The CA²⁺/ATPase Pump in the Endoplasmic Reticulum Affects EPSP Amplitudes and Synaptic Facilitation at the Crayfish Neuromuscular Junction.

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Abstract

There are four mechanisms that remove Ca^{2+} from the presynaptic nerve terminal following an action potential. They are: the Na²⁺/Ca²⁺ exchanger, the Ca²⁺/ATPase pump in the mitochondria, the Ca²⁺/ATPase pump in the plasma membrane, and the Ca²⁺/ATPase pump in the endoplasmic reticulum (E.R.). The objective of this experiment was to investigate the importance of the Ca²⁺/ATPase pump in the E.R. in removing Ca²⁺ from the cell. We tested the relative importance of this pump by measuring excitatory post synaptic potential (EPSP) amplitudes and synaptic facilitation. To do this, we compared normal twin pulse facilitation to twin pulse facilitation in crayfish under the effects of Ochratoxin A, a drug that enhances the functioning of the Ca²⁺/ATPase pump and twin pulse facilitation in the presence of BHQ, a drug that inhibits the functioning of the Ca²⁺/ATPase pump. We found that Ochratoxin A has a depressive effect on both EPSP amplitude and synaptic facilitation, while BHQ had the opposite effect. Our results indicate that the Ca²⁺/ATPase pump in the E.R. plays a significant role in regulating the amount of residual Ca²⁺ in the presynaptic nerve terminal.

INTRODUCTION

Synaptic transmission is the process by which chemical impulses are propagated through the nervous system and occurs when a depolarization causes the voltage gated Ca^{2+} channels in the presynaptic membrane to open. The influx of Ca2+ into the cell causes the synaptic vesicles to fuse with the membrane. After these vesicles have docked, they release neurotransmitter (NT) into the synaptic cleft where they bind with the postsynaptic receptors. Therefore, the amount of Ca2+ that is present in the cell regulates the amount of neurotransmitter that is released and thus affects the amplitudes of the EPSP produced in the postsynaptic cell.

Facilitation is the process by which repeated stimuli produce greater amplitudes of EPSPs in the postsynaptic cell. Katz (1967) showed that facilitation is directly related to the residual Ca^{2+} in the cell. When the first stimulus, or action potential, reaches the presynaptic terminal, Ca^{2+} channels are opened in the presynaptic cell allowing an influx of Ca^{2+} . There is some Ca²⁺ that will then remain in the presynaptic cell for a brief period of time. Facilitation occurs as an increase in the amplitude of the second EPSP when a second stimulus is introduced before the excess Ca^{2+} has had time to diffuse out. Facilitation can be induced in a laboratory setting by use of an artificially stimulated twin pulse, a process in which two stimulus pulses delivered, separated by a set time interval.

There are four main mechanisms that remove Ca^{2+} from the presynaptic cell during synaptic transmission: The Na²⁺/Ca²⁺ exchanger, located in the membrane, which takes three sodium ions into the cell and releases two calcium ions out of the cell, the Ca^{2+}/ATP ase pump within the Mitochondria which takes in Ca^{2+} and breaks down ATP; the Ca^{2+}/ATP ase pump located on the membrane which pumps Ca^{2+} out of the cell and changes ATP to ADP; and the Ca^{2+}/ATP ase pump in the E.R. which absorbs Ca^{2+} while converting ATP back into ADP. Our research concentrated on this last mechanism, the Ca^{2+}/ATP ase pump in the E.R., in order to determine the relative importance of that pump on facilitation of synaptic transmission.

We found some gaps in previous research pertaining to the factors controlling the amount of Ca^{2+} that the E.R. absorbs and how much of an effect it has on facilitation. Our research investigating the function of the E.R. pump could help scientists better understand the role of the E.R. in synaptic transmission. Work done by Jeon, et al (2003) indicated that enhanced synaptic transmission, via increased intracellular Ca^{2+} , played an important role in learning and memory. Similarly, our work could assist the scientific understanding of learning and memory defects that may stem from alterations of synaptic facilitation.

The aim of our experiment was to study the effects of altering the workings of the E.R. $Ca^{2+}/ATPase$ pump in crayfish by using Ochratoxin A and BHQ, two drugs that act to enhance and inhibit

the activity in the pump, respectively. Crayfish were selected as the test subjects due to their easily accessible neuromuscular junctions and their glutamatergic synapses are similar to those found in the central nervous system of humans. We tested the effect of the two drugs on the percent change in facilitation, while varying the delay between the stimuli. We also measured their effects on the amplitudes of the EPSPs.

Jeon et al showed that the lack of a Na^{2+}/Ca^{2+} exchanger enhanced the synaptic facilitation of mice thus enhancing their learning and memory. Another study done in 2002 tested the effects of inhibition of the reverse mode of the Na^{2+}/Ca^{2+} exchanger on the facilitation of paired EPSPs in the crayfish neuromuscular junction (Lin, et al, 2002). We based our hypothesis on these results. We expected that the inhibition of the pump by applying BHQ would result in an increase in EPSP amplitudes and an increase in synaptic facilitation. We also expected to find an inverse relationship between the artifact stimuli delay and synaptic facilitation. Similarly, we hypothesized the opposite when the pump was enhanced. In accordance with our hypothesis, we found that the inhibiting drug, BHQ, caused an increase in EPSP amplitudes and synaptic facilitation while the enhancing drug, Ochratoxin A, had the opposite effect. We also found an inverse relationship between the delay (the time lag between the stimulus artifacts of a twin pulse) and synaptic facilitation using both drugs.

MATERIALS AND METHODS

Dissection

The sides of the exoskeleton of the crayfish (*Procambaris clarkii*) were first removed using a wide incision in order to expose as many nerves as possible. We then removed the flexor muscle and the gut by clearing away as much debris as possible so as to leave only the extensor muscles attached to the dorsal surface of the crayfish.

Electrode Preparation

We used a World Precision Instruments PUL1-K electrode puller to create microelectrodes that had tips approximately 10 micrometers in diameter. We ground down the tips of microelectrodes to make suction electrodes. We filled the microelectrode with 3M KCL, making sure to eliminate any bubbles, and then attached it to a micromanipulator to prepare it for insertion into the crayfish cell. The suction electrode was affixed to a micromanipulator that was connected to a Grass SD9 Stimulator.

Crayfish Ringer's and Drug Preparation

We filled the base of a dish filled with normal Ringer's saline solution. We used low Ca²⁺ in order to prevent the triggering of action potentials while experimenting (See Table 1). We obtained our drugs from Tocris Cookson Inc. (Tocris.com). We mixed 99.9mL of normal Ringer's solution with 0.1mL of 0.1 mM Ochratoxin A to create the drug bath with a similar concentration to the one used by Chong in his Ochratoxin A work in 1992. We also mixed 99.9mL of normal Ringer's solution with 0.1mL of 0.1 mM BHQ to create a bath with similar concentration to that used by Hassessian in 1994. The solution in our dish was changed every 15 minutes in order to keep the crayfish as close as possible to its natural environment.

Chemical	Concentration
KCL	5.4 mM
NaCL	205 mM
MgCl ₂ 6H ₂ O	9.35 mM
NaHCO ₃	2.3 mM
Dextrose	2.0 mM
CaCl ₂ H ₂ O	6.75 mM

Table 1. Chemical composition of normal crayfish Ringer's solution with low Ca^{2+} .

Measuring Membrane Potential and Generating Twin Pulses

We pinned the dissected crayfish tail to the base of the dish that had been filled with the normal Ringer's solution. Reference electrodes were placed in the dish. A suction electrode was used to suck up a nerve on the tail of the cravfish. We ensured that we had a good seal between the suction electrode and the nerve. A microelectrode measured the voltage inside the muscle cell with respect to the outside. A Maclab and its associated equipment along with the Scope 3.0 program were used to measure and record these potentials. Artificial voltage stimuli were sent across the electrode to test for resistance, and if the electrode measured a resistance of $<10M\Omega$, it was probable that the electrode's tip was broken and had to be discarded. If the resistance was too high $>103M\Omega$, it meant that there could be bubbles in the electrode or that the tip was pulled too far. Resistance was checked every other minute to verify the integrity of the electrode.

The stimulator was used to depolarize the cell and induce EPSPs. The frequency of our stimulus was set at 0.5 Hz and the duration of the stimulus was set at ~0.9 ms. Varying voltages were used to induce EPSPs. The delay between the stimulus artifacts was a variable in our experiment and was moved between 200, 140, 80, 40, and 20 ms. The delay value determined the amount of time between the stimuli artifacts of twin pulses.

Data Recording

We took multiple readings at each of the previously listed delays to be used as control data. We then changed solution to each of the drug baths, allowing the crayfish to adapt to the changing conditions for at least 10 minutes. Because neither of the drugs is reversible (Tocris), we changed crayfish after each test. We did multiple trials and took readings at the given delays for each of the drugs.

Data Analysis

We selected our best data for analysis (EPSP recordings with the lowest membrane potentials and the highest EPSP amplitudes). Based on these criteria, we chose data from two neuromuscular junctions from a single crayfish for each drug. The selected data was representative of the rest of our results.

We analyzed our collected data using two methods to reflect our experimental objectives. First, we analyzed our data in order to determine the effects of the drugs on EPSP amplitudes. Because of the difficulty in calculating absolute amplitude values with varying membrane potentials, we used a correction formula in order to standardize our results and make our comparisons. Our formula created a ratio between the amplitude of an EPSP and the given resting potential of that EPSP. We called this ratio the "amplitude factor." The formula for calculating the amplitude factor was:

$$\frac{(\text{amplitude of EPSP1})}{(10 \text{ mV} + \text{membrane potential})}$$

We then compared the amplitude factors of EPSPs generated using normal Ringer's solution and the drug bath saline solutions. Our second data analysis consisted of calculating the percent change in synaptic facilitation at each of the given delays. The formula used to perform this calculation was:

We used this formula to compare the percentage change in facilitation at the given delays in normal Ringer's saline solution and in the drug bath saline solution.

RESULTS

The objective of our experiment was to examine the effects of altering the function of the $Ca^{2+}/ATPase$ pump in the E.R. on synaptic transmission and facilitation. We used twin pulse stimulation to generate EPSPs and examined that data to compare amplitudes and changes in twin pulse facilitation at different inter-pulse intervals.

Our data for Ochratoxin A and BHQ were obtained by examining the best recordings of EPSP amplitudes and changes in synaptic facilitation from two neuromuscular junctions in a crayfish. The rest of our results were representative of this selected data. Because our drugs were not reversible, we used different crayfish for each drug.

We found that with the use of 0.1 mM Ochratoxin A, the pump enhancing drug, the amplitude factor (the absolute amplitude, corrected for changes in membrane potential) decreased from 0.095 for the control to 0.054. A greater amplitude factor represents a greater distance between the peak of an EPSP and its respective resting potential, and thus, a larger EPSP. With 0.1 mM BHQ, the pump inhibiting drug, the amplitude factor increased from 0.116 for the control to 0.168. This represents a 43.2% decrease in amplitude for Ochratoxin A and a 44.8% increase in amplitude for BHQ. (Figure 1) Our results show that Ochratoxin A, by increasing the amount of Ca^{2+} removed by the pump, has a significant depressive effect on the amplitudes of EPSPs, while BHQ, having the opposite effect on the pump, considerably inflates the amplitudes of EPSPs.



Figure 1. Amplitude Factors for Ochratoxin A and BHQ vs. Normals with Corrections for Membrane Potentials. Each bar represents the amplitude factor for the given test conditions. The error bars represent the standard deviation of our average amplitude factor values. The data for Ochratoxin A was obtained from two neuromuscular junctions in one crayfish. The data for BHQ was also collected from two neuromuscular junctions from a different crayfish.

Ochratoxin A caused a decrease in synaptic facilitation. As delay increased, facilitation decreased, showing an inverse relationship between the delay and facilitation. (See Figure 2). At the highest delay, 200 (ms), facilitation was 6.3% for Ochratoxin A and 14.8% under normal conditions. This shows that at delay 200 (ms) there was a 57.5% percent decrease in facilitations comparing normal solution and Ochratoxin A. At the lowest delay, 20 (ms), facilitation increased 31.4% for Ochratoxin A and 56% under normal conditions. This shows that at delay 20 (ms) there was a 44% decrease in facilitations. The average decrease of facilitation for all the times was 55% with a S.E. of 2.8.



Figure 2. Percent Change in Facilitation of Ochratoxin A vs. Normal at varying Twin Pulse Delays. This graph represents a plot of percent change in EPSP amplitude as a function of twin pulse delay. The lines represent a fitted exponential curve to the respective scatter plot data. This data represents two neuromuscular junctions in one crayfish for both control and drug readings.



Figure 3. Percent Change in Facilitation of BHQ vs. Normal at varying Twin Pulse Delays. This graph represents a plot of percent change in EPSP amplitude as a function of twin pulse delay. The lines represent a fitted exponential curve to the respective scatter plot data. This data represents two neuromuscular junctions in one crayfish for both control and drug readings.

BHQ caused an increase in synaptic facilitation. As delay increased, the facilitation decreased, again showing the inverse relationship between the delay and facilitation (See Figure 3). At the highest delay, 200 (ms), facilitation was 15.7% for BHQ and 15.3% under normal conditions. This indicates that at delay 200 (ms) there was a 3.1% percent increase in facilitation when using normal solution and BHQ. At the lowest delay, 20 (ms), facilitation was 105% for BHQ and 53% under normal conditions. This shows that at 20 (ms) delay, there was an increase of facilitation of 100%. The average increase of facilitations for all delays was 29% with an S.E. of 18.

The inverse relationship between the delays of the twin pulses and facilitation can be observed with both Ochratoxin A and BHQ. As the time interval between twin pulses increased, facilitation decreased in an inverse relationship that can be seen in the fitted exponential lines of each figure. The significant difference between the effects of the drugs is seen by examining the percent change of facilitation between the drugs and their respective Normals. Ochratoxin A has a depressive effect and BHQ has an augmenting effect on facilitation. Both drugs had a similar effect on facilitation as they had on EPSP amplitudes.

DISCUSSION

We examined the effects of altering the function of the Ca²⁺/ATPase pump in the E.R.. We discovered that enhancing the pump, using Ochratoxin A, resulted in a 43% decrease in EPSP amplitudes and an average decrease in synaptic facilitation of 55% with a standard error of ± 2.78 . Inhibiting the pump with BHQ resulted in a 44.8% increase in EPSP amplitudes and an average increase in synaptic facilitation of 29.3% with an S.E. of ± 17.7 .

These results indicate several things about the E.R. $Ca^{2+}/ATPase$ pump. We can now state that the pump plays a considerable role in the removal of Ca^{2+} . The general increases and decreases of amplitude factors clearly support our hypothesis that when the functioning of the E.R. Ca^{2+} pump is altered, the amplitudes of EPSPs would be changed in a measurable way. Furthermore, our hypothesis about synaptic facilitation also holds true: when the pump is enhanced, synaptic facilitation showed a marked decrease, and vice versa.

Both of these results support our hypothesis. Our drugs were designed to target only the Ca^{2+} removing mechanism in the E.R.. We can thus infer that when the pump is enhanced, there is a lower concentration of Ca^{2+} in the presynaptic nerve terminal and less neurotransmitter will be released, rendering a smaller EPSP. The opposite is true of inhibiting the pump.

Our results also support work done in 2002 by Casado et al, who showed that depleted calcium stores lead to lowered EPSP amplitudes. With the pump's functioning altering the calcium levels in the cell, EPSP amplitudes were similarly affected.

The residual Ca²⁺ hypothesis states that Ca²⁺ levels in the cell are the cause of synaptic facilitation (Correspondence with Lindgren 2004). Using this information, we can determine that the rate at which the E.R. pump removes Ca²⁺ from the cell has a significant impact on synaptic facilitation. The findings on amplitude factors indicate that the Ca^{2+} level in the cell changes when the E.R. pump is altered, and because of these Ca^{2+} levels, synaptic facilitation will be altered as well. Our findings are similar to work done by Jeon in 2003, who found that inhibiting the sodium/Ca²⁺ exchanger resulted in increased synaptic facilitation and thus higher learning capacity. If Jeon's findings are correct, then we could theorize that in an organism whose E.R. $Ca^{2+}/ATPase$ pump is inhibited, learning capacity could also potentially increase. Bardo et all (2002) found that altering the internal Ca²⁺ stores could lead to changes in neurotransmitter release. Our results support these findings. When the rate of Ca^{2+} removal is adjusted, thereby altering the internal Ca^{2+} , we found changes in EPSP amplitudes, which are directly linked to neurotransmitter release (Campbell).

There are several phenomena in our data that would require future research to explain. The disparity between the standard errors of facilitation change at various times between BHQ and Ochratoxin A is troubling. Ochratoxin A maintains a level of change in synaptic facilitation that is relatively undisturbed by changing the delay between the twin pulses. However, BHO increased synaptic facilitation 105% at 20 (ms) delay, an enormous change, but averaged only a 25.5% increase over the next 4 delay readings, and culminated with only a 3.05% increase at 200 (ms) delay. These results mean that when the pump is inhibited, the cell adjusts its Ca²⁺ removal rate back to normal much faster than when the pump is enhanced. One explanation for this phenomenon: It is possible that when the E.R. pump is inhibited by BHQ, the other Ca^{2+} removal mechanisms begin to work harder to make up the slack and by 200 (ms), the 3 other mechanisms have virtually made up for the fact that the E.R. has slowed its removal of Ca²⁺. Following this thought, it could be that the other three mechanisms do not change their functioning when the pump is enhanced, thus explaining the constant decrease in facilitation.

Another unknown is how exactly these chemicals affect the workings of the pump. They could be having a heretofore undiscovered effect on the functioning of one of the other mechanisms. Future research on the role of the E.R. pump would have to include a wider variety of drugs, all of whom have the effect of enhancing and inhibiting the pump.

Building on this research done on the E.R. pump, we would like to investigate the role of the other four mechanisms in the future. Determining the relative significance of each of the removal mechanisms could provide a working knowledge base that could be used in research dealing with synaptic transmission and learning capacity, or even nerve related diseases and conditions.

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