Ca$^{2+}$ ATP-ase in the Endoplasmic Reticulum has Little Effect on Synaptic Plasticity Following Tetanic Stimulation

BRYAN BERUBE and ANN MCCULLOUGH
Department of Biology, Grinnell College, Grinnell, Iowa

ABSTRACT

Since internal calcium levels influence synaptic transmission, changing the amount of calcium uptake by internal organelles such as the endoplasmic reticulum (ER) should change the magnitude of EPSP amplitudes, a measurement of synaptic transmission. We changed the amount of calcium uptake by the ER by adding two different chemicals that had opposite effects on the Ca$^{2+}$ ATP-ase pump in the ER. In the first experiment, we added the drug, Ochratoxin A, which enhanced this pump, causing an increase in calcium uptake. In the second experiment, we added the drug, BHQ, which inhibited this pump, causing a decrease in calcium uptake. We then compared the percent change in amplitude of EPSPs following tetanic stimulation for each chemical. We expected that Ochratoxin A would decrease the percent change in post-tetanic EPSP amplitude, while BHQ would increase the percent change. However, we found that both chemicals caused an increase in percent change in post-tetanic EPSP amplitude, though neither test displayed results that were significantly different from trials without the addition of either chemical (p<0.05).

INTRODUCTION

Neurons communicate with other cells through the process of synaptic transmission. When an action potential reaches the synaptic terminal, depolarization causes calcium channels to open, allowing calcium into the cell, causing synaptic vesicles to fuse with the cell membrane and release neurotransmitters into the synaptic cleft. These neurotransmitters then bind to receptors on the membrane of the postsynaptic cell, and cause the cell to either depolarize or hyperpolarize. In the crayfish, the release of glutamate causes a depolarization of the postsynaptic muscle cell.

The amount of internal calcium can alter synaptic release, and therefore plays an important role in synaptic plasticity. An increase in internal calcium causes more neurotransmitter to be released, creating a greater depolarization in the postsynaptic cell. Likewise, a decrease in internal calcium causes a reduction in neurotransmitter release, creating a smaller depolarization in the postsynaptic cell (Kamiya, 1994). Immediately after calcium triggers neurotransmitter release, the calcium diffuses away from the presynaptic release site, thus terminating the nerve signal. The calcium is then either extruded from the cell by exchangers in the cell membrane, or is sequestered by Ca$^{2+}$ ATP-ase pumps in the mitochondria or endoplasmic reticulum (ER).

Previous experiments have shown that the accumulation of calcium can be influenced by the modification of these calcium transporters in the presynaptic cell. Studies testing the effects of blocking these transporters demonstrated their importance on synaptic plasticity. In one study, researchers removed the sodium-calcium exchanger from cells in mice through genetic engineering, and discovered an enhanced ability in learning and memory (Jeon, 2003). This study suggested that since such a modification provided fewer means for calcium to leave the presynaptic cell, more calcium remained in the synaptic terminal. The enhanced facilitation was not only evident in standard tests of learning and memory, but was further enhanced by tetanic stimulation, the rapid repetition of electrical impulses in the pre-synaptic cell. Such stimulation caused an accumulation of calcium in the pre-synaptic cell, because the influx of calcium was greater than the efflux of calcium from dispersion and the various ion pumps.

Calcium exchangers in organelles within the cell can also influence the amount of calcium that accumulates in the pre-synaptic cell. Experiments have shown that the inhibition of the calcium pumps in the mitochondria enhanced post-tetanic facilitation (Tang, 1997). This same study, however, found that when a similar calcium pump in the ER was inhibited, no effect on synaptic plasticity was observed. In our study, we explored whether this is truly the case, and attempted to discover in what way the ER affects synaptic plasticity.

By learning the role of calcium up-take by the ER in synaptic transmission, we hoped to gain insight into which internal mechanism has the strongest impact on internal calcium levels. Ultimately, results
from this experiment, as well as those from similar studies, could be used to better understand how learning and memory works in organisms. Since synaptic facilitation is one of the processes responsible for learning and memory, it may be possible to use knowledge about the different effects that chemicals have on this process to help patients suffering from diseases such as Alzheimer’s that reduce learning and memory capabilities.

We studied neurotransmitter release at the crayfish neuromuscular junction, and used intracellular recording to observe changes in excitatory postsynaptic potentials (EPSPs), which is a measure of synaptic transmission. The crayfish neuromuscular junction was an ideal model for studying synaptic transmission. Since glutamate is the neurotransmitter that is released in both the crayfish neuromuscular junction and the central nervous system of mammals, the structures of the pre-synaptic and postsynaptic sites for both organisms are similar. Therefore, results from experiments done on the crayfish neuromuscular junction should be similar to results expected at synapses in the central nervous system of mammals. Additionally, the dissection of the crayfish is easier than mammalian dissection, and the axons of neurons in the crayfish tail are larger than those in the mammalian central nervous system, allowing for more speed and accuracy in measurements.

To study the role of the ER in synaptic transmission, we compared the effects of two different drugs. The first drug, Ochratoxin A, enhanced the Ca\(^{2+}\) ATP-ase pumps in the ER, increasing the up-take of calcium. The second drug, 2,5-di-(t-butyl)-1,4-hydroquinone (BHQ), inhibited the Ca\(^{2+}\) ATP-ase pumps in the ER, decreasing the up-take of calcium. We expected that, since the addition of Ochratoxin A should decrease the amount of calcium in the cytoplasm, it would decrease synaptic transmission, resulting in smaller EPSPs. In contrast, we expected that the addition of BHQ, since it should increase the amount of calcium in the cytoplasm, would increase synaptic transmission, resulting in larger EPSPs.

We found that the addition of Ochratoxin A resulted in larger EPSP amplitudes, rather than the hypothesized decrease in amplitude. We did obtain the expected increase in EPSP amplitude with the addition of BHQ. These results suggest that the Ca\(^{2+}\) ATP-ase pumps in the ER do not have as direct an impact on synaptic transmission as was expected.

**MATERIALS AND METHODS**

**Preparation of Extensor Muscle Cells of Crayfish**

The crayfish used in our experiments were placed in an ice bath ten to fifteen minutes before dissection. This procedure makes dissection easier because the colder environment slows the reaction time of the crayfish. To dissect the tail, we cut along the sides of the tail and peeled back the ventral portion so that only the extensor muscle cells remained attached to the exoskeleton. We then pinned the tail to a Petri dish filled with crayfish saline. We started each experiment by using standard crayfish saline (Table 1), which we replaced with fresh saline every fifteen minutes to ensure that regular environmental conditions were maintained.

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>5.4</td>
</tr>
<tr>
<td>NaCl</td>
<td>205</td>
</tr>
<tr>
<td>MgCl(_2) (\cdot) 6H(_2)O</td>
<td>9.35</td>
</tr>
<tr>
<td>NaHCO(_3)</td>
<td>2.3</td>
</tr>
<tr>
<td>Dextrose</td>
<td>2.0</td>
</tr>
<tr>
<td>CaCl(_2) (\cdot) 2H(_2)O</td>
<td>6.75</td>
</tr>
<tr>
<td>pH is adjusted to 7.4</td>
<td></td>
</tr>
</tbody>
</table>

*Table 1. Concentrations of Components in Standard Crayfish Saline Solutions*

**Preparation of Electrodes**

Using a PUL-1 (World Precision Instruments) electrode puller, glass capillary tubes were pulled to create micropipettes with fine tips measuring less than 0.1\(\mu\)m. We filled the micropipette and the micropipette holder with a 3M KCl solution to carry the charge (Akaike 1973), and ensured that there were no air bubbles, which would interfere with the flow of the electrical impulse. When inserting this microelectrode into the holder, we left a meniscus of KCl on the holder to eliminate the possibility of air bubbles entering the microelectrode. We used the same electrode puller to create suction electrodes. After pulling micropipettes, we broke and sanded the tips, using Emory paper, to create an opening through which a nerve could fit with a tight seal. We adjusted the micromanipulators to ensure that the angle of insertion of the microelectrode into the cell was as vertical as possible.

**Measurement of Excitatory Post-Synaptic Potentials of Extensor Muscle Cells Using Intracellular Recording**

Before penetrating the cell, we lowered the microelectrode into the saline to determine and eliminate the junction potential between the microelectrode and two reference electrodes, which were also inserted into the saline (Akaike 1973). The resistance of the microelectrode was tested and maintained at a range of 10-20 M\(\Omega\). After locating a nerve along the edge of the exoskeleton, the suction electrode was used to capture a nerve for stimulation. We then inserted the microelectrode into the lateral
bundle of fast extensor muscles close to the nerve in order to measure the EPSP following the stimulation of the nerve by the Grass SD9 Stimulator.

Tetanic Stimulation of Extensor Muscle Cells
A stimulus of four volts was applied to the nerve at a frequency of 0.5 hertz to measure and record several EPSPs prior to tetanic stimulation. These measurements were the basis on which the results following tetanic stimulation were compared. Without changing the voltage, we then increased the frequency of the stimulus to 20 hertz for a duration of 10 seconds, after which we returned the frequency to 0.5 hertz. Directly following the tetanic stimulation we recorded EPSPs at a two second interval until the amplitude of the EPSP appeared to decrease at a slower rate. Measurements were then taken every minute until the amplitude of the EPSP returned to measurements prior to tetanic stimulation. Once the amplitudes reached these levels, the microelectrode was removed and inserted into another cell, and the same procedure was carried out.

Addition of Chemicals
After performing several control experiments with standard crayfish saline, we modified the saline by adding the chemicals. For each experiment, we added a chemical to the saline at a concentration of 0.1 μM. The crayfish remained in the new saline for ten minutes prior to experimentation, so that the chemical would have time to take affect. The same procedure as above was used to measure the EPSPs, with several trials being taken in different cells.

Analysis of Data
After measuring the amplitudes of the EPSPs, we calculated the percent change in EPSP amplitude by subtracting the average pre-tetanic EPSP amplitude from the post-tetanic EPSP amplitude and dividing by the pre-tetanic EPSP amplitude. To compare this percent change with the addition of the drug to that of the control, we obtained a ratio of these two percent changes. Different crayfish appeared to have different reactions to tetanic stimulation and to the chemicals. To account for the differences between individual crayfish, we used a paired t-test. We used the ratio of the experimental percent change to the control percent change and compared this to a value of 1, which would be the value of the ratio if there was no difference. This negated variance between different crayfish and looked only at the change due to the chemicals.

RESULTS
Since internal cellular calcium affects synaptic transmission, we expected that when the ER removes more calcium from the cytoplasm, a decrease in synaptic transmission would occur. On the other hand, when the ER is prevented from removing the calcium, synaptic transmission would increase. Under conditions of elevated calcium, such as following tetanic stimulation, these changes in amplitude should be exaggerated.

We applied two drugs that affected the calcium uptake of the ER. The first, Ochratoxin A, enhanced the Ca²⁺ ATP-ase pump in the membrane of the ER, thereby decreasing internal calcium. The second chemical, BHQ inhibited the pump, thus increasing the amount of calcium that accumulated in the cytoplasm.

Since we were mainly interested in the impact these drugs had on synaptic plasticity, we tested their effects following tetanic stimulation. Tetanic stimulation, a brief stimulation at a high frequency, caused a buildup of internal calcium, which led to an increase in the magnitude of EPSPs (Figure 1).

![Figure 1. EPSP amplitude before and after tetanic stimulation.](image)

Following the stimulus, shown in this model as a sharp increase and subsequent decrease, the post-tetanic amplitude is greater than the pre-tetanic amplitude.

After applying Ochratoxin A, we expected to observe less of an increase in post-tetanic EPSP amplitude. However, when calculating the percent change, we observed that Ochratoxin A caused a greater increase (figure 2). To compare this percent change with the addition of Ochratoxin A to that of the control, we obtained a ratio of these two percent changes \( \frac{\text{Post-Tetanic}}{\text{Pre-Tetanic}} \).

Using a two-tailed paired t-test, we found that the increase in EPSP amplitude with Ochratoxin A was close to significant (\( p=0.058 \)), but less than the normally accepted significance level (\( p<0.05 \)). Furthermore,
the effects of Ochratoxin A did not appear to be reversible, though we did not collect enough data to test this mathematically.

When we added BHQ to the crayfish saline, we expected to observe a greater increase in post-tetanic EPSP amplitude. By obtaining a ratio of the average change in EPSP amplitude with the addition of BHQ and without any drug, we found that BHQ did increase the EPSP amplitude (\( \bar{x} \pm SE = 2.2 \pm 1.1, n = 5 \)) (figure 3). This increase was also close to significant (\( p=0.063 \)). Furthermore, BHQ did not display any reversibility. The percent changes in amplitude remained elevated after the crayfish was rinsed with standard saline.

DISCUSSION

In the work reported here, we hoped to study the endoplasmic reticulum’s role in synaptic transmission by measuring EPSP amplitudes following tetanic stimulation. These magnitudes are an indicator of internal calcium accumulation in the pre-synaptic cell because higher amounts of internal calcium release more neurotransmitters, causing an increase in post-synaptic EPSP amplitude.

To study the role of the ER, we added two chemicals that normally have opposite effects on the Ca\(^{2+}\) ATP-ase pump in the membrane of the ER. The first drug, Ochratoxin A, enhanced this pump, thereby removing more calcium from the cytoplasm, leaving less calcium available to aid in the release of neurotransmitters. Thus, we predicted a decrease in the amplitude of post-synaptic EPSPs. The second drug, BHQ, has been shown to block the Ca\(^{2+}\) ATP-ase pump in the ER (Takami 1997), causing an increase in calcium accumulation, which should lead to more neurotransmitters being released and a subsequent increase in post-synaptic EPSP amplitude.

By measuring post-tetanic EPSP amplitude, we were also able to study the effects of the ER on synaptic plasticity. Contrary to our hypothesis, Ochratoxin A showed no decrease in post-tetanic EPSP amplitude, and in fact seemed to increase the amplitude. However, t-tests revealed that these observations were not statistically significant. These results differed from previous experiments, in which Ochratoxin A caused a significant increase in the Ca\(^{2+}\) ATP-ase pump of the rat renal cortex for up to six hours (Chong 1992).

There are several possibilities for why our results with Ochratoxin A differed so greatly from our hypothesis. First, we may have used faulty chemicals. However, other researchers in our laboratory used chemicals from the same batch and preliminary observations suggested that the chemical did in fact have the expected results. Second, the tetanic stimulation that the crayfish underwent before we added Ochratoxin A could have resulted in an accumulation of internal calcium that could have influenced our results. We could not test this possibility, because we found that Ochratoxin A is not reversible, so we would not have anything to compare to unless the control was done prior to the addition of the chemical.

Although t-tests comparing the percent change in amplitude of post-tetanic EPSPs with the addition of BHQ to the control did not display significance (\( P<0.05 \)), our results do suggest that further studies may show significance. We did experience an increase in percent change for every trial, but our lack of significance could be due to our high variability between trials. However, other studies have also shown that the addition of BHQ does not significantly affect synaptic transmission (Tang 1997).

Our results suggest that the endoplasmic
Ca\textsuperscript{2+} ATP-ASE IN THE E.R. FOLLOWING TETANIC STIMULATION

The role of the endoplasmic reticulum in synaptic transmission is discussed. However, the lack of statistical significance implies that other means of calcium exchange may play a more important role. Furthermore, BHQ appears to have a stronger effect on the Ca\textsuperscript{2+} ATP-ase pump than Ochratoxin A. These findings could mean that the Ca\textsuperscript{2+} ATP-ase pump is more easily inhibited than enhanced. Future experiments should be done to determine if this is indeed the case and if there is a physiological advantage for neurotransmitter release to be more easily increased than decreased.

ACKNOWLEDGEMENTS

We thank our professor, Clark Lindgren, and our lab assistant, Sue Kolbe, for their assistance in the laboratory and out. We thank Priya Malik and Laura Dobbs for aiding us in our times of need. We would also like to especially thank Tirza Costello and Lilly Radoshevich for listening to our constant complaining. Lastly we would like to thank the maker of the Mac computer for making us do a great deal of our work not once, but twice. Lastly, we would like to acknowledge Bryan Berube and Ann McCullough for all their hard work and dedication to this experiment.

REFERENCES


