Bio219 Home

Comparison of Rates of Endocytosis in Sea Urchin Eggs and Blastulae

Jenna L. Gustafson, '04
jgustafs@wheatonma.edu
508.286.5365

image courtesy of:
http://www.seaotter.com/marine/research/stronglyocentrotus.jpg.html

I. Introduction

Endocytosis is the inward bulk transport of extra cellular fluids and particles that involves forming a small infolding of the cell membrane and the pinching off to the membrane to form a membrane bounded vesicle inside the cell. The vesicles formed, known as endosomes, move inward from the cell surface, meeting and fusing with other vesicles, such as lysosomes, and increasing in size (Loewly, 1991). The process of endocytosis is of interest to me because I find it fascinating that cells can take in portions of the extra cellular fluid and particles in the extra cellular matrix by simply pinching off part of their membrane to form a vesicle for transport. Also, I was curious about whether there is a difference in rate of endocytosis in a static cell as compared to a dividing cell.

Sea urchins are fascinating creatures to study and have a particularly interesting and easy to study developmental process. Following fertilization of sea urchin eggs, within two minutes the appearance of the fertilization envelope is present and after about 50 minutes, the first cell goes through its first round of mitosis, (Sidwell Research, 2003). Approximately 7-8 hours after fertilization, the blastula stage is reached, which can be studied for such processes as the formation of tight junctions, endocytosis, and exocytosis. I plan to study the process of endocytosis in the sea urchin blastula. I chose to study this cell behavior in the sea urchin blastomeres because the sea urchins are easily available, interesting to watch develop and produce beautiful images. I hypothesize that the rate of endocytosis in sea urchin eggs is greater than the rate in urchin blastulae. I believe this to be true because as the blastulae are undergoing mitosis, the cytoplasm and cell membrane are constantly changing and increasing the overall surface area of the dividing cell. The cell must utilize the process of exocytosis to increase the surface area of the cell which will balance out the process of endocytosis, producing an apparent decrease in the rate of endocytosis, (Morris, 2003). In the unfertilized urchin egg,

the cell membrane remains unvarying as the cell is not undergoing mitosis.

The water-soluble fluorescent sugar molecule, rhodamine dextran, the sea water it is in, (Terasaki, 1995). Fluorescent dextran is used to study endocytosis because it is not produced by the urchin cell that it will label. The dextran will fluoresce under green fluorescent light and reveal where it is present in the cell. Dextran can enter the cell only by endocytosis, (Karp, 1999) and therefore serves as an excellent indicator of this process.

The experiment I performed was to culture sea urchin embryos and allow them to develop for approximately one day and to culture unfertilized urchin eggs. After a day of development, the blastulae and eggs were labeled with the fluorescent marker rhodamine dextran, washed numerous times, then imaged using fluorescence light microscopy. Image analysis using Adobe Photoshop allowed me to quantitate the data and compare the rate of endocytosis in the urchin egg versus the blastula.

II. Materials and Methods

Materials

Lytechinus pictus embroys
Lytechinus pictus eggs
Filtered natural sea water (FNSW)
FNSW-PABA
  FNSW supplemented with 10mM 0.5% p-aminobenzoic acid (Dufort, 2000)
Nitex mesh funnel : 80µm pore size
Rhodamine Dextran : 1.0 ml of 50 µM in methanol
Deciliating solution
  2.9g NaCl in 50ml FNSW (Stephens, 1986)
Spot insight camera
Spot computer imaging program
Nikon Eclipse E400 Fluorescence microscope
Adobe Photoshop 7

Methods

Shedding and Fertilization

Both a male and a female urchin were given an intra-coelomic injection of 0.5 M KCl (Dufort, 2000) which releases either sperm or eggs from the urchin. Eggs were collected in filtered sea water and sperm was collected dry on parafilm. Next, eggs were washed twice by removing their FNSW and approximately 10 volumes of fresh FNSW. Then, washed one last time with FNSW-PABA. Sperm was activated by a 1:1000 dilution of dry sperm in FNSW. Within five minutes, activated sperm is added to eggs in a ratio of one drop activate sperm per one milliliter of egg suspension. Successful fertilization was confirmed by viewing the formation of a fertilization envelope around each egg under a light microscope.

Fertilization Envelope Stripping

The envelope surrounding the fertilized eggs was removed by washing the eggs three times in FNSW-PABA to remove the sperm, then passing the eggs once through 80 µm Nitex mesh to strip away the envelope. The eggs were then washed in FNSW without PABA to wash away the fertilization envelopes. To confirm that the fertilization envelopes were removed, the eggs were view under a light microscope.

Incubation and Staining

The fertilized eggs were placed in a Petri dish and allowed to develop for approximately a day at room temperature, to permit for a number of rounds of cell division to occur. After 23 hours, the sea urchin blastulae were added to a prepared solution of rhodamine dextran in a concentration of 50 µM. Using a Pasture pipette, one drop of blastulae suspension was added to an epindorph tube containing 0.5 ml of the rhodamine dextran solution. After a pulse of 6 minutes, the blastulae that settled to the bottom of the tube were removed and washed with 40 ml of FNSW in a 50 ml capped tube. The tube was then centrifuged to settle the blastulae to the bottom. The FNSW was then removed and replaced with 40 ml of fresh FNSW. The sea urchin blastulae were washed and centrifuged a total of 5 times. Unfertilized sea urchin eggs were stained using the same process, but were pulsed for 10 minutes instead of 6 minutes.

Deciliating

Blastulae were deciliated to allow for imaging. Sea urchin blastulae were added to a highly concentrated prepared solution of 2.9g NaCl in 50 ml FNSW and centrifuged. The NaCl solution was removed and 30 ml of FNSW was used to wash the blastulae, (Stephens, 1986).

Preparation of Controls

Two controls were prepared, unstained eggs and unstained, deciliated blastulae. The unstained eggs and blastulae will not glow under green fluorescent light and will therefore show that fluorescence in stained samples is a result of the presence of rhodamine dextran in the cell. A 1.0 ml suspension of each was placed in separate epindorph tubes and set aside.

Imaging

Slide chambers were prepared using a technique suggested by Prof. Morris. Two thin pieces of double stick tape were placed lengthwise at the top and bottom of a slide in the area which would be covered by the upper and lower edge of the cover slip. A cover slip was then placed on top, creating a chamber that will pull under it the drop of suspension to be imaged. Four slides were prepared using this method: stained urchin blastulae, stained urchin eggs, unstained urchin blastulae and unstained urchin eggs. Slides were viewed on a mercury lamp Nikon Eclipse E400 light microscope using a Spot© camera and software on a Mac G4 computer in the ICUC at Wheaton College. All images were captured using a 40x objective lens and viewing under green fluorescent light on the microscope.

A total of 3 trials were run in which sea urchin eggs were fertilized, allowed to develop to a time point of approximately a day, then labeled with rhodamine dextran. Each experiment took about one hour for fertilization and an hour and a half for staining, deciliating and imaging. Data was quantitated by measuring the brightness of fluorescence in the images. Using Adobe Photoshop 7, images were analyzed for mean luminosity, a feature unique to the image toolbar in the program. Mean luminosity was determined over the selected area of the cell image for each of the following images: rhodamine labeled egg, rhodamine labeled blastula, control egg and control blastula. The image area selected was a the largest possible square that could fit inside the circular image. Averages were taken for the data gathered in each image group, as approximately two images were selected to represent each group, excluding the controls.

III. Results

The sea urchin eggs that were labeled with rhodamine dextran were viewed under a 40x objective lens under green fluorescent light. The eggs showed up as a dim, red glowing sphere in a field of black on the slide. About 6 eggs were found on the slide and all had similar results. The red is indeed dim but as compared to the surrounding black space it does stand out as a clear and definite difference. As shown in Figure 1, the red glow is uniform across the entire surface of the egg and there is no appearance of spots or speckling anywhere on the egg. The general trend across this slide of labeled eggs is the consistency between all the eggs, showing a uniform red glow on each. Image analysis of

the two pictures captured using Photoshop gave mean luminosities of 26.07 and 25.96 respectfully. The average value across these two images gives a mean luminosity of 26.02. Mean luminosity is measured on an arbitrary scale of units.

The unlabeled sea urchin eggs were viewed under the same settings as the labeled eggs. There were about 8 eggs on the slide which all showed an extremely faint presence of red, almost undetectable. As seen in Figure 2, the edges of the egg against the black background of the unlabeled water was the only indication of where the egg was located and upon a closer look, the egg could be noticed. The very faint glow of red was similar in all eggs on the slide, and the general trend across the slide was the consistency of red in each egg. Only one image was captured and upon analysis in Photoshop, the mean luminosity was 4.66.

The sea urchin blastulae labeled with rhodamine dextran were viewed using the green fluorescent light and the same settings as described above. The slide viewed only had three visible eggs on it. As seen in Figure 3, the blastulae showed up as red, glowing spheres in a field of black on the slide. Each of the three blastulae had the same red glow accompanied by brighter red speckling pattern on the blastulae. Two of the three blastulae had the very similar speckling patterns, with small red specklings more concentrated around the periphery of the blastula. The third blastula had larger speckles, which could be classified more correctly as dots. These dots of brighter, more intense fluorescence were located across the entire blastula and did not seem to have a particular grouping anywhere. The general trend across this slide was the noticeable speckling on the rhodamine dextran labeled blastulae. Two of the three blastulae were captured as images. Image analysis of the two images using Photoshop gave mean luminosities of 91.50 and 73.66 respectively. The average value across these two images gives a mean luminosity of 82.58.

The unlabeled urchin blastulae were viewed using the same settings as in the previous case. The slide showed 4 blastulae on the slide and each had a very faint red glow to them, similar to the slide of the unlabeled urchin eggs. The faint glow only slightly stood out from the black background of the unlabeled sea water, as seen in Figure 4. The general trend across the slide was the consistent, faint red glow in each of the blastulae, and the absence of any sort of speckling or dotting. Only one image was captured and upon image analysis in Photoshop, the mean luminosity was 11.58.

Figure 1 is an image of a sea urchin egg labeled with rhodamine dextran viewed under a 40x objective lense taken on a mercury lamp Nikon Eclipse E400 light microscope using a

Spot© camera and software. (The scale bar can be applied to the following three images).

Figure 2 is an image of unlabeled sea urchin eggs under a 40x objective lense taken on a mercury lamp Nikon Eclipse E400 light microscope using a Spot© camera and software

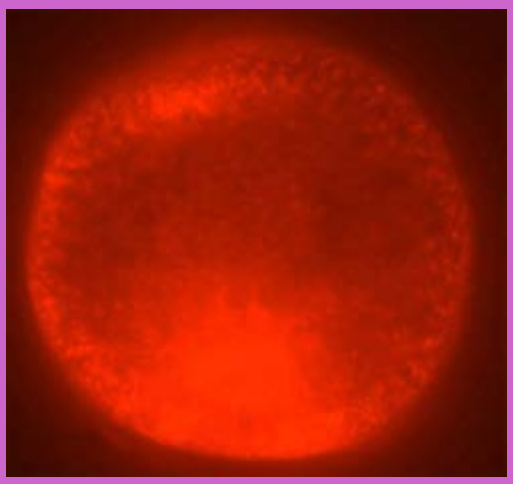

Figure 3 is an image of a sea urchin blastula labeled with rhodamine dextran viewed under a 40x objective lense taken on a mercury lamp Nikon Eclipse E400 light microscope using a Spot© camera and software.

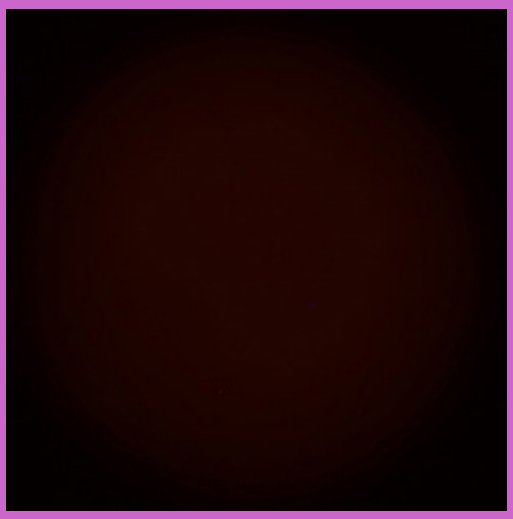

Figure 4 is an image of an unlabeled 23 hour old sea urchin blastula viewed under a 40x objective lense taken on a mercury lamp Nikon Eclipse E400 light microscope using a Spot© camera and software.

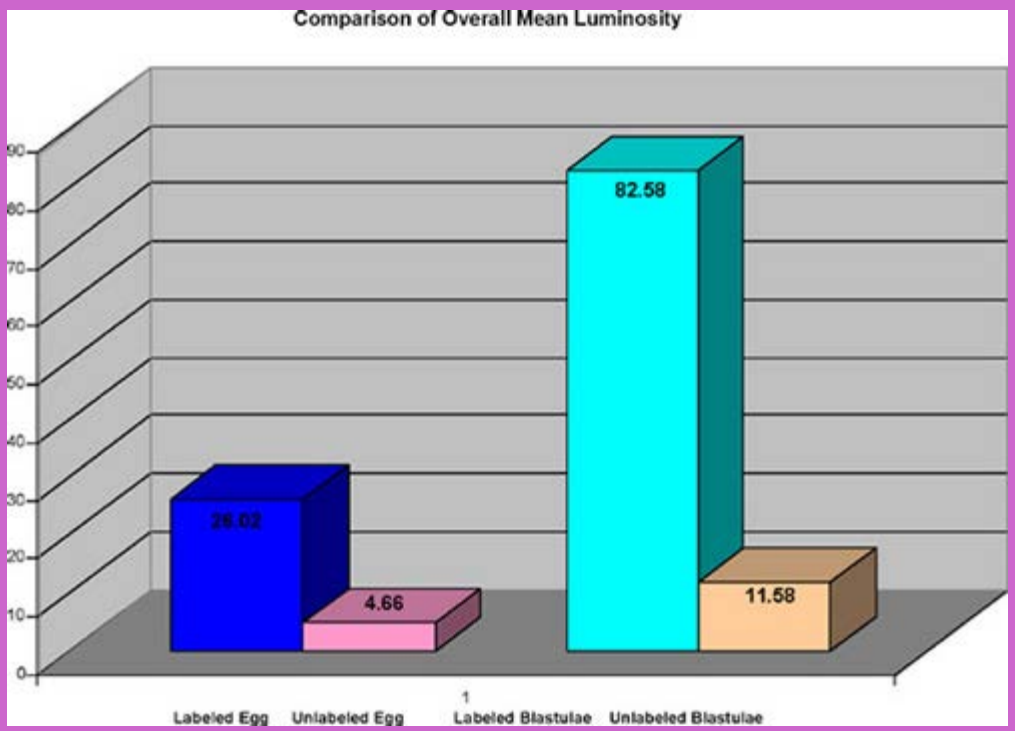

Figure 5 shows the comparison between the overall mean luminosities in the labeled and unlabeled urchin eggs and urchin blastulae.

IV. Discussion and Conclusions

The presence of the red fluorescence inside both the labeled egg cells and blastulae indicated that the rhodamine dextran successfully labeled the cells. The red in both the labeled egg cells and blastulae (Figures 1 and 3) indicate that endocytosis is occurring in the cells, as this is the only way in which rhodamine dextran can be present inside the cell. Rhodamine dextran is unable to diffuse across the cell membrane to enter the cell because it is a hydrophilic polysaccharide with a high molecular weight, (Molecular Probes, 2001). The controls were also successful, since they did not show significant fluorescence under the green fluorescent light (Figures 2 and 4), which indicates that without the application of rhodamine dextran to the cells, no noteworthy fluorescence was produced.

Comparison of figure 1 to figure 3 shows the comparison in rate of endocytosis between the urchin egg and blastula. As can be seen in figure 3, the sea urchin blastula is overall a much brighter red than the urchin egg in figure 1. Analysis of figures 1 and 3 in Photoshop gave mean luminosities of 26.02 and 82.53 respectfully. These numbers show that the overall luminosity in the blastulae is greater than the overall luminosity in the eggs, directly corresponding to the rate of endocytosis. As you can see in figure 5, the difference between the mean luminosity in the labeled egg versus the labeled blastulae is great.

Both control images (Figures 2 and 4) show that the unlabeled eggs and blastulae do not notably glow under green fluorescent light. Figure 2 produced a mean luminosity of 4.66, which, in comparison, is only approximately 1/6 of the mean luminosity of the labeled urchin egg. Likewise, figure 4 produced a mean luminosity of 11.58, approximately 1/7 of the mean luminosity in the labeled blastulae. As seen in Figure 5, comparison of the control images to those of the images labeled with rhodamine dextran, one can note that there is a remarkable difference in luminosity and therefore conclude that rhodamine dextran successfully labeled the endocytic pathway in the cells.

The data gathered does not support my hypothesis that the rate of endocytosis in the eggs is greater than the rate of endocytosis in the blastulae; it shows completely the opposite of what was expected. The urchin blastulae showed an overall greater mean luminosity, corresponding to a higher rate of endocytosis, while the urchin egg showed a considerably lower overall mean luminosity. It was expected that the urchin egg have an overall greater mean luminosity since it is not undergoing mitosis, but the results do not support this. I suspect that the reason my data did not support the hypothesis is because the unfertilized eggs were not given an adequate pulse in the rhodamine dextran labeled sea water, and were unable to effectively endocytosis the labeled water as a result. Another possible explanation for the observed results lies in the process of exocytosis which increases the surface area of dividing cells. As discussed in the introduction, the cell must utilize the process of exocytosis to increase the surface area of the cell which will balance out the process of endocytosis, producing an apparent decrease in the rate of endocytosis, (Morris, 2003). However, it is possible that the rate of endocytosis in the cell was greater than the rate of exocytosis, causing the dividing urchin blastulae to glow brighter than expected.

One possible source of error in my experiment could lay in the step involving fertilization envelope stripping. The 80 _m Nitex mesh does not strip 100% of the fertilization envelopes from the eggs, and when view under the microscope to check the success of stripping, I did notice that approximately a quarter of the fertilized eggs still retained their fertilization envelope. I suspect that this could have been a source of error because the two images captured of the blastulae labeled with rhodamine dextran had mean luminosities of 91.50 and 73.66. I believe that the difference in luminosities could be because one of the fertilized eggs had its fertilization envelope successfully stripped while the other did not. This might account for the difference, as there is a possibility that it is more difficult for the rhodamine dextran to cross the membrane in a blastulae that retained its fertilization envelope than a blastulae that did not.

My collaborator, Meghan Tracewski, studied the formation of tight junctions in sea urchin blastulae and followed a similar protocol to mine. Meghan's results only pertained to fertilized sea urchin blastulae labeled with rhodamine dextran, as she had no need to study eggs when inquiring about the formation of tight junctions. The results Meghan

obtained did however corresponded with my results; a number of her images had the same profuse red glow with a speckling and spotting pattern that we believed to be the presence of endosomes and lysosomes.

To refine my experiment, I would allow the unfertilized eggs to pulse longer in the rhodamine dextran. In my experiment I pulsed them for 10 minutes and the results showed a lower mean luminosity than I had expected. Possibly allowing more time for the eggs to endocytosis the rhodamine dextran would produce a higher overall mean luminosity. Also, I would dejelly the eggs, which I did not do. Dejellying the eggs might allow for the rhodamine dextran to be readily endocytosed. Finally, in the preparation of the slide chambers, I would used a double thickness of the double stick tape. This would allow for more eggs to be pulled under the cover slip and would give more data to be analyzed. More images could be taken and I could overall gather more data, because it is hard to draw adequate conclusions from just two or three experimental images.

Further experiments I would like to perform to expand my experiment in a new direction would be to study the process of exocytosis. I would like to see if there is correspondence in the rates of endocytosis and exocytosis in the sea urchin blastulae. I would do this by following the same protocol as in my previous experiment, but after the final washing, I'd place the eggs in a depression chamber slide and take a time lapse video under green fluorescent light to image the decrease in overall mean luminosity. Using Photoshop to analyze the data I could plot the difference in mean luminosity over the time period and see if it shows a consistent loss as a result of exocytosis.

V. Bibliography

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