

A Question-Based Project That Uses A MAP Kinase Western Blot To Examine The Role Of Calcium In Sea Urchin Fertilization

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Extended Abstract

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. 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 (Colledge *et al*, 1994). 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 (Carroll *et al*, 2000). At fertilization, sperm binding triggers the activation of phospholipase C, leading to rapid production of the second messengers inositol triphosphate (IP₃) and diacylglycerol (DAG), and the opening of Ca²⁺ channels on the endoplasmic reticulum. The resulting Ca²⁺ influx is a central node in fertilization signal transduction and it stimulates many of the events of egg activation, including exocytosis of the cortical granules and elevation of the fertilization envelope. 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)?

To answer these questions, the students artificially produce a cytoplasmic Ca²⁺ influx in unfertilized eggs with the calcium ionophore, A23187. On day one of the project the students examine eggs that are fertilized, treated with 5 μM A23187, or left untreated in sea water alone (unfertilized). Using microscopes, the students can see elevation of the fertilization envelope in both fertilized eggs and eggs treated with A23187, and at 2.5 hours post-fertilization observe cleavage of fertilized eggs but not eggs treated with A23187. From this the students can conclude that the calcium influx is not sufficient for cell division because treatment with A23187 did not produce parthenogenic cleavage in the absence of fertilization. The students also prepare samples for Western blot analysis of MAP kinase to determine if it is active (phosphorylated) or inactive (dephosphorylated) under each experimental condition. On day two of the project the students perform SDS-PAGE on these samples, and then perform electroblotting to transfer the proteins to a nitrocellulose membrane. The immunodetection step is done on day three, with an antibody that specifically recognizes MAP kinase phosphorylated on threonine 202 and tyrosine 204 (requisite for activity) or an antibody recognizes both phosphorylated and dephosphorylated MAP kinase (total MAP kinase). The absence of a band for A23187-treated samples detected with the phospho-specific antibody shows that MAP kinase is indeed inactivated by the calcium ionophore, and therefore that the calcium influx 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 their results and 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.

Gametes from the California purple sea urchin, *Strongylocentrotus purpuratus*, were extracted by injection of 0.55 M KCl into the coelomic cavity, as described previously (Carroll *et al*, 2000). The calcium ionophore A23187

(Sigma-Aldrich, St. Louis, MO) was prepared as a 1 mM stock in DMSO. To prepare egg lysates for Western blot analysis, 2 ml of egg suspension in sea water (1% volume of settled eggs /volume seawater) was equilibrated at 16° C for 10 minutes and then was either fertilized (1 µl dry sperm/ml), treated with 5 µM A23187, or was left untreated (sea water alone). The sample was then incubated at 16°C with periodic gentle mixing for 35 minutes, after which the eggs were pelleted from the sea water and 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 Na₂VO₄). The antibodies to detect phosphorylated MAP kinase and total MAP kinase were both purchased from Cell Signaling Technology (Beverly, MA), and MAP kinase bands were detected by enhanced chemiluminescence (ECL).

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