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The Calcium Dependence of Kiss-and-Run Exocytosis at the Crayfish Neuromuscular Junction

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ABSTRACT

The classical mode of neurotransmitter release, full collapse fusion (FCF), occurs when a vesicle is completely incorporated into the presynaptic membrane. Growing evidence supports a novel additional mode of neurotransmitter release known as "kiss-and-run" (K&R) in which the vesicle transiently fuses with the membrane, maintaining its basic morphology. In this study we sought to determine whether K&R occurs at the crayfish neuromuscular junction (NMJ) and, if it does exist, whether its prevalence is dependent on the extracellular calcium concentration. We loaded the dye FM1-43 and the fluorescence quencher bromophenol blue (BPB) into synaptic vesicles and looked for a fluorescence increase corresponding to the segregation of FM1-43 from BPB through K&R. We observed K&R at a low extracellular calcium concentration, but it was ambiguous whether K&R occurred at normal extracellular calcium levels. Thus, extracellular calcium appears to play a role in determining the mode of neurotransmitter release.

INTRODUCTION

Secretion of neurotransmitters from presynaptic vesicles is essential for neuronal communication. Full collapse fusion (FCF), the classical mode of neurotransmitter release, is an exocytic mechanism in which the fusion pore dilates and the vesicle collapses into the plasma membrane, releasing its entire contents into the synaptic cleft (Harata et al. 2006). Exocytosis necessitates a slow, compensatory endocytosis to maintain constant membrane area and replenish the vesicle pool. Growing evidence suggests that neurotransmitter can also be released via a transient membrane fusion mechanism termed "kiss-and-run" (K&R). In this alternate mode of exocytosis, the fusion pore opens and closes rapidly, releasing its contents—often incompletely—but retaining basic vesicle morphology and preserving vesicles for reuse (Doreian et al. 2008).

FCF and K&R may occur simultaneously at the nerve terminal, but research has shown that certain conditions affect the relative probabilities of FCF and K&R events. For example, increased neuronal firing rates favor FCF in adrenal chromaffin cells (Doreian et al. 2008), and raising extracellular hypertonicity promotes K&R in hippocampal synapses (Stevens and Williams 2000). The relationship between extracellular calcium concentration and exocytic mode, however, is unclear. Ales et al. (1999) found that high extracellular calcium levels increased the fraction of K&R events in rat chromaffin cells; however, Elhamdani et al. (2006) found opposite results in bovine chromaffin cells. These results show the prevalence of K&R, but reflect the lack of a complete mechanistic model that would explain why certain conditions favor K&R over FCF.

Evidence suggests that vesicles undergoing K&R are rapidly retrieved and reloaded with neurotransmitter and are thus able to support multiple instances of transmitter release. To detect reuse, however, investigators must be able to distinguish between first fusion and subsequent reuse. This distinction is not possible with readings of presynaptic capacitance, postsynaptic currents or ampertometry (Harata et al., 2006). By contrast, the exocytosis of synaptic vesicles loaded with fluorescent dyes allows some differentiation of K&R and FCF depending upon release and diffusion rates. Cochilla et al. (1999) found that upon stimulation, hippocampal synaptic vesicles partially retained the lipophilic dye FM1-43, but released neurotransmitter. This suggests that the fusion pore closed rapidly before much dye could escape, a characteristic indicative of K&R. Fluorescence-quenching dyes such as bromophenol blue (BPB) can be co-applied with fluorescent dyes in various ways; for example, Harata et al. (2006) observed the uptake of BPB into FM1-43-loaded vesicles via the corresponding fluorescence loss.

In our study, we used FM1-43 and BPB to determine the prevalence of K&R events at different extracellular calcium concentrations at the crayfish neuromuscular junction (NMJ). In contrast with the experiments of Harata et al. (2006), we loaded both dyes into synaptic vesicles. Since BPB is hydrophilic while FM1-43 is lipophilic, we reasoned K&R would allow BPB to pass rapidly through the fusion pore while FM1-43 would be much slower to diffuse. FCF, however, would result in the exocytosis of both dyes. Increasing fluorescence could

only be accomplished via the segregation of the two dyes and was taken to indicate the occurrence of K&R.

Many studies on K&R have been done using chromaffin and hippocampal cells, but few have been performed using the NMJ. To our knowledge, none have examined the crayfish NMJ, a convenient invertebrate model of synaptic transmission. In the present study we examined the effects of altering extracellular calcium concentration on the prevalence of K&R at the crayfish NMJ. We found preliminary evidence that K&R is more likely to occur at the crayfish NMJ at low extracellular calcium levels.

MATERIALS AND METHODS

Solution preparation

Normal Ringer's bathing solution contained in mM): 5.4 KCl, 13.5 $CaCl_2$, 196 NaCl, 2.6 $MgCl_2$, and 10 mM HEPES, pH 7.4. Due to the small size of the preparation and the need for it to be inverted for microscopy, ionic stimulation was employed in place of electrical stimulation. For ionic stimulation to load and release FM1-43 and BPB, high-potassium Ringer's solutions were created by raising KCl to 60 mM with a corresponding decrease in NaCl. For experiments at low extracellular calcium concentrations, $CaCl_2$ was decreased to 8.1 mM with a corresponding increase in NaCl in both normal- and high-potassium solutions.

To distinguish between K&R and FCF, FM1-43 and BPB were purchased from Invitrogen. Fresh stock solutions of each (both 8 mM) were prepared before each experiment. FM1-43 and BPB were both diluted to a final concentration of 8 µM in solutions used in dye equilibration and loading but not release.

Crayfish preparation

Crayfish were anesthetized with ice for at least 15 min. The tail was excised and the swimmerets and abdominal flexor muscle were removed to reveal the extensor muscle cells. The left or right half of one tail segment was then selected for testing. The preparation was inverted, fixed to a microscope dish, and bathed in normal Ringer's solution.

Dye equilibration, loading, and release

Dye was added at normal potassium concentration (13.5 mM) for 5 min to equilibrate the preparation. The preparation was then rinsed with dye-free normal Ringer's solution. Dye was loaded at high potassium (60 mM) for 10 min, and the preparation washed again. To stimulate dye release from nerve terminals, the preparation was bathed in dye-free high-potassium Ringer's solution.

Image collection and analysis

Images were collected using an Olympus IX81F-3 confocal microscope with a 40x water immersion objective and a GFP dichroic excitation emission filter. Using Slidebook (Intelligent Imaging Innovation, Inc.), a series of photos were taken at 15 s intervals for 8 min. Synaptic regions were located in time-lapses and the fluorescence intensity determined in Slidebook.

RESULTS

In an initial experiment, we loaded vesicles with FM1-43 only. Dye loading was accomplished by ionic stimulation of the presynaptic cells in a dye-containing bathing solution. Because exocytosis of synaptic vesicles is balanced by endocytosis, the dye and extracellular fluid were taken up into new, 'loaded' vesicles. After the bathing solution was replaced with dye-free solution, the exocytosis of these new vesicles could be observed via fluorescence microscopy.

We observed fluorescence intensity changes in regions of interest (ideally containing one or more boutons); an example for the initial experiment is shown in Figure 1. In this experiment, FM1-43 fluorescence gradually decreased upon stimulation, indicating that successful release of neurotransmitter and dye occurred (Figure 2).

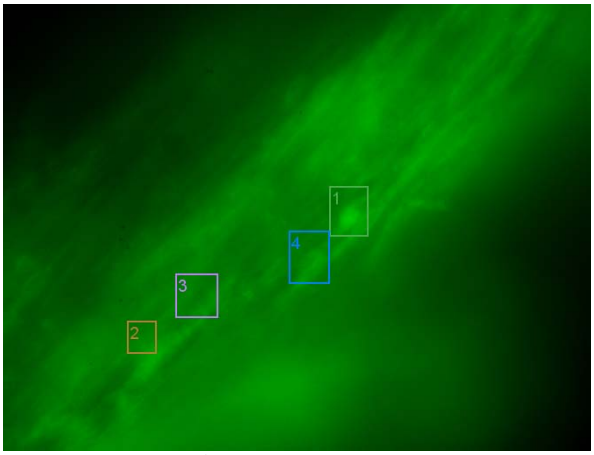

Figure 1. Regions of interest chosen to measure changes in fluorescence intensity during stimulation. Localized bright regions correspond to synaptic boutons.

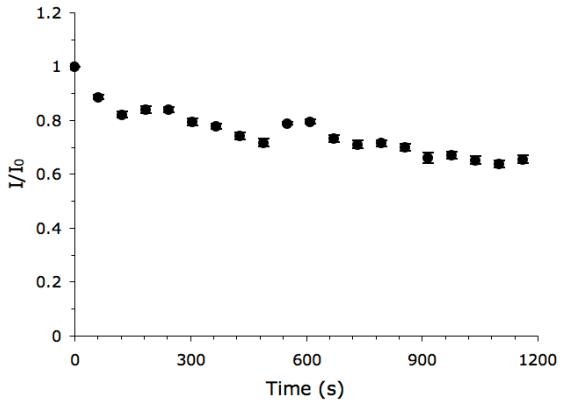

Figure 2. Release of FM1-43 with standard extracellular calcium concentration (13.5 mM). A gradual decrease in fluorescence was observed, indicating synaptic vesicle release. Error bars represent ± SEM (n = 4).

In order to determine the prevalence of K&R at the NMJ of the crayfish, we loaded synaptic vesicles with FM1-43 dye with and without a BPB quencher. The only conceivable explanation of increasing fluorescence intensity is separation of dye and quencher. Based on the difference in hydrophilicity between the two dyes, we reasoned that separation could occur during a transient period of diffusion out of the vesicle followed by rapid closure of the vesicle—namely, during K&R. Accordingly, we considered increases above initial fluorescence intensity to be indicative of K&R.

Upon the addition of 8 µM BPB quencher, an initial increase in fluorescence occurred during the first five minutes of stimulation, followed by a decline and then a leveling off of fluorescence change (Figure 3), indicating that K&R occurred initially, followed by FCF.

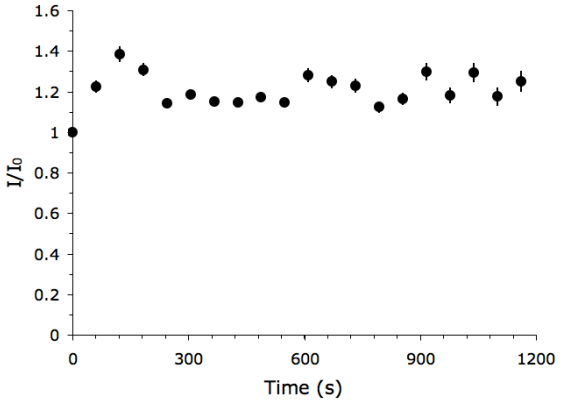

Figure 3. Release of FM1-43 and BPB with standard extracellular calcium concentration (13.5 mM). An initial (0-180 s) increase in fluorescence occurred, indicative of K&R. Error bars represent ± SEM (n = 4).

A second trial of 8 µM FM1-43 and 8 µM BPB, however, showed a gradual decline in fluorescence with no increase in fluorescence, suggesting that K&R failed to occur (Figure 4). When calcium concentration was lowered by 40% an initial increase in fluorescence was again observed followed by a leveling off and then a decline, suggesting that K&R was occurring at initial time points (Figure 5).

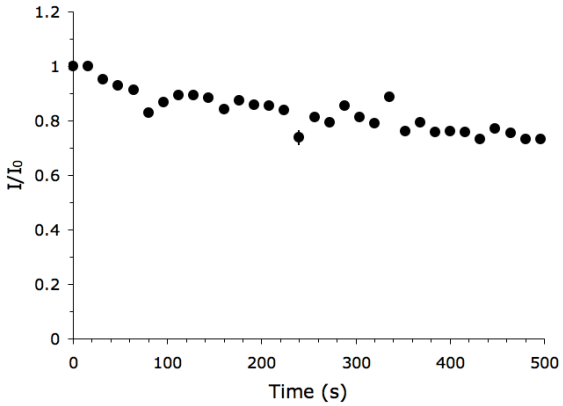

Figure 4. Second trial of release of FM1-43 and BPB with standard extracellular calcium concentration (13.5 mM). A gradual decrease in fluorescence was observed, indicating that K&R did not occur. Error bars represent ± SEM (n = 4).

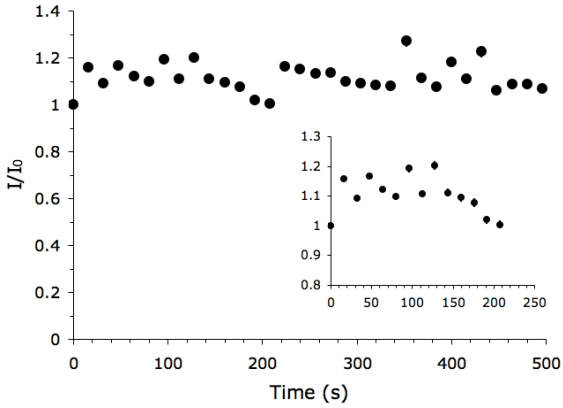

Figure 5. Release of FM1-43 and BPB with lowered extracellular calcium concentration (8.1 mM). An initial increase in fluorescence was observed in the first minute of stimulation, suggesting the occurrence of K&R. Inset highlights fluorescence increase. Error bars represent ± SEM (n = 4).

DISCUSSION

In the word reported here, we followed the techniques of Harata et al. (2005), using a novel quenching method with bromophenol blue (BPB) and FM1-43 in order to clearly distinguish between K&R and FCF. We successfully observed and measured the release of FM1-43 dye and BPB quencher at the crayfish NMJ. Our results suggest

that K&R may occur at the crayfish NMJ but is rare under normal ion concentrations in the extracellular bathing solution. We observed an increase in fluorescence, indicative of K&R in one trial, but a gradual decline in fluorescence in a second trial. By using a high potassium concentration to induce neurotransmitter release, we used a sustained stimulation analogous to high frequency stimulation. Thus, our results are not unexpected even if K&R is highly prevalent at the crayfish NMJ, as several studies have found that high frequency stimulation favors FCF (Doreian et al. 2008; Elhamdani et al. 2006; Harata et al. 2006). This frequency dependence is thought to occur in order to preserve vesicles for high stress situations (Doreian et al. 2008).

When external calcium was reduced by 40%, we observed an initial increase, followed by a leveling off and decline of fluorescence. This suggests that K&R occurred initially and was followed by FCF and simultaneous release of both BPB and FM1-43, causing a loss of fluorescence. These results agree with those of Zhang et al. (2009), who found that K&R was more likely to occur at shorter latencies.

K&R occurred at low extracellular calcium levels, but only one of two experiments at normal extracellular calcium levels showed evidence of K&R. These results lend credence to the finding of Elhamdani et al. (2006) that a moderate reduction in external calcium favored K&R transient fusion. When calcium influx was blocked, vesicles were more likely to fuse with the plasma membrane for a short period of time through a narrow fusion pore with a high conductance, characteristics indicative of K&R (Elhamdani et al. 2006).

Future research should test the effects of high extracellular calcium concentrations on the prevalence of K&R at the crayfish NMJ. In future studies the prevalence of K&R could be described quantitatively (e.g. by the amplitude of fluorescence increase above initial value) rather than qualitatively (presence or absence of fluorescence rise). The procedure carried out in our experiment of high potassium stimulation may not be representative of the physiological conditions involved at the crayfish NMJ. Therefore, this experiment should be repeated testing the effects of extracellular calcium with the stimulation of a single neuron using an electrode.

In summary, we have found evidence that K&R is favored at low extracellular calcium levels. The discovery of K&R has yielded a more complete understanding of the exocytosis of synaptic vesicles. Exocytosis is fundamental to neuronal communication, and its regulation—and potential pathologies thereof—may have macroscopic consequences.

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