The Effect of the Cytoplasmic Ca^{2+} Influx on Cellular Processes and Metabolic Events During Sea Urchin Fertilization.

Jessica Mikerina
Professor Aaron Coleman, Ph. D.
BIBC 103, Section A02: Lab TA: Huizi, Lab Partners: Ryan Chatterji and Cole Parker
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Abstract:

Sea urchin fertilization initiates the process of egg activation which involves many metabolic events including a cytoplasmic Ca^{2+} influx. This influx prompts the inactivation of the enzyme MAP Kinase (MAPK), which is believed to be the protein responsible for keeping the unfertilized egg in mitotic arrest before activation. Fertilization is also followed by DNA synthesis, the procession into G_1/S phase, and finally cell division. In our experiments, we carried out procedures to test whether the cytoplasmic Ca^{2+} influx was sufficient to prompt the inactivation of MAPK and the beginning of cell division. We injected unfertilized sea urchin eggs with the calcium ionophore A23187 to produce an artificial cytoplasmic Ca^{2+} influx and compared the resulting egg activation and cleavage data with normally fertilized eggs. We then ran a Western blot on the samples, using fluorescent immunodetection to identify which samples contain inactivated MAPK. Both samples were run with solvent controls. We concluded that the cytoplasmic Ca^{2+} influx was sufficient to inactivate MAPK on its own, but was not sufficient to initiate cell division in the absence of actual fertilization.

Introduction:

The fertilization of a sea urchin egg prompts several biochemical cascades of events that lead to the exhibition of certain behaviors in the newly fertilized egg. This egg activation is signified by signal transduction events that occur after a sperm binds to an egg, which involve the activation of the enzyme Phospholipase C (PLC) due to the secretion of small molecules by the sperm (BIBC 103 Lab Manual). This event is critical because it prompts the rapid, cytoplasmic Ca^{2+} influx that transmits second messenger signals throughout the cell, indicating the beginning of various biochemical processes. One of these processes is the exocytosis of vesicles and release of cortical granule contents into the vitelline envelope, which expands because of untethering and osmotic pressure to become the fertilization envelope (BIBC 103 Lab Manual). Another critical event that occurs during fertilization is the inactivation of the enzyme Mitogen-Activated Protein Kinase (MAPK) which releases the egg cell from its arrested state of being perpetually kept in the G_0 phase of the cell cycle (BIBC 103 Lab Manual).

This system is of great importance to the understanding of the interconnection between fertilization, inactivation of MAPK, and cell division. In nature, these events may appear to follow a linear flow, however, biochemically speaking the series of events that occurs is more akin to a maze or the branches of a tree than a linear cascade. Countless small molecules, second messengers, and electrochemical signals act and overlap to promote the events occurring during fertilization to promote egg activation, cell division, and the eventual healthy growth and development of the new sea urchin individual. The event that is most of interest to us in the context of our research is the cytoplasmic Ca^{2+} influx. In our experiments, we sought to determine the relationship between the cytoplasmic Ca^{2+} influx and the metabolic event of MAPK inactivation and the cellular process of cell division. Previous literature has explored the relationships between these biochemical processes in various organisms including sea urchins (Carroll, 2000; Kumano, 2001; Tachibana, 1997). Through our experiments, we sought to enhance the current understanding of MAPK inactivation and cell division and enriched the hypothesis positing the relationship between the cytoplasmic Ca^{2+} influx and MAPK inactivation and cell division.

In order to study the cytoplasmic Ca^{2+} influx and its relation to MAPK inactivation and cell division we completed two main experiments. First, we injected samples of unfertilized sea urchin egg cells with four experimental conditions, these are: seawater, sea urchin sperm cells, the calcium ionophore A23187, and DMSO. (BIBC 103 Lab Manual). This experiment allowed us to observe the activation and cell division occurring in the different samples relative to each other. We determined the percent of egg activation and cell division of the normally fertilized eggs and the eggs injected with an artificial cytoplasmic Ca^{2+} influx. Comparing this data allowed us to make conclusions about the sufficiency of the cytoplasmic Ca^{2+} influx in metabolic processes. Second, we prepared Western blot samples of our four experimental conditions and ran an SDS-PAGE on them in order to create a Western blot visualized by fluorescent immunodetection (BIBC 103 Lab Manual). The Western blot allowed us to observe the relative

concentration of activated and inactivated MAPK in each of our samples further enhancing our understanding of the relationship of the cytoplasmic Ca^{2+} influx in metabolic processes.

The background and experimental techniques given above allow us to answer the two experimental questions that we are interested in: Is the cytoplasmic Ca^{2+} influx sufficient to inactivate MAP kinase even in the absence of actual fertilization? Is the cytoplasmic Ca^{2+} influx sufficient to induce cell division in the absence of actual fertilization?

Materials and Methods:

Experiment 1: Examination of Sea Urchin Egg Activation and Cell Division

The gametes used were collected from the California purple sea urchin (*Strongylocentrotus purpuratus*). Four samples were prepared with one of the four experimental conditions each: seawater, sea urchin sperm cells (in seawater solvent), 1 mM A23187 (stock in DMSO solvent), and 1mM DMSO (stock). The samples were equilibrated at the set temperature 16 degrees C for 10 minutes and then incubated at the set temperature 16 degrees C for 2.5 hours, inverted every 20 minutes. These eggs were observed for cleavage and cell division under light microscopes. (BIBC 103 Lab Manual).

Experiment 2: Western Blot

For the Western blot sample preparation: The samples were prepared with twice the volume of each experimental condition and were only incubated for 35 minutes, everything else was the same as above. These eggs were observed for egg activation under light microscopes. After the incubation the samples were centrifuged to isolate the eggs from the seawater and MAPK lysis buffer with protease/ phosphatase inhibitor was added.* The samples were centrifuged again and the supernatant preserved (BIBC 103 Lab Manual). *MAPK lysis buffer: 1% NP-40, 20 mM HEPES (pH = 7), 15 mM EGTA, 150 mM NaCl. Inhibitor: 2 mM Pefabloc, 10 ug/mL pepstatin, 100 mM beta-glycerophosphate, 4 mM NaF, 2 mM Na_3VO_4 (BIBC 103 Lab Manual). For the SDS-PAGE: A 10% polyacrylamide gel was used and 15 ug total protein was loaded into each well (BIBC 103 Lab Manual). For the Immunodetection: The primary antibody was a polyclonal IgG raised in a rabbit against a human MAPK fragment. The secondary antibody was a polyclonal IgG raised in a goat against the rabbit IgG, the goat IgG had a fluorescent quantum dot nanocrystal attached to its constant region that was detected using a UV transilluminator (BIBC 103 Lab Manual).

Results:

Group	Treatment	% Egg Activation (Experiment 2)	% Cleavage (Experiment 1)
1	1 Seawater	0%	0%
	2 Fertilized	98.6%	94.4%
2	3 A23187	N/A	N/A
	4 DMSO	N/A	N/A
3	5 Seawater	0%	0%
	6 Fertilized	50%	70.7%
4	7 A23187	91%	65%
	8 DMSO	0%	0%
5	9 Seawater	0%	0%
	10 Fertilized	94%	100%
6	11 A23187	83.3%	4.8%
	12 DMSO	0%	0%
7	13 Seawater	0%	0%
	14 Fertilized	95%	94%
8	15 A23187	88%	15%
	16 DMSO	0%	0%
9	17 Seawater	0%	0%
	18 Fertilized	100%	98%
10	19 A23187	92.8%	8.9%
	20 DMSO	37%	8.8%

Table. 1. Percent egg activation and cleavage in the different experimental conditions. The data presented describes the experimental conditions used and the corresponding egg activation and cell cleavage that was observed after 35 minutes, and 2.5 hours, respectively. The data indicated that the cytoplasmic Ca^{2+} influx is sufficient to produce egg activation and insufficient to produce cell cleavage.

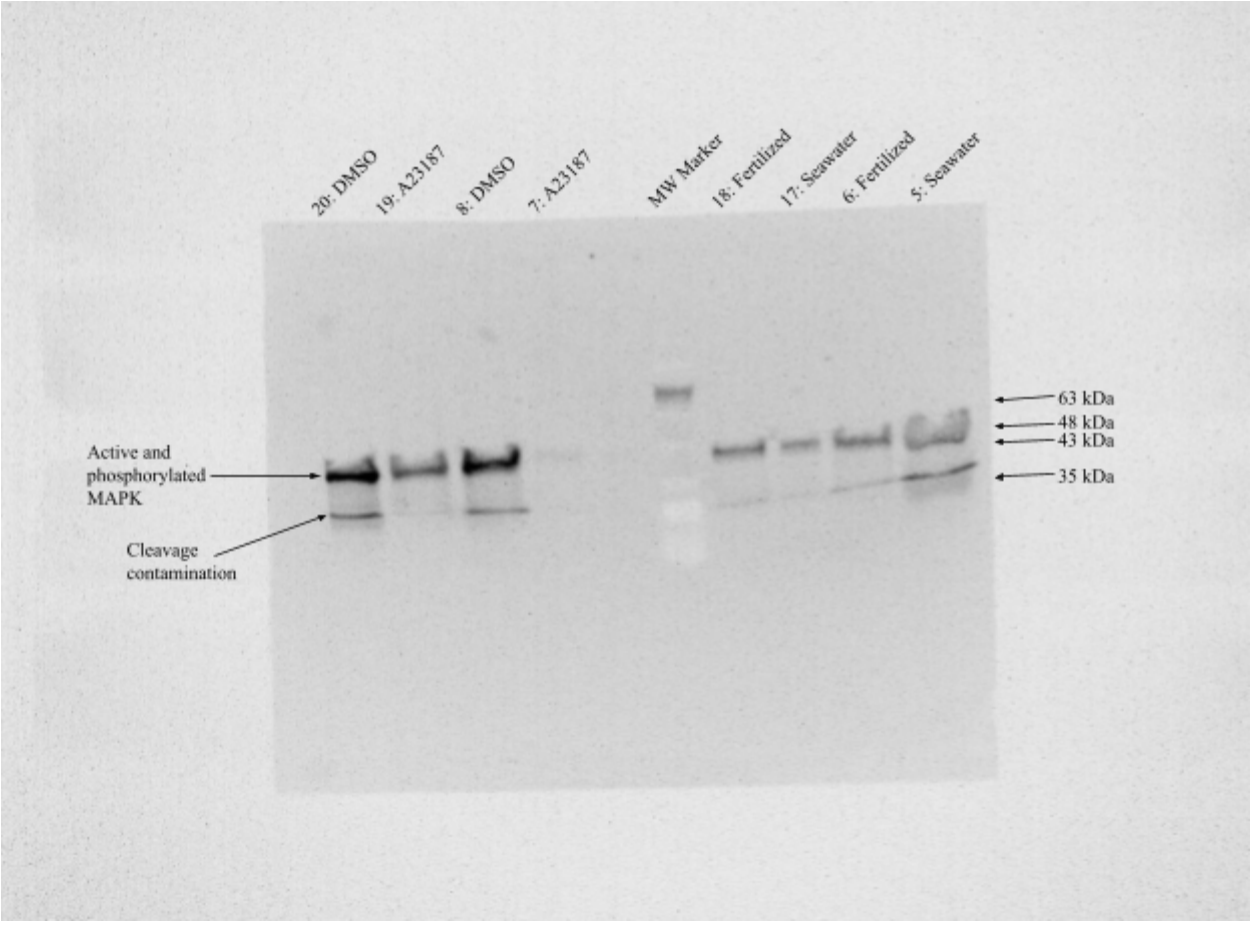

Figure. 1. UV-illuminated Western Blot of MAPK. The data presented shows the relative inactivation of MAPK in each of the experimental conditions. We expected that the fertilized samples would have inactivated and dephosphorylated and thus invisible MAPK bands and that the seawater samples would have still active and phosphorylated MAPK and thus visible MAPK bands. We also expected that the A23187 samples would have inactivated and dephosphorylated and thus invisible MAPK bands and that the DMSO samples would have still active and phosphorylated MAPK and thus visible MAPK bands. Unfortunately, due to experimental error, human error, pipetting error, contamination, or all of the above, the bands appear not as expected. There is still valuable information in the above image. For example, the bands for samples 7 and 8 appear correctly, with DMSO having a strong, dark band, and A23187 having a faint, invisible band. Additionally, samples 20 and 19 have some merit, with DMSO having a strong, dark band, and A23187 having a lighter band. The fertilized/seawater samples are a bit harder to distinguish. These results do not match popular consensus reached by the rest of the lab, who were able to visualize bands that support our experimental hypothesis and answer the experimental question as expected.

The first experiment we completed was the observation of the sea urchin eggs for activation and cleavage. This experiment allowed us to gain an understanding of how an artificial cytoplasmic Ca^{2+} influx affected egg activation and cell division as opposed to the natural cytoplasmic Ca^{2+} influx induced by actual fertilization. The experiment involved preparing and then observing four samples, each with a different experimental condition as follows: seawater, sea urchin sperm cells, the calcium ionophore A23187, and DMSO. (BIBC 103 Lab Manual). It was important to do this experiment because it provided us with information that aided us in answering our experimental questions regarding the sufficiency of the cytoplasmic Ca^{2+} influx in inactivating MAPK and stimulating cell division. The results of this experiment showed that the normally fertilized eggs were activated and proceeded to cell division, with the average fertilized egg activation being 87.52% and the average fertilized egg cleavage being 91.42%. These numbers indicate that the fertilization of the eggs proceeded normally and incited the proper response. The results also showed that the eggs treated with A23187 were activated, but did not proceed to cell division, with the average A23187-treated egg activation being 88.77% and the average A23187-treated egg cleavage being 9.57% (Note: this excludes the outlier in the data: one group obtained an A23187-treated egg cleavage of 65%, which is an outlier and will not be assessed as a valid data point seeing as it does not correspond to the general consensus).

The second experiment we completed was the Western blot, which also included the original SDS-PAGE in addition to electroblotting, and the following procedure to use immunodetection to make the Western blot results visible and interpretable. This experiment allowed us to visualize the inactivation of MAPK in the various samples we prepared, and analyze how the difference in results reflects back onto our experimental questions regarding the sufficiency of cytoplasmic Ca^{2+} influx in inactivating MAPK. The experiment involved preparing four samples, each with a different experimental condition as follows: seawater, sea urchin sperm cells, the calcium ionophore A23187, and DMSO and then putting them through an SDS-PAGE gel, an electroblot, and then make it fluorescently visible through immunodetection. (BIBC 103 Lab Manual). It was important to do this experiment because it allowed us to physically see how much MAPK was inactivated in each of the samples under the different experimental conditions. The results of this experiment showed that

Discussion:

We observed that eggs treated with A23187 underwent activation 88.77% of the time, which was more so than eggs that were treated with sea urchin sperm which underwent activation 87.52%. This indicates that the cytoplasmic Ca^{2+} influx is sufficient to induce the cellular response of egg activation. Egg activation however, also integrally involves MAPK inactivation, this must occur for the egg to be properly activated. The Western blots that were carried out in the lab provide evidence that supports the hypothesis that the cytoplasmic Ca^{2+} influx is sufficient for MAPK inactivation. When MAPK is inactivated it is dephosphorylated and the immunodetection technique that was used only detected phosphorylated MAPK, thus we would expect that the bands for the fertilized and A23187 samples would be invisible or very faint. This was true for the majority of the class. Unfortunately, not all of the bands that our group visualized were as easily decipherable as those of the rest of the class. And yet, the A23187 for one duplicate (#7) reacted correctly, indicating that MAPK was inactivated and only a very faint band was observed. Additionally, the DMSO indicated a strong, dark band, supporting the hypothesis that it was the artificial cytoplasmic Ca^{2+} influx caused by the A23187 that caused MAPK inactivation.

We observed that eggs treated with A23187 did not undergo cleavage a significant amount of the time. The A23187-treated eggs could be observed undergoing cell division 9.57% of the time as compared to the sperm-treated eggs which underwent cleavage 91.42% of the time. This indicates that the cytoplasmic Ca^{2+} influx is not sufficient to induce cleavage and promote further cell division in the absence of actual fertilization.

We conclude that the artificial cytoplasmic Ca^{2+} influx is sufficient for the inactivation of MAPK, however it is insufficient for the induction of cell division. This conclusion is consistent with the previously published literature. Previous experiments support the hypothesis that MAPK is inactivated by the cytoplasmic Ca^{2+} influx, whether it is naturally produced from fertilization, or artificially induced by adding A23187. Furthermore, research shows that the cytoplasmic Ca^{2+} influx is both necessary and sufficient for MAPK inactivation (Carroll, 2000). Although this is outside the scope of our study, it supports our hypothesis regarding the importance of the cytoplasmic Ca^{2+} influx for cellular processes and metabolic events during fertilization.

We have concluded that the cytoplasmic Ca^{2+} influx is sufficient to induce MAPK inactivation, however, research shows that the cytoplasmic Ca^{2+} influx is also necessary for MAPK inactivation and that other biochemical factors are not sufficient to incite MAPK inactivation in the absence of the cytoplasmic Ca^{2+} influx (Kumano, 2001). This can be further tested with a procedure involving the addition of a cytoplasmic Ca^{2+} influx inhibitor, that would artificially decrease the cytoplasmic Ca^{2+} concentration even in the event of fertilization, thus testing whether the cytoplasmic Ca^{2+} influx is the prime determining factor of MAPK inactivation. An experiment that can be performed to test this is the treatment of both fertilized and unfertilized sea urchin eggs with a chelator, such as BAFTA, which would block the cytoplasmic Ca^{2+} influx, then a MAPK activity assay can be performed to test the presence of

activated and phosphorylated protein as opposed to inactivated and dephosphorylated protein in the samples (Carroll, 2000). If the cytoplasmic Ca^{2+} influx is necessary for MAPK inactivation, then we would see high activity of MAPK after BAFTA treatment because it would remain activated in the absence of the cytoplasmic Ca^{2+} influx. However, if the cytoplasmic Ca^{2+} influx is not necessary for MAPK inactivation, then we would see low to no activity of MAPK after BAFTA treatment because it would be inactivated by other metabolic processes in the absence of the cytoplasmic Ca^{2+} influx.

Based on the data gathered from our experiments in the duration of our study, as well as the knowledge gained from the published literature, we can conclude that the inactivation of MAP kinase at fertilization is necessary and sufficient to get the egg to enter S-phase of the cell cycle. Both natural inactivation of MAPK by fertilization and artificial inactivation of MAPK in starfish egg samples yielded eggs that entered S-phase and underwent DNA synthesis; however, constitutive activation of MAPK, even in fertilized eggs, produced eggs that remained in an arrested state and did not enter S-phase (Tachibana, 1997). Inactivation of MAP kinase at fertilization is necessary but not sufficient to get the zygote to undergo cleavage. As stated previously, inactivation of MAPK in an egg cell is necessary for it to enter S-phase and begin DNA synthesis (Tachibana, 1997); however, from our own experiment, we can say that inactivation of MAPK alone is not sufficient to initiate cleavage and further cell division.

Citations and Bibliography:

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