A Question-Based Laboratory Project for Examining the Role of Calcium in Sea Urchin Fertilization

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Introduction

Sea urchins are a well-established model system for studying fertilization and early development. Urchin gametes are also an excellent tool for teaching cell biology in the laboratory, allowing students to make direct connections between biochemical changes in the egg and the cellular events of fertilization that can be observed under the microscope. In this project, the students examine some of the signal transduction events that occur at fertilization, and answer two questions regarding the role of the cytoplasmic calcium influx in entry of the zygote into the cell cycle. The central experiment of the project is a Western blot that allows the students to determine the activity of mitogen-activated protein (MAP) kinase under different conditions. MAP kinase becomes active during the maturation of oocytes in most animals, and this activity is thought to maintain cell cycle arrest and prevent parthenogenesis (4). Sea urchin eggs, which complete meiosis during maturation, contain active MAP kinase that becomes inactivated upon fertilization, presumably removing the cell cycle brake and allowing the zygote to undergo cleavage (5, 6).

At fertilization, sperm binding triggers the activation of phospholipase C, leading to rapid production of the second messengers inositol triphosphate (IP_3) and diacylglycerol, and the opening of Ca^{2+} channels on the endoplasmic reticulum (see figure 1)(1). The resulting Ca^{2+} influx is a central node in the signal transduction that occurs at fertilization and it stimulates many of the events that occur during egg activation (7). Cytoplasmic Ca^{2+} initiates exocytosis of the cortical granules, causing the fertilization envelope to elevate around the egg. The students are introduced to the major aspects of fertilization signal transduction in lecture, and are then directed to answer the following two questions with their laboratory experiments:

1. Is the calcium influx sufficient to inactivate MAP kinase?
2. Is the calcium influx sufficient to produce cell division (first cleavage)?

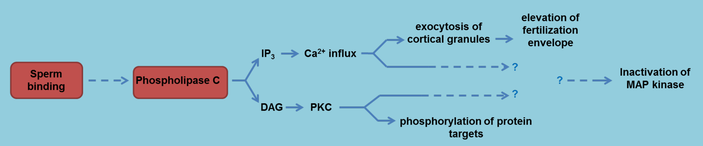

Figure 1. Sea Urchin Fertilization Signal Transduction. Established signaling steps are indicated with solid arrows; incompletely understood steps are indicated with dashed arrows. Sperm binding initiates the activation of phospholipase C, which cleaves phosphatidyl-inositol 4,5-bisphosphate to inositol triphosphate (IP_3) and diacylglycerol (DAG)(1). Soluble IP_3 triggers the opening of Ca^{2+} channels on the endoplasmic reticulum, and the resulting cytoplasmic calcium influx induces exocytosis of the cortical granules leading to elevation of the fertilization envelope. DAG and Ca^{2+} activate protein kinase C (PCK) isoforms that are known to phosphorylate specific protein targets in the zygote (2, 3).

Experiment Design

To answer these questions, the students artificially produce a cytoplasmic Ca^{2+} influx in unfertilized eggs with the calcium ionophore, A23187. This compound renders the ER membrane permeable to Ca^{2+}, and will produce elevation of the fertilization envelope and other aspects of egg activation in the absence of fertilization (8). Fertilized eggs serve as a positive control for answering both questions, and a solvent control for A23187 (dimethylsulfoxide, DMSO) is also included, so that eggs treated under four experimental conditions are examined: 1. sea water alone; 2. fertilized; 3. A23187; 4. DMSO.

On the first day of the project the students treat egg suspensions in sea water with the different conditions, and observe the eggs under the microscope for egg activation (raised fertilization envelope) at 35 minutes and for cleavage at 2.5 hours. The students expect egg activation in both the fertilized and A23187-treated samples, and by looking for cleavage in A23187-treated eggs can answer if Ca^{2+} is sufficient to induce this. Egg lysates are also prepared for the Western blot that will determine the amount of active (phosphorylated) MAP kinase present under the different conditions, allowing the students to determine if Ca^{2+} is sufficient to inactivate MAP kinase. The concept of necessary vs. sufficient in experimental design and interpretation of results is discussed in lecture, and is emphasized throughout the project. At the end of the project, the students write a lab report where they present and analyze their results to answer the two questions. As part of the report they are directed to read some of the relevant literature, and from this must design an experiment to determine if calcium is necessary for MAP kinase inactivation. Finally, they are also asked to discuss their reasoning for whether MAP kinase inactivation is necessary, sufficient or both for cleavage of the zygote at fertilization.

An additional experiment can also be added to the project where the students examine the activation of phospholipase C under the same four experimental conditions. To do this, the students use a commercially available ELISA kit that measures the concentration of an IP_3 breakdown product, inositol monophosphate (IP_1). They are asked to explain whether they expect to see phospholipase C activation (high IP_1 concentrations) in eggs treated with A23187, and whether their results match this expectation.

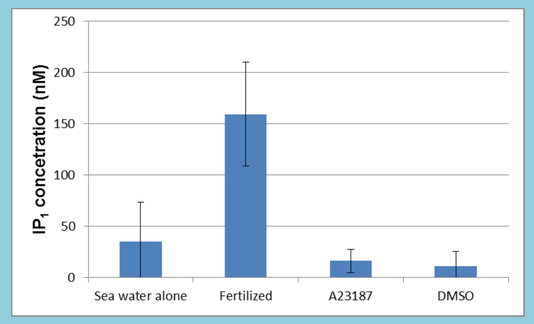

Figure 4. Detection of Phospholipase C Activity in Sea Urchin Egg Samples Using an ELISA that Measures an IP_3 Breakdown Product: Egg suspensions were treated as in figure 2 for 35 minutes at 16 C, and then whole-cell lysates were prepared. A commercially available ELISA kit was used to measure lysate inositol monophosphate (IP_1) levels. IP_3 has a half-life of approximately 30 seconds and is rapidly broken down to IP_2 and IP_1 by specific phosphatases, and lithium chloride is added during experimental treatments to stabilize IP_1 and prevent its break down to free myo-inositol. Therefore, the IP_1 concentration indicates the level of phospholipase C signaling that has occurred. The experiment shown was performed by students during spring quarter 2010. Bars represent the average IP_1 concentration from 6 ELISA wells utilizing 4 independent samples, and the error bars indicate standard deviation.

Based on what they know about the signal transduction that occurs at fertilization, the students are asked to predict whether they expect A23187 treatment to activate phospholipase C. In their lab reports they discuss whether the experiment gave the expected result with each treatment.

Experiment Schedule

Day 1
- Observe fertilization and egg activation
- Score experimental samples for egg activation at 35 minutes
- Score experimental samples for cleavage at 2 ½ hours
- Prepare samples for Western blot and ELISA

(Requires approximately 3 to 3 ½ hours total)

Day 2
- Perform SDS-PAGE and electroblotting on Western blot samples—block nitrocellulose filter overnight

(Requires approximately 3 to 3 ½ hours)
(The following day the blots are placed in the primary antibody for the students and incubated overnight until the next lab period.)

Day 3
- Immunodection: Add secondary antibody, wash, perform ECL detection

(Requires approximately 3 hours)
(Optional)

Day 4
- Perform ELISA to detect phospholipasae C activation
- Examine and discuss Western blot films during ELISA incubation

(Requires approximately 3 ½ hours)

Ca2+ is Sufficient to Inactivate MAP Kinase

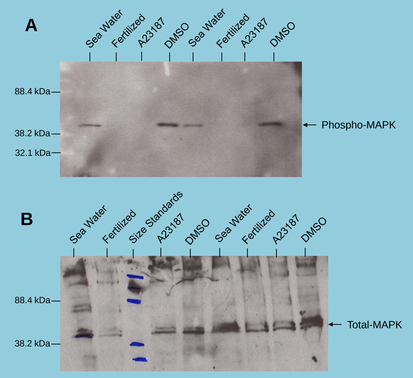

Figure 2. Western Blot to Detect the Inactivation of MAP Kinase Following Fertilization or Treatment with A23187: Egg suspensions were left untreated (Sea Water), fertilized, treated with 5 µM A23187, or DMSO for 35 minutes at 16°C. Lysates were prepared and analyzed by Western blot. **A.** Blots were probed with an antibody that specifically recognizes MAP kinase phosphorylated on Thr202/Tyr204 (active). **B.** Blots were probed with an antibody against total MAP kinase (active and inactive). Lab groups share samples so that identical samples are analyzed for phosphorylated and total MAP kinase. Both A and B are student data generated over the last two quarters, although from different classes using different samples.

Ca2+ is Not Sufficient to Induce Cell Division

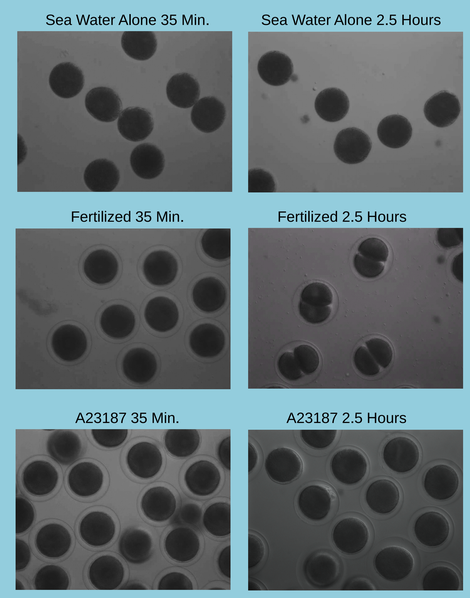

Figure 3: Eggs in filtered sea water were left untreated, fertilized, treated with 5 µM A23187, or DMSO (not shown) for the indicated times. The students observed which treatments produced activation (elevation of fertilization envelope) and/or cleavage (presence of cleavage furrow). These images were taken using differential interference contrast microscopy at an original magnification of 200X. The students use phase contrast microscopes (Motic BA300) to observe egg activation and cleavage, however standard light microscopes also work well to observe these events.

Materials and Methods

Preparation of Sea Urchin Gametes: Gametes from the California purple sea urchin, *Strongylocentrotus purpuratus*, were extracted by injection of 0.55 M KCl into the coelomic cavity. Gametes used in our Biochemical Techniques course are generously provided by the Hamdoun Lab, Scripps Institute of Oceanography, University of California, San Diego. Sperm is stored dry and kept on ice until use. Eggs are diluted into filtered sea water to a concentration of 1% (approx. 1 ml of settled eggs per 100 ml) for use in experiments, and are kept refrigerated.

Treatment of Egg Suspensions: Our Biochemical Techniques laboratory sections have 24 students who break up into 8 lab groups of three students each. Each lab group performs two treatments (sea water alone and fertilized, or A23187 and DMSO) for the Western blot, ELISA, and observation of cleavage, and data and samples are shared. For each sample, 1 ml of egg suspension (2 ml for Western blot samples) is taken in a 2 ml microfuge tube and equilibrated at 16°C, after which the indicated treatment is added. Fertilized samples receive 1 µl dry sperm/ml. The calcium ionophore A23187 (Sigma-Alderich, St. Louis, MO) is prepared as a 1 mM stock in DMSO and added to egg suspensions at a final concentration of 5 µM. Solvent control samples receive an equivalent volume of DMSO. Egg samples are incubated at 16°C with periodic gentle mixing.

Western blot for phosphorylated and total MAP kinase: Samples are centrifuged to pellet the eggs (30 sec. full speed in microfuge), the sea water is removed, and the egg pellet is dissolved in 30 µl of MAP kinase lysis buffer (1% NP-40, 20 mM HEPES, pH 7, 15 mM EGTA, and 150 mM NaCl, with a protease/phosphatase inhibitor cocktail of 0.2 mM Pefablock, 1 µg/ml pepstatin, 10 mM β-glycerophosphate, 0.4 mM NaF, and 0.2 mM Na_2VO_4), and kept on ice. Lysates are centrifuged for 10 min. at 4°C, the pellet is discarded, and an equivalent volume of 2X SDS-PAGE sample buffer is added before storing at -20°C until the next lab.

Egg lysates are separated by SDS-PAGE using 10% precast gels on Bio-Rad Mini-Protean 3 minigel electrophoresis units. Proteins are electroblotted to nitrocellulose membranes using Bio-Rad Trans-Blot semi-dry transfer units, 2 blots per unit at 75 mA for 50 minutes. The membranes are then blocked overnight in TBST (10 mM tris-HCl, pH 7.4, 150 mM NaCl, 0.1 Tween-20) with 5% BSA. The following day, either a rabbit polyclonal antibody against phospho-Thr202/Tyr204 MAP kinase, or a rabbit polyclonal antibody against total MAP kinase (both from Cell Signaling Technology, Beverly, MA) is added at a 1:1000 dilution of the manufacturer's concentration in TBST, and incubated overnight. The blots are washed and incubated with a goat, anti-rabbit IgG peroxidase conjugate antibody (1:1000 dilution in TBST) for 1 hour. MAP kinase bands are detected by enhanced chemiluminescence (ECL).

IP_1 ELISA to Detect Phospholipase C Signaling: IP_1 ELISA kits were purchased from Cisbio Bioassays (Bedford, MA). The assay is a competition ELISA with a monoclonal antibody that recognizes the IP_3 breakdown product IP_1. Lithium chloride at a concentration of 45 mM was added to the egg suspension during the initial equilibration at 16°C and during the treatment incubation to inhibit breakdown of IP_1 to free myo-inositol. Whole cell lysates of egg pellets were dissolved in 50 µl lysis buffer (supplied with kit), kept frozen until the ELISA experiment, and were diluted 1:2 for the assay.

Discussion and Assessment

We have taught this version of the project in our Biochemical Techniques course for one year now, and have had great success with it. The project has several elements that make it appealing to students and which promote the learning of scientific reasoning skills. During the first lab, the students enjoy observing fertilization of the sea urchin eggs and cleavage of the zygotes, and this helps to engage them in the project. The ability to tie visual events to the biochemistry that they cannot "see," *e.g.* elevation of the fertilization envelope from a Ca^{2+} influx initiated by A23187, is the key element in this. Also, we believe that framing the project with two central questions that must be answered, and asking these questions in a way that requires an understanding of necessary vs. sufficient, requires the students to exercise a higher level of reasoning that better fosters the development of these skills. Two groups of lab sections were assessed for their ability to use necessary vs. sufficient reasoning to analyze data, one before completion of the lab report and the other after. Both groups were taught these concepts in lecture before the assessment. Students performed significantly better on moderate-difficulty problem set after completing the lab report and going through the required reasoning, with a pre-report average of 66.2% and a post-report average of 79.2% (p = 0.048). However, there was no improvement in performance on a more difficult problem set; the pre-report average was 38.5% and the post-report average was 33.2% (p = 0.296). This sort of scientific reasoning is critical for students to understand how experiments are designed and how data is analyzed in any field of biology.

References:

1) Ciapa and Chiri, 2000. Egg activation: Upstream of the fertilization calcium signal. *Biology of the Cell* 92:215-233.
2) Heinecke *et. al.*, 1990. A specific requirement for protein kinase C in activation of the respiratory burst oxidase of fertilization. *J. Biol. Chem.* 265:7717-7720.
3) Collas *et. al.*, 1997. Protein kinase C-mediated interphase lamin B phosphorylation and solubilization. *J. Biol. Chem.* 272:21274-21280.
4) Colledge *et. al.*, 1994. Disruption of *c-mos* causes parthenogenetic development of unfertilized mouse eggs. *Nature* 370:65-68.
5) Carroll *et. al.*, 2000. The relationship between calcium, MAP kinase, and DNA synthesis in the sea urchin egg at fertilization. *Dev. Biol.* 217:179-191.
6) Kumano *et. al.*, 2001. Calcium-mediated inactivation of the MAP kinase pathway in sea urchin eggs at fertilization. *Dev. Biol.* 236:244-257.
7) Battaglia and Gaddum-Rosse, 1987. Influence of the calcium ionophore A23187 on rat egg behavior and cortical F-actin. *Gamete Res.* 18:141-152.
8) Steinhardt and Epel, 1974. Activation of sea-urchin eggs by a calcium ionophore. *Proc. Nat. Acad. Sci. USA* 71:1915-1919.