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Methyl-beta-cyclodextrin induced cholesterol depletion facilitates synaptic transmission at the crayfish neuromuscular junction

MARIA VENNERI, KAI VORHIES, and ANDREW ZDECHLIK
Department of Biology, Grinnell College, Grinnell, Iowa

ABSTRACT

Methyl-beta-cyclodextrin (MβCD) is a toxin that has been found to act as a cholesterol inhibitor within the cell membrane. Research has indicated that the removal of cholesterol from the cellular membrane causes an increase in excitatory postsynaptic potentials (EPSPs) in neuromuscular junctions when the presynaptic cell is stimulated. This rise in EPSPs is due to the increase of calcium within the presynaptic cell when the membrane becomes more fluid. We investigated the effects of increasing membrane fluidity by removing cholesterol from the cellular membrane and the consequences of doing such within the crayfish neuromuscular junction (NMJ). This research is important due to its possible insights into neurodegenerative diseases such as Niemann-Picks disease. Based on prior research, we hypothesized that increasing membrane fluidity by removing cholesterol from the presynaptic membrane will increase the amplitude of the first EPSP in paired-pulse stimulation therefore creating a short term plasticity, via an increase of calcium within the cell. Our second hypothesis was that there would be a decrease in paired-pulse facilitation, coming from a diminished second EPSP caused by a lack of readily-releasable pool of neurotransmitter. Contradictory to our hypotheses, our results showed an increase in facilitation when paired-pulse stimulation was used in combination with MβCD.

INTRODUCTION

Neuronal communication is conducted between the membranes of pre- and postsynaptic cells via neurotransmitters. As such, neuronal communication depends on the many factors of the cellular membrane, including receptor proteins, ion permeability, and most importantly, one of its structural components, cholesterol. Cholesterol regulates membrane fluidity, with more cholesterol equating to a more viscous membrane. Cholesterol also regulates many of the key properties of synaptic transmission, such as promoting the formation of lipid rafts at synaptic sites, which contain many signaling molecules and ion channels, and modulating the efficacy of neurotransmitter release (Jia et al. 2006).

When a cell is introduced to MβCD, a toxin that depletes membrane cholesterol, the levels of reactive oxygen species increase in both intra- and extracellular spaces (Petrov et al. 2014). Reactive oxygen species are chemically active molecules containing oxygen that bind to lipids and deteriorate them, changing and reducing the viscosity of the membrane. The production of reactive oxygen species increases cytosolic calcium, which activates calcineurin, and in turn enhances synaptic vesicle exocytosis (Petrov et al. 2014; Silverman-Gavrila 2013). This chemical process also causes spontaneous synaptic exocytosis to occur more frequently. Spontaneous release of neurotransmitters can lead to the desensitization of neurotransmitter receptors and a decrease in protein synthesis at the synapses, which could provide possible explanations for neurodegeneration (Petrov et al. 2014; Tashiro et al. 2004). Therefore, purposeful inhibition of cholesterol allows for researchers to observe the effects of neurodegenerative diseases and retroactively find ways to alleviate them.

Using single pulse stimulation, Zamir and Charlton (2014) determined that applying MβCD to the neuromuscular junction of crayfish appendages causes a facilitation of the EPSP. When crayfish are exposed to MβCD, the neuromuscular synapses undergo short term plasticity, a brief change in neuronal expression, therefore suggesting that a paired-pulse stimulation could be effective in measuring whether a facilitation or depression occurs (Fioravante & Regehr 2011).

The purpose of our experiment was to test the effects of removing cholesterol from the cell membrane on short-term plasticity and synaptic transmission. Therefore we decided to test paired-pulse stimulation in order to see the effect of MβCD on the percentage change between EPSPs. We decided to use crayfish as our test organism because crayfish have hardy nervous systems that are easy to manipulate, and do not decay under stressors like MβCD. We used paired-pulse stimulation to determine if the enhanced quanta release, induced by greater membrane fluidity, would result in a smaller

second EPSP amplitude, due to a lack of neurotransmitters in the readily-releasable pool.

We hypothesized that increasing membrane fluidity by removing cholesterol from the presynaptic membrane would increase the synaptic transmission due to an increase of calcium within the cell. We then also hypothesized that there would be a reduced paired-pulse facilitation, evidenced by a diminished second EPSP. We expected the first EPSP amplitude to be larger due to a higher concentration of cytosolic calcium, resulting from the increased membrane fluidity, and thus an increase in the amount of neurotransmitters available for release. (Petrov et al. 2014). Consequently, we expected the second EPSP amplitude from paired-pulse stimulation to be smaller, because the readily-releasable pool of neurotransmitters would be depleted from the initial, enhanced release. As a result of these factors, we expected to observe a reduced facilitation because the second EPSP would be unable to reach the same amplitude as the first EPSP. Our data do not support our hypothesis, demonstrating a facilitation rather than a depression as a result from paired-pulse stimulation. We found this because there was a statistically significant positive percent change between the first and second EPSPs in the MβCD solution as compared to that of the control.

MATERIALS AND METHODS

Crayfish Abdomen Preparation

Data were collected from a total of 9 crayfish, with 4 used for experimental conditions and 5 for separate control trials. We followed the same crayfish abdomen preparation procedure for all trials. Before beginning dissection, each crayfish, *Orconectes*, was anesthetized by submergence in a container filled with ice water. Once immobilized, we separated the abdomen from the body by fully extending it and then cutting with dissection scissors at the base of the torso. We prepared the abdomen for experiment by using scissors to cut along the ventral surface on each side, stopping just before the telson. In order to expose the dorsal extensor muscle groups, we grasped the swimmerets and pulled back the flexor muscle groups, and removed the flexor muscle mass and intestines. We pinned the prepped abdomen ventral side up to Sylgard in a glass petri dish. Once securely pinned, we covered it with standard crayfish saline solution to mimic extracellular fluid. During the experiment, we replaced the saline with fresh saline, or corresponding solution based on the trial type, in 30-minute intervals. Time interval was chosen based on previous research (Zamir & Charlton 2006) and has no factor in the experiment.

Solutions

For our extracellular fluids, we submerged the crayfish in a solution created out of stock MβCD powder of 10 mM MβCD and a low calcium standard crayfish saline solution containing 5.4 mM KCl, 196 mM NaCl, 2.6 mM $MgCl_2$, 6 mM $CaCl_2$, and 10 mM HEPES buffer with a pH of 7.4. We used 10 mM MβCD because the same concentration was used in the experiment done by Zamir and Charlton (2006). We filled the petri dish with 100 mL of the standard crayfish saline solution as the control. For our experimental extracellular solution, we combined 13.1 mg of MβCD for every 1 ml of the standard saline solution to dilute MβCD to the experimental concentration of 10 mM.

Microelectrode preparation and experimental setup

Microelectrodes were made from borosilicate tubes, using a PUL-1 microelectrode puller by World Precisions Instruments. Delay was set at 2, and the heat was set at 7.1. Using a fine syringe, we filled one recording microelectrode with 3.0 M KCl. We tapped on the tube of the microelectrode, near the tip, to release any air bubbles. We created the suction electrode by blunting the microelectrode tip with sandpaper, making the opening large enough to suck the crayfish nerve bundle into place. We then put the recording electrode, which would eventually allow for EPSP readings to be processed and analyzed, in the electrode holder and the suction electrode into the electrode holder connected to the syringe. We placed the petri dish containing our crayfish abdomen underneath a Leica Zoom 2000 microscope, and focused the zoom on a deep phasic extensor muscle and nerve bundle. Using the micromanipulators, we positioned the recording microelectrode above the standard crayfish saline solution in preparation to penetrate the muscle cells. We then positioned the suction electrode in the solution to suck in the crayfish nerve bundle using the connected syringe.

Electrophysiology and Data Collection

Before recording intracellular data for each trial, we placed the microelectrode in the saline solution to check for an acceptable resistance (10-20 mΩ) and used an A-M Systems, Inc. model 3000 ACDC amplifier to zero the membrane potential. Using the micromanipulator, we inserted the recording microelectrode into the crayfish muscle cell and used Analog Digital Instruments PowerLab 4/25 to record an adequate membrane potential (between -50 mV and -80 mV) to ascertain that the electrode had successfully entered the cell. After successfully sucking a nerve into the suction electrode, we stimulated the nerve using a Grass Instruments Division SD9 Stimulator. We produce

our paired-pulse stimulation with a delay of 50 ms, and a duration of 0.02 ms. We used paired-pulse stimulation because other research has not explored its effects on the NMJ. Voltage varied between trials, set just above the threshold to generate an EPSP. Upon stimulation, we used the computer program Lab Chart v. 8.0.5 to record the resulting paired EPSPs and their amplitudes. We did this without the MβCD in the solution at first to gain a specific control for the crayfish. We had to use different crayfish for our control and our experiment to account for the decomposition of neurological function over time. We measured EPSPs for thirty minutes, with the control using the entire thirty minutes, and the experimental trials utilizing the first ten minutes for a control, and then used the last twenty for experimental data collection after switching out the low calcium saline solution with one containing MβCD.

Data Analysis

To account for individual differences between crayfish due to natural variation, we used the percent difference in amplitude between EPSPs, which eliminates the confounding variable of differing initial cellular resting membrane potential (mV). We then used the mean of the percent changes to find the average percent change between the control and the experimental tests. We found the percent change in amplitude by using the equation: ([AMP2-AMP1]/AMP1) x100. We also conducted an unpaired, two-tailed t-test to determine whether or not our experiment had significance using Microsoft excel. We used the mean percentage change of each trial from the control and the experimental tests to conduct the t-test, and it produced a value of 0.02, meaning that our results are statistically significant. The data are presented as the mean and standard error of the mean.

RESULTS

We investigated the role of cholesterol on synaptic transmission and synaptic plasticity in the crayfish neuromuscular junction by inhibiting cholesterol within the cell membrane. This inhibition was achieved through the addition of MβCD to the extracellular solution of the crayfish. We measured EPSP amplitudes for a control and drug condition, while stimulating the nerves with a paired-pulse stimulation. EPSPs were measured at a 30-second or 1-minute interval for 30 minutes. Time between stimulations did not create a difference in our results; therefore we combined the data and reported them together.

MβCD increases paired-pulse facilitation

We observed a positive difference in percent change in EPSP between the control and drug conditions. Both showed a paired-pulse facilitation (Fig. 1), but the drug condition showed an enhanced facilitation. In the control condition, the change from the first to the second EPSP was 22.2% (SE = 3.2), while the MβCD condition, showed a change of 38.8% (SE = 6.5). The standard errors show that, the EPSPs recorded during the experimental condition were more varied than those recorded in the control. An independent unpaired, two-tailed t-test showed that the differences in percent change from one condition to the other were significant ($p < 0.05$). Based on this information, we concluded that MβCD causes a greater paired-pulse facilitation in the crayfish neuromuscular junction, and thus the cell is not limited by a lack of readily-releasable neurotransmitter.

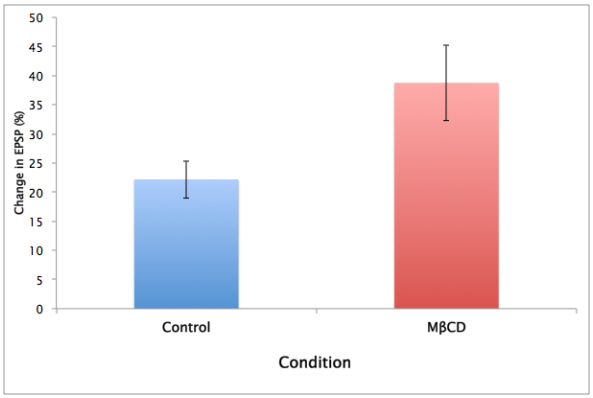

Figure 1. Mean percent change in EPSP from the first EPSP to the second EPSP via paired-pulse stimulation for the control condition (saline extracellular solution) and experimental condition (MβCD extracellular solution). The amplitude of the percent change in EPSP for the MβCD condition was significantly larger than the control condition ($p < 0.05$). Paired-pulse facilitation was greater in the experimental condition. Error bars represent standard error of the mean. n = 159 for control condition, n = 85 for MβCD condition.

MβCD does not increase synaptic transmission

After we observed a significant difference between the percent changes in the control and the MβCD conditions, we compared the first EPSP in both conditions in order to see if an initial facilitation was occurring (Fig. 2). By taking the mean amplitude of the first EPSP of the three stimulations before MβCD was added in each trial (n = 4), we determined the mean amplitude for the control condition. The three stimulations after MβCD was added to the extracellular solution for each trial (n = 4) allowed us to determine the mean amplitude in the MβCD condition. The control condition showed a mean EPSP amplitude of 17.8 mV (SE = 1.6), while the MβCD condition showed a mean EPSP amplitude of 25.5 mV (SE = 6.6). Similar to our other analysis, the results measured in the MβCD condition were more varied than the control condition. We conducted an unpaired, two-

tailed t-test to see if the differences in amplitude of the first EPSP from the control to the experimental were significant. The mean amplitude of the first EPSP in the control condition (M = 17.8 mV) was not significantly lower than the mean amplitude of the first EPSP in the MβCD condition (M = 25.5 mV, $p > 0.05$, Fig. 2). Thus, we concluded that synaptic transmission is not significantly affected by MβCD.

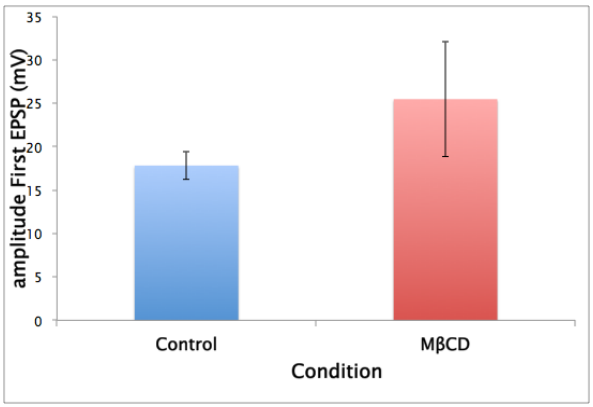

Figure 2. Mean amplitude for first EPSP of paired-pulse stimulation in control condition (saline extracellular solution) and experimental condition (MβCD extracellular condition). There was no significant difference between the mean first EPSPs of the control and the experiment conditions ($p > 0.05$). Error bars represent the standard error of the mean. n = 4 for both conditions.

DISCUSSION

Using paired pulse stimulation and intracellular recording techniques; we examined the role of cholesterol in synaptic transmission at the NMJ in crayfish deep phasic extensor muscles. We found that the MβCD solution caused a significant difference in the EPSP amplitudes between the control and experimental conditions. The control data depict the standard percent change in EPSP amplitude in response to paired-pulse facilitation, while the percent change in the experimental solution shows a trend of being relatively greater. These results contradict our initial hypotheses.

Our results corresponded with the similar research conducted by Petrov et al. (2014), which found that facilitation occurs during synaptic transmission due to an increased influx of calcium into the cell, despite cholesterol depletion. Based on this knowledge, we predict that the facilitation displayed by our data reflected a similar influx of calcium into the presynaptic cell, as the membrane became more fluid. Specifically, applying the results of research done by Silverman-Gavrila et al. (2013), the influx of calcium likely activated calcineurin and thus the mechanism for quanta release into the synapse. These mechanisms combined with the ability of vesicles to bind with greater ease to a more fluid membrane may explain the paired-pulse facilitation represented in our results by the relatively greater experimental EPSP amplitude.

This possible explanation is further supported by the information reviewed and discussed in Fioravante and Regehr's (2011) paper, which reported that rather than the paired-pulse stimulation depleting the readily-releasable-pool of vesicles in the presynaptic neuron, the increased membrane fluidity increases endocytosis, allowing the vesicles to be rapidly reformed and regenerated inside the presynaptic cell (Hosoi et al. 2009). The fact that the time interval between stimulations had no significant effect on the data, indicates that the readily-releasable-pool was not stressed as we hypothesized it would be.

However, future research is needed as our first hypothesis, which stipulated that the increased membrane fluidity would lead to an increased neurotransmitter release, was rejected due to a p-value that was greater than 0.05. We believe that with a larger sample size, there is a possibility for our hypothesis to be supported by the data. The small sample size was due to the limited amount of crayfish on which experimentation was conducted. A larger sample size would decrease the standard error and account for natural variance among the data.

Our research produces several questions, such as whether a largely extended period of time will reduce facilitation, or even if over long amounts of time the facilitation stops, or falls into a depression because of the eventual dissemination of calcium from the cell. Therefore, future research is required in order to consider long periods of time as a variable. A separate question is whether or not a different concentration of MβCD could be utilized to artificially create premature apoptosis in the cell, or whether over time the effect of increased levels of MβCD could change the observed results of the experiment. Another question that our research creates is whether or not the facilitation we observed is related to the neurodegeneration found in Niemann-Picks disease. If it is, future research can be conducted on ways to return the cell to normal, in which case our research could eventually lead to a cure based on the cause of the disease.

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