Calcium and sperm relationship to sea urchin egg cleavage through competing MAPK and PLC pathway

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Abstract: Sea urchin fertilization produces many different changes in the cell that allow a cell to divide and grow. To view this growth, we used a microscope, Western Blot, and Elisa to see two different ways in which a cell changes to see which way causes the egg to begin dividing and growing. The viewing of the treatments under the microscope showed that fertilization and A23187 caused egg activation while only fertilization caused cell cleavage. The result of the Western Blot was inconclusive due to a lack of following a loading control of alpha tubulin, yet showed a potential observation that MAPK is dephosphorylated in fertilized egg cells. The Elisa showed that IP_1 concentrations of fertilized eggs and calcium introduced eggs are quite the same showing an activation of the PLC pathway in both treatments. The results held conclusive to state that calcium can not cause cell division. But due to no loading control it is inconclusive to state that cytoplasmic calcium influx was enough to inactivate MAPK, also if MAPK is inactivated by dephosphorylation or degradation.

Introduction:

When a male sperm and female egg from sea urchins fuse together, fertilization occurs. Fertilization facilitates the activation of a mature egg by causing a brief intracellular wave of calcium (Ca^{2+}) [1]. The Phospholipase C (PLC) activation is essential for the initial influx of Ca^{2+} as seconds after insemination, the Inositol 1,4,5-triphosphate (IP_3) concentration rise from 0.2-0.3μM in 20-30 seconds to approximately 1μM at 2 minutes[1]. IP_3 is responsible to bind to the IP_3 receptor on the endoplasmic reticulum (ER) allowing Ca^{2+} to be released into the cytoplasm[2]. Thus the PLC signal transduction pathway produces IP_3 which binds to the ER to increase the concentration of Ca^{2+} in the cytoplasm. Another competing pathway during initial fertilization is the enzyme, mitogen activated protein kinase (MAPK). Phosphorylated MAPK is found in unfertilized sea urchin eggs which arrests the eggs from entering the cell cycle[3]. MAPK is regulated upstream by a dual specific kinase ERK kinase (MEK) that phosphorylate threonine and tyrosine to active MAPK and regulate transcription factors[4]. MEK inhibitors cause MAPK to remain dephosphorylated thus allowing sea urchin eggs to undergo cell division[5]. In the experiment, we are looking to understand sea urchin fertilization through analysis of MAPK and Ca^{2+} as the two competing pathways that play a role in cell division. Isolating each pathway, we aim to test if fertilization is the only method to cause cell division. We aim to answer three questions: Is a cytoplasmic calcium influx sufficient to produce cell division in an unfertilized egg?, Is cytoplasmic calcium influx sufficient to inactive MAPK in sea urchin eggs?, and at fertilization, is MAPK inactivated by dephosphorylation of by degradation? To test these questions, we ran 2 Western Blots: one for total MAPK activity, and one for total phosphorylated MAPK. Each western blot had untreated sea urchin eggs from seawater as the negative control, fertilized sea urchin eggs as the positive control, calcium ionophore (A23187) introduced into sea urchin eggs as the experimental trial, and dimethylsulfoxide (DMSO) as a solvent control for each experimental condition besides the seawater treated sea urchin eggs. We also ran a competitive Elisa to measure the amount of inositol monophosphate (IP_1) present in the sea urchin egg. The presence of IP_1 comes from the degradation of IP_3 following the release of Ca^{2+} from the ER[6]. For the competition Elisa we used each four treatments separately to test for IP_1concentration. Nonspecific binding (NSB) was used as the negative control, and total activity (TA) as the positive control. From these techniques, we hypothesize that calcium influx will not be sufficient to produce cell division in an unfertilized sea urchin egg. We hypothesize that the calcium influx is not sufficient to inactivate the MAPK in sea urchin eggs. Lastly, we hypothesize that MAPK is inactivated by dephosphorylation rather than degradation.

Materials + methods:

Induced cell division:

- Sperm
- A23187
- DMSO

Unfertilized sea urchin eggs were harvested in the winter from Sripps Institute of Oceanography. Use a P-1000 micropipette with a cut wide tip to place 1ml of seawater suspended unfertilized eggs into four 2ml microfuge tube. Incubate the tube in 16˚C water bath for 10 minutes to equilibrate. Add treatments to each microfuge tube: add nothing (seawater), add 1μl sperm (fertilized), add 5μl of 1mM A23187 stock in DMSO (Ca^{2+}), and add 5μl DMSO (DMSO). Incubate treatments for 2.5 hours at 16˚C with inverting samples every 20 minutes. Place a large drop of each treatment separately under a light

microscope and view if cell division took place in the egg and record percent cleavage.

Western Blot preparation:

- MAPK lysis buffer
    1. 1% NP-40
    2. 20mM HEPES, ph 7
    3. 15mM EGTA
    4. 150mM NaCl
- protease/phosphatase inhibitor cocktail
    1. 2mM Pefabloc
    2. 10µg/ml pepstatin
    3. 100mM B-glycerophosphate
    4. 4mM NaF
    5. 2mM Na_3VO_4

Use a P-1000 micropipette with a cut wide tip to place 2ml of seawater suspended unfertilized eggs into four 2ml microfuge tube. Incubate the tube in 16˚C water bath for 10 minutes to equilibrate. Add treatments to each microfuge tube: add nothing (seawater), add 2µl sperm (fertilized), add 10µl of 1mM A23187 stock in DMSO (Ca^{2+}), and add 10µl DMSO (DMSO). Incubate at 16˚C for 35 minutes with inverting samples every 5 minutes. Place a large drop of each treatment separately under a light microscope and view the percent activation of the egg and record percent activation. Pellet the eggs in a micro-centrifuge at full speed for 30 seconds in a 1.5ml microfuge tube. Make a solution of 90µl MAPK lysis buffer and 10µl of protease/phosphatase inhibitor cocktail and vortex. Add 50µl of the MAPK/inhibitor cocktail to the pellet and homogenize using pipette tip. Centrifuge sample in a cold room microfuge at full speed for 10 minutes. Save the suspensions separately as the Western Blot samples in the freezer.

Elisa preparation:

- 0.5M LiCl
- Elisa lysis buffer (premade from company)

Use a P-1000 micropipette with a cut wide tip to place 1ml of seawater suspended unfertilized eggs into four 2ml microfuge tube. Add 100µl of 0.5M LiCl into Elisa egg samples. Incubate the eggs at 16˚C for 10 minutes. Add treatments to each microfuge tube: add nothing (seawater), add 1µl sperm (fertilized), add 5µl of 1mM A23187 stock in DMSO (Ca^{2+}), and add 5µl DMSO (DMSO). Incubate at 16˚C for 35 minutes with inverting samples. Transfer samples into 1.5ml microfuge tubes and centrifuge at full speed for 30 seconds and remove supernatant. Add 50µl of lysis buffer to each egg pellet and homogenize with pipette tip. Store lysates as Elisa samples in the freezer.

Western Blot gel electrophoresis:

- Tank buffer
    1. Tris (pH 8.3), glycine, SDS
- Stacking gel
    1. Tris (pH 6.8), 3% acrylamide, SDS, low [Cl^-]
- Running gel
    1. Tris (pH 8.8) 10% acrylamide, SDS, low [Cl^-]
- Buffer
    1. 600ml 1X from 10X stock buffer
- Bradford reagent (premade from company)

Set up the gel by adding buffer to the chamber and then the tank. Thaw samples and leave on ice. Run a Bradford assay where for each treatment add 5µl of lysate with 95µl of dH_2O with 900l Bradford reagent and a blank of 100µl dH_2O and 900µl Bradford reagent. Measure absorbance at 595nm and calculate 10µg of protein from each sample. Make and mix 20µl samples for 2 SDS-PAGE's with 10µg of protein and adding H_2O to a volume of 16µl then add 4µl of the 5x SDS-PAGE sample buffer. Heat the samples at 95˚C, load the wells according to which SDS page will be treated for total MAPK vs total phosphorylated MAPK and let the gel run until the tracking dye is 1cm from the bottom of the gel.

Western blot with semidry blotting apparatus:

- Nitrocellulose sheet
- 2 transfer stacks
- electroblotting buffer (premade from company)
- Tris-Buffered Saline with Tween-20 (TBST)
- TBST/BSA+rabbit anti-MAPK (Total or Phosphorylated) antibody diluted 1:1,000 (1˚ Antibody)

Take gel out of electrophoresis unit and cut the stacking gel off. Place gel in electroblotting buffer and nitrocellulose transfer stacks separately. Place a transfer stack followed by the nitrocellulose, gel, and other transfer stack sandwiched together into the cassette for the electroblotting unit with 1.3 AMPS running through. Mark the molecular weight bands on the nitrocellulose, block with 20ml TBST. Incubate at 4˚C with gentle agitation and add 15ml of 1˚ antibody.

Immunodetection of MAPK

- Phosphate-buffered saline (PBS)
- Anti-rabbit IgG fluorescent-tagged antibody (2˚ antibody)
- E-gel Imager from Invitrogen-Life Technologies (imager)
- Image J

Discard of the 1˚ antibody and add 20ml PBS with slight agitation as a PBS wash. Do 3 PBS washes with agitation for 5 minutes. Immediately after add 10ml of 2˚ antibody and incubate for 1hour with the rotator speed at slow speed. Do 4 PBS washes each taking 10 minutes. Add diH_2O to rinse the filter and use the imager set to the UV transilluminator and receive your digital image where you can then use image J to normalize the results from lane to lane and quantify band intensities from one another.

Competition Elisa:

- IP_1- peroxidase conjugate (premade from company)
- Anti-IP_1 monoclonal antibody (premade from company)
- Dilution buffer
- Orbital shaker
- Nonspecific binding (NSB)
- Wash buffer
- Total Activity (TA)
- 3,3',5,5'Tetramethylbenzidine (TMB)
- Sulfuric acid (stop solution)

Thaw Elisa samples and add 60µl of dilution buffer to each treatment and mix. Pipette 50µl of lysate into competitive Elisa wells, add 25µl of IP_1-peroxidase conjugate followed by 25µl of anti-IP_1 monoclonal

Results:

antibody. For the NSB as the negative control add 25µl of the IP_1-peroxidase conjugate with 75µl of dilution buffer. Secure wells with clay and cover with a wet paper towel before incubating on the orbital shaker for two hours at room temperature. Remove the solution from each well by aspirating using a pasture pipette tip. Add 250µl of wash buffer to each well. Agitate and aspirate for a total of 6 times. Add 100µl of TMB to each treatment including a well for TA and blank. Add 5µl of IP_1- peroxidase conjugate to TA and incubate all for 20 minutes on the orbital shaker with a wet paper towel on top. Add 100µl of stop solution and then transfer 175µl of each treatment into a 96 well plate to measure the absorbance at 450nm. Analyze the data in regards to a standard curve of known IP_1 concentrations.

	Percent cell activation				Percent cell cleavage			
	Seawater	Fertilized	DMSO	A23187	Seawater	Fertilized	DMSO	A23187
Group 1	0	40	-	-	0	20	-	-
Group 2	-	-	0	20	-	-	0	0
Group 3	0	64.9	-	-	0	49	-	-
Group 4	-	-	0	20	-	-	0	0
Group 5	0	22	-	-	0	33	-	-
Group 6	-	-	0	11	-	-	0	5
Group 7	0	86	-	-	0	17	-	-
Group 8	-	-	0	16	-	-	0	0

Fig 1. Sea urchin egg activation and cleavage using treatments of just seawater, a sperm introduced into an egg, DMSO treated, and A23187 added into the egg. The results show the recorded values of measurements taken by a light microscope and viewed by the eye.

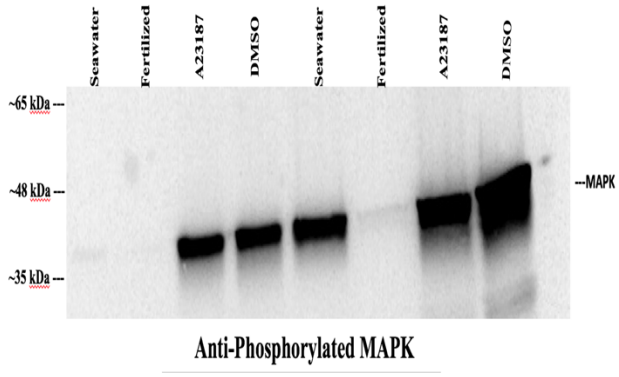


Fig 2. Western blot of anti-phosphorylated MAPK from Gel A with the 4 treatments from 2 groups.

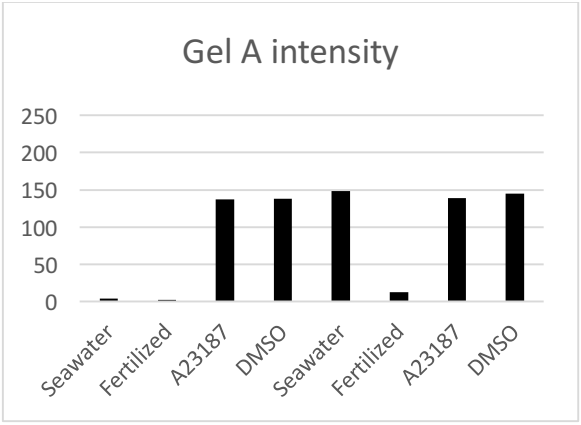
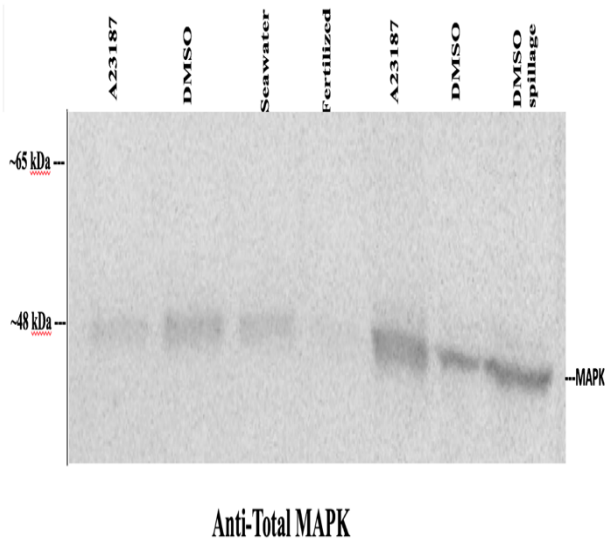
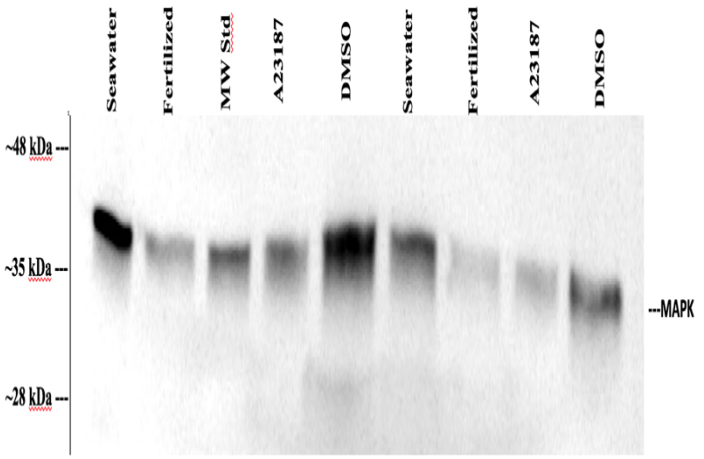


Fig 3. Intensity of the bands produced from the western blot in fig 2 on the color scale from 0-255.



Anti-Total MAPK

Fig 4. Western blot of anti-total MAPK from Gel B with the 4 treatments from 2 groups.



Anti-Phosphorylated MAPK

Fig 6. Western blot of anti-phosphorylated MAPK from Gel C with the 4 treatments from 2 groups.

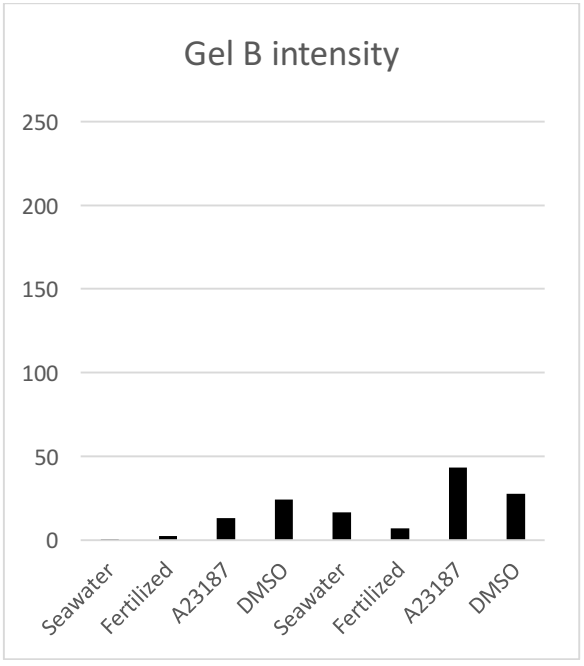


Fig 5. Intensity of the bands produced from the western blot in fig 4 on the color scale from 0-255.

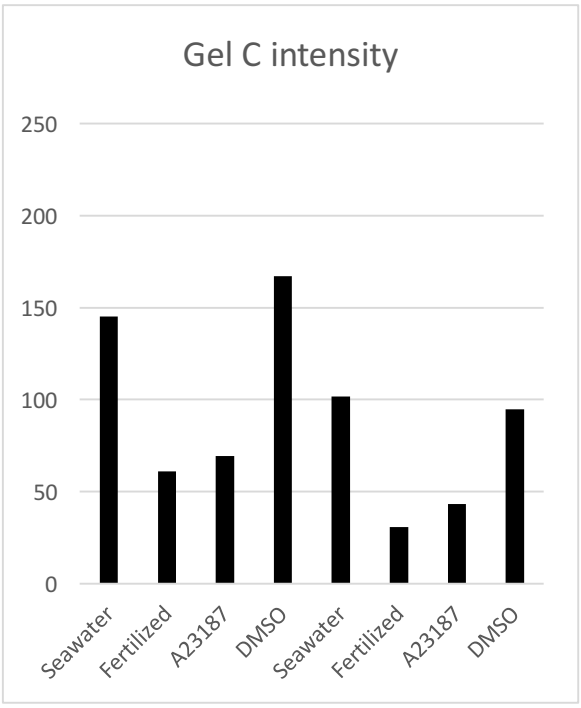


Fig 7. Intensity of the bands produced from the western blot in fig 6 on the color scale from 0-255.

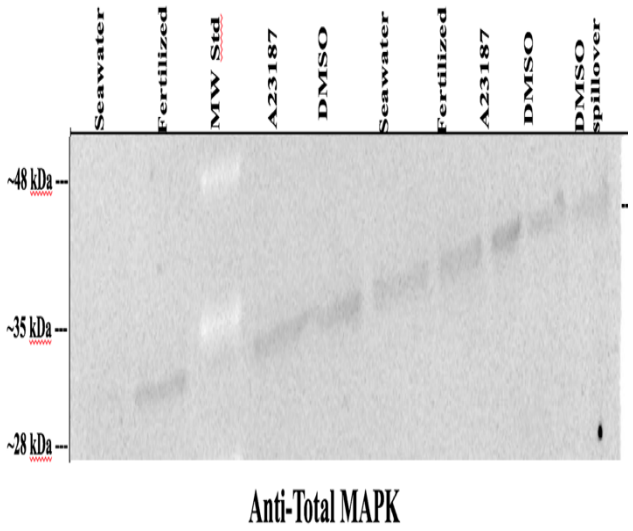


Fig 8. Western blot of anti-total MAPK from Gel B with the 4 treatments from 2 groups.

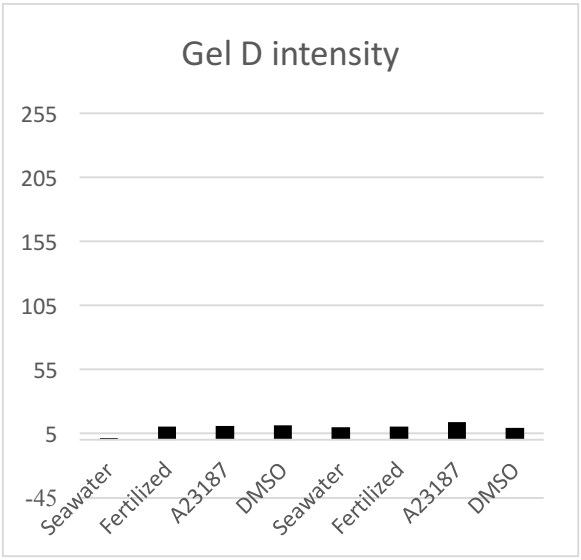


Fig 9. Intensity of the bands produced from the western blot in fig 8 on the color scale from 0-255.

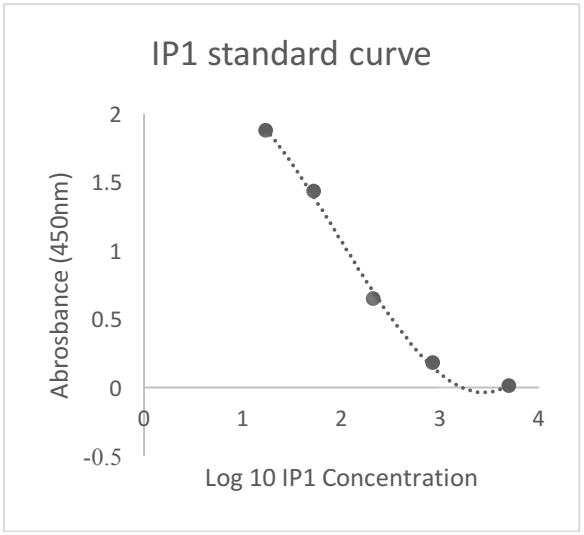


Fig 10. IP_1 standard curve $y=0.1789x^3-1.0548x^2+0.9112x+2.0316$, $R^2=0.99784$.

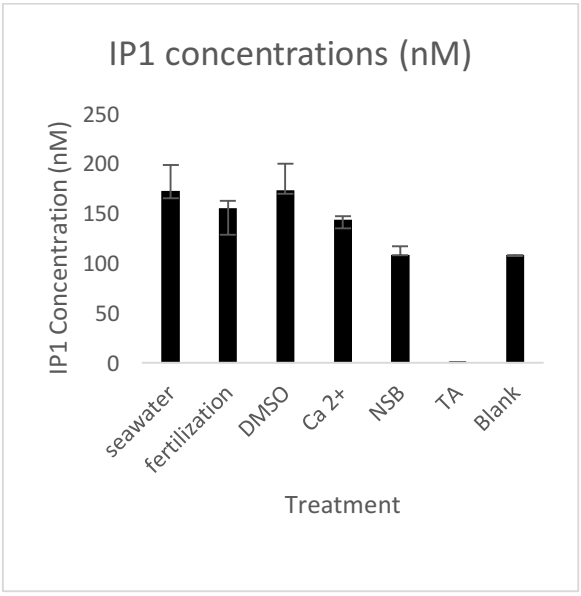


Fig 11. IP_1 concentrations of the 4 treatments with NSB as the negative control and TA as the positive control.

Discussion: The results of the activation vs cleavage of the sea urchin eggs (Fig 1) gave compelling evidence towards our first hypothesis. The negative control of the sea water showed that each egg was indeed unfertilized and therefore inactive. The positive control of fertilized eggs was viewed as there was clear activation across all group trials. The DMSO as a solvent control showed no activation as expected to not partake in any activation pathway. The A23187 too showed activation in smaller amounts in all groups. The cleavage data for seawater and DMSO was 0 across all trials as expected due to the inactivity of the egg. Fertilization followed the expected trend that a portion of the eggs would divide and show cleavage after 2.5 hrs., in all cases but one where there was an increase in cleavage compared to activation. The A23187 also followed the expected trend of having no cleavage except in one case where there was a smaller number of cleaved eggs compared to activated. The anomaly of the fertilized case was due to inaccurate measuring of activated eggs. The slide if viewed under a further scope would have shown other areas of activation not accounted for in the activation data while a more through job was performed during recording of the cleaved eggs. The A23187's error could possibly be due to contamination of the sample with sea urchin sperm which would account for the found cleaved eggs. Sperms function is to supply the DNA for the synthesis is centrosomes,[1] without centrosomes mitosis can not occur to produce cleavage. Therefore, there was a human error that must have occurred to see such cleavage. (Fig 1) answers the first question such that, a cytoplasmic calcium influx is not sufficient to produce cell division in an unfertilized egg. The results from the western blot show staggering differences among the data. (Fig 2 and Fig 3) The data from the fertilized columns are consistent and show that there is little to no phosphorylated MAPK which along with the cell division seen from Fig 1, is what we expect to see. The fertilization results from (Fig 6 and Fig 7), should be disregarded as there was molecular ladder spillage from lane 3 potentially into lanes 1-5. The fertilization in both total MAPK was very low (Fig4,5,78,9) degradation of MAPK would have been the case had we had a loading control for alpha tubulin that was followed through the experiment. Since there was no loading control to ensure equal loading of amount of protein none of the results can be conclusive. Variations thus seen by the blots and intensity graphs are very likely due to the vast differences in percent activation among all samples as these eggs were harvested in the winter and the experiment has been run in the summer. Sea urchin eggs are known to be harmed by freezing[7]. (Fig 2,3) The A23187 being so high shows that the influx of Calcium into the egg is not enough to inactivate MAPK as MAPK would then not be in its phosphorylated form. The results from the competition Elisa have yielded unexpected results. All the samples gave a greater intensity reading than the positive control of the NSB. (Fig 11) The fertilized egg sample had less IP_1 than the seawater and DMSO and almost equal levels to the A23187. The NSB intensity may have been due to the incomplete binding of the IP_1-peroxidase conjugate to the antibody thus producing a loss of signal. Assuming that NSB has a higher intensity than what is seen, the fertilized egg sample did have some IP_1 as the intensity is lower than that of the seawater and DSMO. The interesting result is that the A23187 has a lower IP_1 level almost equal to that of the fertilized egg sample. This shows that the PLC pathway must also be active in the case of the A23187 including in the fertilized egg sample which was expected. Our scope of the question is to take into account the insertion of A23187 to mediate the plc pathway, but intracellularly the egg has calcium levels that are being maintained, the introduction of additional calcium can turn on isozymes of PLC[8] to therefore start the plc pathway and make IP_1. Therefore, the results hold that PLC is activated by fertilization and A23187 while seawater and DMSO are held constant.

Conclusion:
The experiments performed only gave conclusive results to show that Calcium influx is not capable of undergoing cell cleavage. And due to the lack of tracing of a loading control in the Western Blot, we have inconclusive evidence to support the claim that the calcium influx was able to inactivate MAPK, and if MAPK was inactivated or degraded at fertilization. Although there were inconclusive results many trends showed up that with proper adherence to a loading control can lead to probing new questions about MAPK and the PLC pathway in embryonic development. Further western blots would aid in these trends. Future experiments into pre fertilization or calcium influx of intracellular calcium would allow for a better understanding of calcium's role in the egg and development and growth of the zygote due to seeing results showing calcium activating the PLC pathway[8].

Citations:

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