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Kiss-and-Run Fusion Can be Detected at the Crayfish Neuromuscular Junction

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ABSTRACT

Kiss-and-run fusion is a recent theory about exocytosis whereby the vesicles do not fully collapse into the membrane. We attempted to show that kiss-and-run fusion occurs at the neuromuscular junction of crayfish by comparing the release rates of two different FM dyes (FM 1-43 and FM 2-10). We predicted that if kiss-and-run fusion occurred at the crayfish neuromuscular junction, there would be a discrepancy between the rates of release of the dyes. We found that FM 1- 43 was released slower than FM 2-10, supporting the possibility that kiss-and-run fusion occurs at the crayfish neuromuscular junction.

INTRODUCTION

Kiss-and-run is a concept that has recently been proposed and remains a topic of much debate. Full collapse fusion is the premier method by which neurotransmitter is released from the vesicle into the synaptic cleft (Rizzoli, S. O. et al. 2007). In full collapse fusion, the vesicle fuses with the presynaptic cell membrane and loses its identity, becoming part of the presynaptic membrane. During this process, the neurotransmitter is completely released into the synaptic cleft. This is followed by a clathrin-mediated regeneration of a new vesicle some distance from the release site (Zhang 2007). The regeneration process takes about 10 seconds, which could, at high stimulation rates, potentially cause a deficiency in neurotransmitter release because a large percentage of the vesicles have been incorporated into the cell membrane and have yet to be recycled (Zhang 2007; Nobutoshi 2006). In addition, full fusion allows for the uptake of larger molecules.

Similar to full collapse fusion, the process of kiss-and-run begins with the vesicle fusing with the presynaptic cell membrane. The vesicle brushes against the membrane, creating a small fusion pore that allows for some neurotransmitter to be released. However, unlike the vesicles in full collapse fusion, the vesicles in kiss-and-run fusion do not lose their identity. The fusion pore detaches from the membrane and the vesicle reforms (Nobutoshi 2006). This process of regeneration is much shorter than that of full collapse fusion (Rizzoli 2007). If kiss-and-run fusion were shown to exist, this process would solve the issue created by full collapse fusion at high rates of stimulation (Nobutoshi 2006).

In previous studies, kiss-and-run has been detected at neuromuscular junctions of frogs (Gaffield, M. A. et al. 2007). To our knowledge, the existence of kiss-and-run fusion has not been explored in crayfish. Therefore, we focused on the neuromuscular junctions found in the extensor muscles of the crayfish tail.

Previous studies on frogs have used FM dyes to observe the process of kiss-and-run fusion (Gaffield, M. A. et al. 2007). FM dyes fluoresce when harbored in vesicle membranes and not in aqueous solutions (Zhang 2007). Thus, FM dyes can report fusion events because once released, the dye will enter an aqueous solution in the synaptic cleft and the fluorescence will disappear. Two particular fluorescent dyes were used in our study: FM 1-43 and FM 2-10. FM 1-43, a styryl dye, fluoresces green (581 nm) when excited by blue light (471 nm). In addition, FM 1-43 has hydrophobic properties that will cause it to be released relatively slowly since it has a tendency to remain bound to the membrane. FM 2-10 is more hydrophilic, which would result in faster release from the membrane than that of FM 1-43. However, FM 2-10 fluoresces red (620 nm) when excited by green light (506 nm); thus making it possible to differentiate between the two dyes. Given the amphiphatic properties of the FM 1-43 and FM 2-10 dyes, we hypothesized that if kiss-and-run fusion was occurring at the crayfish neuromuscular junction, the rates of release of the dyes would be different. In full collapse fusion, the two dyes should leave at equal rates despite these properties because the vesicle releases all of its contents and becomes part of the membrane. Thus, a discrepancy between the two rates would imply that there was a fusion pore that didn't force all the vesicle contents to leave and that the contents left based on either their hydrophilic or hydrophobic properties. Our experiment showed that FM 1-43 dye was released more slowly than FM 2-10 dye, thus supporting our hypothesis.

MATERIALS AND METHODS

Crayfish Preparation

The crayfish selected were chosen for their small size (4-10 cm), so that they could be handled and viewed with more ease, while allowing us to condense the area into which the dye could diffuse, therefore reducing the necessary amount of dye. A dissection dish was modified to have a small volume (7 mL) using dental wax to fill in the excess area.

A tail (2 cm) was cut off a live crayfish that had been submerged in ice for at least 15 minutes. The swimmerets were removed by cutting along the ridge on the dorsal side from anterior to the posterior ends of the tail and the abdominal flexor muscle was removed using a thumb revealing the extensor muscle cells below. A thumb was used because there was less risk of damaging the extensor muscle cells during dissection.

The crayfish tail was placed into a dissection dish and pinned down to prevent movement. Ringer's solution was immediately added to prevent cells from dying.

Solution Preparation

We used two different solutions: Ringer's solution and a high K^+ concentration Ringer's solution. The Ringer's solution contained the following concentrations: 5.4 mM KCl, 196 mM NaCl, 2.6 mM MgCl, 13.5 mM CaCl, and 10 mM Hepes Buffer. The high K^+ concentration Ringer's solution was the same as the above but consisted of 60 mM KCl and 131.4 mM. This high K^+ Ringer's solution was created in order to stimulate the neuromuscular junction, depolarizing the nerve terminal and causing the neurotransmitter-containing vesicles to undergo exocytosis (followed by compensatory endocytosis).

Dye Preparation

Two different FM dyes, purchased from Invitrogen, were used to detect kiss-and-run fusion: FM 1-43 and FM 2-10. The FM dyes were diluted with water to create stock solutions. The FM 1-43 stock solution was prepared by diluting 0.1 mg of the dye with 82 µL of water. The FM 2-10 stock solution was prepared by diluting 5 mg of FM 2-10 with 450 µL of water and dividing it into 9 aliquots, which were then frozen.

The dye solutions that were used for loading were composed of 30 µL of FM 1-43, 20 µL of FM 2-10 and 10 mL of either the 60mM K^+ Ringer's solution or normal Ringer's solution. Based on Hirata (1999), exposing the crayfish to the solution of the two dyes and the normal Ringer's solution would allow the dye to infiltrate the extracellular matrix.

Experimental Procedure

The loading process was completed in three separate steps. In the first step, we placed the solution with both dyes and the normal Ringer's solution on the crayfish and allowed in to sit for 20 minutes. The solution was then removed and replaced with the solution containing the two dyes and the 60 mM K^+ Ringer's solution and left for 10 minutes. Finally, this solution was removed and the first solution of the two dyes and the regular Ringer's solution was reintroduced for 15 minutes.

Once the crayfish was washed, 60 mM K^+ Ringer's solution was placed on the crayfish to create stimulation, causing exocytosis and therefore releasing the dye into the synaptic cleft. During this unloading process, a series of photos wer taken at 30-second intervals while alternating FITC and RDM dichroic excitation emission filters (see below).

Imaging Data

The images collected in the experiment were taken using a Nikon Eclipse 50i Epifluorescence microscope with a 40x water-immersion objective (n.a. 0.8) and with either a FITC or RDM filter cube. An X-Cite 120 PC fluorescence illumination system was used to excite the fluorophores. The different filter cubes were used to observe either FM 1-43 or FM 2-10 dye. The FITC cube emits blue light, exciting the FM 1-43 fluorophores to emit green light. The RDM cube emits green light, exciting the FM 2-10 fluorophores to emit red light. Using Image Pro, by MediaCybernetics, images of the crayfish were automatically taken every 30 seconds for a period of 15 minutes.

The amount of dye present in the vesicles of the presynaptic cell corresponds to the amount of fluorescence in the images. By using MetaMorph, produced by MDS, to determine the intensity of the light present in areas of interest, and then comparing these areas of interest over the time intervals, we were able to detect any changes in dye concentration.

RESULTS

The synaptic vesicles at crayfish neuromuscular junction were loaded with FM 1-43 and FM 2-10 dyes using the protocal described in Harata (1999). The solution of the dyes and the normal Ringer's solution was followed by adding the solution of the dyes and the 60 mM K^+ Ringer's solution to allow for the uptake of the dyes via endocytosis. The 60 mM K^+ solution with the dyes would increase uptake of the dyes by inducing exocytosis of synaptic vesicles, followed by compensatory endocytosis. The unloading processes of theses dyes were observed through imaging.

Our study agrees with previous experiments that indicate the presence of kiss-and-run fusion. Once the images for the trial were obtained, we used MetaMorph to find the intensities of light for the FM 1-43 dye images (14) and the FM 2-10 dye images (15) at specific areas of interest (Figure 1 and 2).

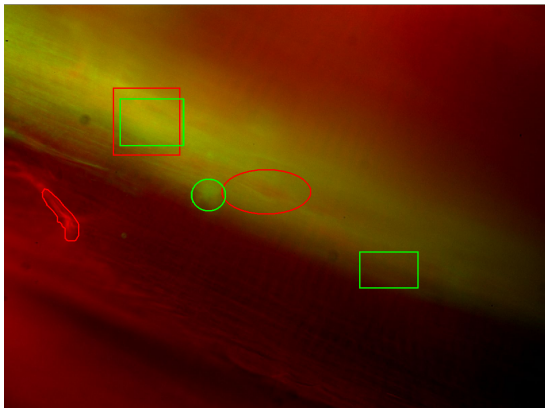

Image A

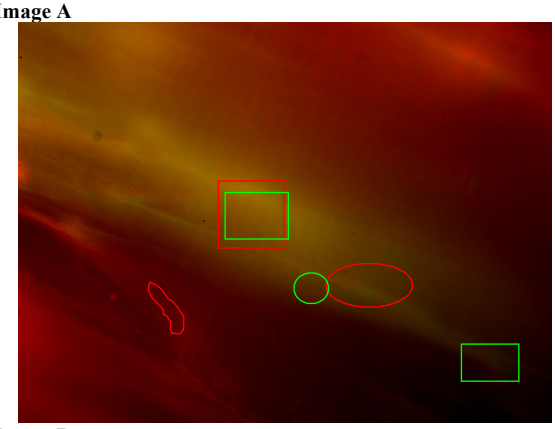

Image B

Figure 1. Alternating images were collected at 30 second intervals and overlaid producing images of both FM 1-43 (green regions) and FM 2-10 (red regions) images. Image A was image 2 of 14 images once all the images were overlayed with each other. Image B was image 5 of 14 images.

For each set of images, we found three different areas of interest, resulting in six areas of interest. We then normalized the data (F/F_i) and found the average of the three areas of interest ($[F/F_i]/3$). Using Microsoft Excel, we created a graph showing the intensities of light in the areas of interest for both FM 1-43 and FM 2-10 over 840 seconds (Figure 2).

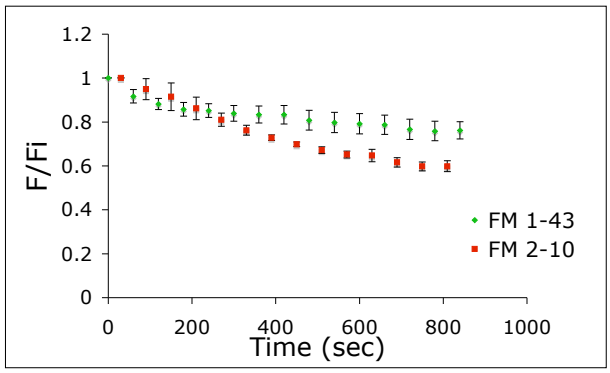

Figure 2. The intensities of both FM 1-43 and FM 2-10 dyes over the course of 840 seconds while stimulating the neuromuscular junction with a high K^+ concentration. Each data point represents the average of the normalized intensities of three areas of interest. The error bars show the standard deviation from the mean of the three normalized areas of interest.

Based on Figure 2, we can see that over time, the FM 1-43 fluorescence intensity decreased at a slower rate than the intensity of the FM 2-10 dye. In addition, the error bars do not vary greatly and almost never intersect with each other. This shows that there is likely a significant difference between the rates of release of the dyes.

DISCUSSION

We successfully detected the uptake of FM 1- 43 and FM 2-10 dyes in the crayfish neuromuscular junction by observing their fluorescence. Likewise, we quantified FM 1- 43 and FM 2-10 unloading from the presynaptic cell through exocytosis into the synaptic cleft. Detecting both the uptake and unloading of the dyes allowed us to recognize kiss-and-run fusion at the crayfish neuromuscular junction. We predicted that if kiss-and-run fusion were occurring at the crayfish neuromuscular junction, then the FM 1- 43 dye would be released at a slower rate than the FM 2-10 dye due to their respective amphiphatic properties. From the data that we collected and analyzed (Figure 2), we see that FM 1-43 dye left at a slower rate than the FM 2-10 dye, supporting our hypothesis and implying that kiss-and-run fusion is occurring.

Prior to aquiring the final data shown in Figure 2, it was necessary to change loading process, including the concentrations of the FM dyes and time intervals. First, we experimented with loading the crayfish neuromuscular junction during one 10-minute time interval with a high K^+ concentration. However, after researching loading procedures in more depth, we changed our loading procedure to have three time intervals with dyes in both high K^+ concentration solutions and regular Ringer's solutions (Hirata 1999). The new loading process increased visibility of the dye, but the concentrations of the dyes were not high enough to see visible changes. We then decided to increase the dye concentrations to not only to see the changes better, but also to be able to decrease the time of exposure. This change in concentration led to our final loading procedure and thus our final results.

Our final results suggest that kiss-and-run fusion occurred because the FM 2-10 left more quickly than the FM 1-43 dye. FM 2-10 dye has more hydrophilic properties therefore the dye more readily leaves the vesicle into the hydrophilic synaptic cleft, while FM 1-43 has more hydrophobic properties and thus will not leave the cell as quickly. In full collapse fusion, the two dyes should leave at equal rates despite these properties because the vesicle releases all of its contents and becomes part of the membrane. Thus, the discrepancy between the two rates implies that there was a fusion pore that didn't force all the vesicle contents to leave and that the contents left based on either their hydrophilic or hydrophobic properties.

Through our data, we have determined that kiss-and-run fusion occurs in another model organism. We had not found any previous research regarding kiss-and-run fusion occurring at the crayfish neuromuscular junction; the only research available to us was done with the neuromuscular junction of frogs (Gaffield 2007). In future studies, to eliminate possible variables that could interfere with the exocytosis of the contents of the vesicles, we would recommend the use of quantum dots. While expensive, the size of quantum dots would prevent them from leaving the vesicle during kiss-and-run fusion, but allow them to exit the vesicle during full collapse fusion (Zhang 2007).

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REFERENCES

Gaffield, M. A., Rizzoli, S. O., Betz, W. J. "Mobility of Synaptic Vesicles in Different Pools in Resting and Stimulated Frog Motor Nerve Terminals." Neuron 51 (2007): 317-325.

Harata, N. C., Choi, S., Pyle, J. L., Aravanis, Al. M., Tsien, R. W. "Frequency-Dependent Kinetics and Prevalence of Kiss-and-Run and Reuse at Hippocampal Synapses Studied with Novel Quenching Methods." Neuron. 49 (2006): 243-256.

Hirata, K., Nakagawa, M., Urbano, F. J., Rosato, M. D., Moreira, J. E., Uchitel, O. D., Sugimori, M., Llinas, R. "Reduced facilitation and vesicular uptake in crustacean and mammalian neuromuscular junction by T-588, a neuroprotective compound." Proceedings of the National Academy of the Sciences of the United States of America. 96 (1999): 14588 - 14593.

Rizzoli, S. O., Reinhard J. "Kiss-and-Run, Collapse and 'Readily Retrievable' Vesicles." Traffic 8 (2007): 1137-1144.

Vardjan, N., Stenovec, M., Jorgacevski, J., Kreft, M., Zorec, R. "Subnanometer Fusion Pores in Spontaneous Exocytosis of Peptide Vesicles." The Journal of Neuroscience 17 (2007): 4737-4746.

Zhang, Q., Cao, Y.-G., Tsien, R. W. "Quantum dots provide an optical signal specific to full collapse fusion of synaptic vesicles." Proceedings of the National Academy of Sciences of the United States of America 104 (2007): 17843-8.