could argue that the early and late changes are regulated by different factors.

The results of this paper suggest that one of these regulating factors is a protein (or proteins) released by cells upon activation and which reversibly controls at least one of the late events, a post-transcriptional increase in protein synthesis. These proteins appear to act in a suppressive fashion, and their loss from the cell surface promotes an increased synthetic rate. Kinetic data of this paper and a similar study by Shapiro (20) indicate that during activation by fertilization or by ionophore a maximal release of labeled protein occurs coincident with or shortly after the cortical reaction. Trypsin-like proteases of the cortical granules do not appear to be involved in the release, since inhibitors such as N-α-p-tosyl-L-lysine chloromethyl ketone (10 mM concentration), L-1-tosylamide-2-phenylethyl chloromethyl ketone (1 mM concentration), and phenylmethyl sulfonyl fluoride (5 mM concentration) do not prevent the release of the labeled surface protein and do not affect activation of protein synthesis. Partial metabolic activators such as ammonia, nicotine, and procaine result in a slower release of this protein, but in the absence of cortical granule breakdown. No apparent structural analogy exists among these three agents except that all are amines (primary, secondary, or tertiary amines). These activating agents may perturb the plasma membrane, releasing the proteins.

It is not yet known whether protein synthesis is regulated by one or several of the nondialyzable components, nor is it clear whether any of these components regulate the other late events, such as DNA synthesis and chromosome condensation. Correlative arguments support the concept that all late events are regulated by some similar mechanism; agents releasing iodinatable protein or various treatments perturbing the surface (21) activate all the late events. Assuming surface proteins are the regulators, it is most provocative that the metabolism of the egg can literally be “titrated” by ammonia; low concentrations will increase protein synthesis, but higher concentrations are needed to induce chromosome condensation (3). In light of the present results, this could mean that increasing concentrations of ammonia release increasing amounts of some general regulatory molecule, i.e., that the regulation is quantitative and depends on the amount of cell surface component that is released; or this could mean that increasing concentrations of ammonia release different species of regulatory molecules, i.e., that there are qualitatively different cell surface regulators for cell processes such as protein or DNA synthesis.

Concepts of membrane-mediated regulation of cell activity, as through hormone receptors, are widely accepted (22). Recent work has suggested that the promotion of cell activity, as occurs during various types of transformation, is related to the loss of a large molecular weight glycoprotein (8, 9). Also, glycopeptides derived from the surface of HeLa cells will repress the protein synthesis of these cells (23).

We see two major possibilities as to how loss of the surface components of the egg might be translated into activation of the metabolic processes of the cell. One of these is that this surface molecule is regulating some enzyme or enzyme system. In analogy to models of hormone action (22), this enzyme could be a nucleotide cyclase and the effect mediated through cyclic nucleotides to protein-modifying enzymes (e.g., protein kinase). Although cAMP and cGMP do not change after fertilization (24–26), the possibility of a transient increase at the time of the late changes (5 min after insemination) has not been eliminated. Alternatively, regulation could be directly on a protein kinase. This enzyme is present in the cortex of sea urchin eggs (27).

A second possibility is that the surface components regulate cell structure and that their loss during fertilization or activation results in cell recompartmentation and increased availability of enzymes to their substrates. Glucose-6-phosphate dehydrogenase (28) and aldolase (29) are present in particular fractions in unfertilized eggs and are translocated to the soluble fraction in the fertilized egg. Also, studies on enzyme activation suggest that substrates and enzymes are unavailable to each other until after fertilization (30, 31). Such recompartmentation may occur in two dimensions, as by changes in membrane fluidity, or in three dimensions, as through changes in the structure of the cytoplasm. A recompartmentation mechanism is appealing since it would explain the generalized promotion of many processes as are induced during partial activation by ammonia or during fertilization by sperm.

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