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BHQ, heparin sodium reduces or eliminates EPSPs at crayfish neuromuscular junctions

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ABSTRACT

Internal calcium stores have been shown to have an effect on synaptic transmission. Our objective in this experiment was to see how the depletion or inhibition of internal calcium stores would affect synaptic transmission. We added two drugs to our crayfish preparation: Butylhydroquinone, a food preservative, and sodium heparin, a common anticoagulant. We used Butylhydroquinone (BHQ) to inhibit the sarcoplasmic-endoplasmic calcium ATPase (SERCA) pumps on the endoplasmic reticulum (ER) to deplete the internal Calcium stores in the cell. We blocked the IP_3 receptors on the ER that are responsible for releasing calcium using sodium heparin. We used BHQ and heparin together to see how their combined effects would affect the EPSPs. Finally, we washed the BHQ and heparin off the crayfish and took EPSP recordings to determine if a calcium rebound occurs. We found that both BHQ and heparin decrease EPSPs. When used together, the drugs decrease the amplitude as well. We were unable to demonstrate a calcium rebound into the ER.

INTRODUCTION

Discovering more about internal calcium stores' role in the cell is important because synaptic transmission plays a large role in the development of medicines and chemicals. If we can discover more about how internal calcium stores affect synaptic transmission, then the knowledge can be applied to gain a better understanding about how medicines can help to cure or aid maladies, especially those that occur because of miscommunication or the lack of communication between neurons in the brain.

Organelles such as the endoplasmic reticulum (ER) serve as calcium storage compartments for the cell. Neurons use the ER to store calcium to be used during synaptic transmission. The ER has many types of receptors, two of which are ryanodine and inositol 1, 4, 5-triphosphate receptors (RyRs and IP_3Rs) that regulate the release of calcium from the ER. There is also a pump that takes in calcium into the ER from the cytoplasm known as the sarcoplasmic-endoplasmic calcium ATPases (SERCA). Once an action potential travels down the axon, it causes a depolarization in the presynaptic terminal. This depolarization causes an influx of calcium through voltage-gated calcium channels (VGCCs,) which can lead to calcium-induced calcium release (CICR) from the ER. The Ca^{2+} ions that flow through the VGCCs in the cell membrane activate the VGCCs on the ER which then allow for the release of further Ca^{2+} (Collin, et al. 2005).

The role of the IP_3Rs is a little more difficult to pinpoint because of the lack of a chemical that can inhibit IP_3 without affecting the RyRs. When a G protein is activated, it then activates phospholipase C, produces IP_3 and diacylglycerol (DAG) through the process of hydrolysis with phosphatidyl inositol-bisphosphate (PIP_2). Inhibiting this process with heparin can activate the RyRs, meaning that using heparin can actually cause an increase in calcium. Scientists have found evidence to support the role of IP_3Rs in synaptic transmission when using xestospongin C, an IP_3 inhibitor (Cong Y-L, et al. 2004).

Studies have shown that certain drugs like thapsigargin and caffeine block the SERCAs in the ER, which blocks the re-intake of calcium into the ER. When stimulated repeatedly, this can deplete the internal calcium stores. At first, this can result in increased EPSPs, but over time, the EPSPs become nearly obsolete (Krizaj D et al. 2003).

We used Butylhydroquinone (BHQ), a reversible, common food preservative, to block the re-intake of calcium into the ER by blocking the SERCAs. We used sodium heparin to inhibit IP_3 to see if it activated the RyRs on crayfish ERs.

The affect of reintroduction to calcium after internal storage depletion has been studied sparingly. We hypothesized that when we reintroduced our specimen to normal saline solution that the internal stores would experience a rebound. We thought this would cause a delay in the normalization of the EPSPs because calcium would be flowing into the ER to make up for its depletion in our experiments. We thought that this would take time. In Krizaj's experiment, it took 84±4 minutes for the calcium levels to return to their normal state after they depleted the cells of their internal stores using caffeine (2003).

Also, little is known about what happens when both IP_3Rs and SERCAs are blocked. We intended to see how the effects of applying both BHQ and heparin affect the EPSPs. It has been shown that both decrease EPSPs in other organisms (Saliares, 2007; Collin, 2005). We thought that the addition of both BHQ and heparin to our preparation would more effectively decrease the amplitude of EPSPs in the crayfish. We measured the effectiveness of our chemicals in terms of the biggest decrease (i.e. the biggest decrease will be the most effective). We also thought that adding heparin to the BHQ solution would reduce the increase in EPSPs that result from blocking the SERCA pumps.

In our experiment, we found that both BHQ and heparin decrease EPSPs significantly. The EPSPs did increase after we washed off the chemicals, but they did not return to normal levels. This suggests either that the chemicals have a more permanent affect on the crayfish neuromuscular junction or we did not give the drugs enough time to wear off completely. We were not able to conclusively prove if there was a calcium rebound into the internal stores after we washed off our chemicals for the same reason. When using BHQ and heparin, we found that the EPSPs were significantly decreased as well. However, we did not find that this was due to the combination of BHQ and heparin; it could be due to one chemical's effects more than the other's.

MATERIALS AND METHODS

Preparation

The crayfish were provided by Carolina Biologicals, a company based in North Carolina, and were of the species *Procambarus clarkii*. The superficial extensor muscle cells we used were dissected by cutting off the tail from the body (as close to the body as possible), cutting along the ridge that runs parallel to the length of the tail (closer to the ventral side), and pushing off the muscle tissue and organs to reveal the long extensor muscles running along the dorsal side of the tail. We then pinned the tail on the anterior and posterior ends onto a silicon layer in a dish. We filled with crayfish solution (directions below) that was pH of 7.4, until the muscle was completely submerged in the solution.

Solutions and Chemicals

The control solution we used was a modified Ringer's solution made by mixing 5.4 mM KCl, 196 mM NaCl, 2.6mM $MgCl_2$ * $6H_2O$, 10mM Sodium Hepes Buffer, and 13.5mM $CaCl_2$ * $2H_2O$. It was mixed 24 hours prior to use and has a pH of 7.4.

We used 10 µM dilutions of butylhydroquinone and heparin sodium, made by mixing 10 mM concentrations of both in 100 mL of our control solution.

Instruments and Set Up

The microelectrodes were made by pulling 2 mm glass pipette tubes using a microelectrode puller. We aligned the pipette in the grooves in the rotaries and then heated the center for approximately 7 seconds. We then filled them with 3M KCl solution and dipped the tips into a saline solution. The resistance of the electrodes was approximately 10 M ohms.

We measured our membrane potentials by using a voltage amplifier that was connected to the computer, and used the program Scope and its function, Bridge Amplifier, to record the changes in voltage. We stuck the ground wire in the solution with dental wax. We outfitted the amplifier with a microelectrode that we made earlier, using the electrode holder as a bridge between the amplifier and the microelectrode.

To create EPSPs, we used a Grass Stimulator and a suction electrode to send an electrical impulse to the nerves. Using our suction electrode and microscope, we pinpointed the location of a nerve on the crayfish tail and used the suction electrode to suck it up. We then used the Grass Stimulator to send an electrical pulse to the nerve. We used Scope again to monitor and record the resulting EPSPs. Our stimulus ranged from 3-10 x 10 mV.

RESULTS

We did three different experiments: (1) to test if BHQ increased and then decreased the amplitude of the EPSP, (2) if Heparin decreased the amplitude of the EPSP and (3) the effect on the EPSP's amplitude when both BHQ and Heparin are added. In every experiment we measured the EPSPs before the drug was added (baseline reading), the drug was added and finally washed off to see if there is a calcium rebound. Averaging the amplitude of the EPSP in every condition to be able to compare the differences between the different conditions gave us the data.

After introducing BHQ, the EPSP's amplitude significantly decreased (p=0.00025), as shown in Figure 1 (blue line). The percent change of the amplitude was -58.4% from the baseline reading to when BHQ is added. After we removed BHQ from the preparation, the amplitude of the EPSP increased, as shown in Figure 1 (blue line), but not significantly (p=0.12390) nor to the initial readings. The percent change of the EPSP's amplitude from BHQ to the wash off of BHQ was +31.1%.

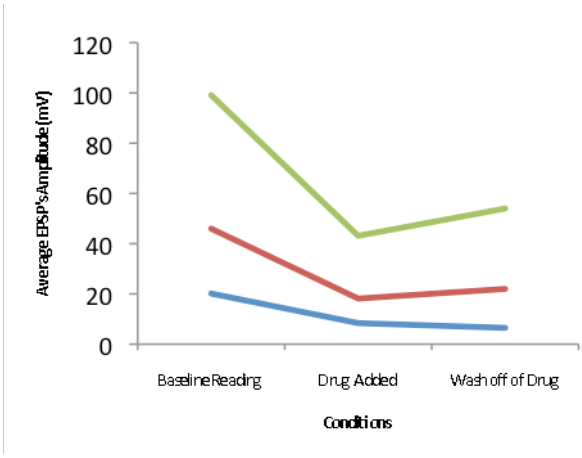

Figure 1. The Effect of Drugs on the Amplitude of the EPSPs
In the graph, the average of various measurements of EPSP's amplitude in conditions: Baseline Reading, Drug Added, and the Wash off of Drug. The green line represents the combination of Heparin and BHQ experimental group, the red line corresponds to Heparin experimental group and the blue line represents BHQ experimental group. You can see that the average amplitude when the drugs were added decreased in comparison to the baseline readings, but once the drugs were washed off, the amplitude increased again but not as high as before.

The amplitude of the EPSPs significantly decreased as predicted when we added heparin to our preparation (p=0.00028), as shown in Figure 1 (red line). The percent change was -40.7% amplitude decrease from the baseline to when heparin was introduced. After heparin was washed off of the preperation, the ampltude of the EPSP did increase but not significantly (p=0.21361) nor to initial levels, as shown in Figure 1 (red line). The percent change of increase of the EPSP's amplitude was +1.1% when heparin was washed off compared to when the preparation had heparin.

When we introduced both BHQ and Heparin, the EPSP amplitude decreased significantly (p=0.00365) as shown in Figure 1 (green line). The percent change was -53.0% decrease in amplitude when both the drugs were added from the original readings. After washing both BHQ and Heparin off of the crayfish, the amplitude of the EPSPs increased, but not significantly (p=0.06927) nor to the initial readings, as shown in Figure 1 (green line). The percent change was +22.0% increase in amplitude from when the drugs were in effect to when the drugs were washed off of the crayfish.

In all three experiments, we found what we predicted would happen, but we did not catch the increase of amplitude when BHQ was added. After the drugs were washed off, there was an increase in EPSP amplitude, but not significantly, based on α=.05.

DISCUSSION

We utilized both heparin and BHQ in the investigation of the effects of internal calcium stores on EPSPs in a post-synaptic cell, namely the crayfish muscle cell.

BHQ was predicted to block the re-intake of Ca^{+2} in the ER by blocking the SERCA pumps that are responsible for the circulation of Ca^{+2} ions in and out of the ER. By doing so, the concentration of calcium in the ER would decrease and would not reach initial levels even after providing the muscle cell with the initial amount of calcium (Krizaj et al. 2003). The concentration of calcium in the cell would be higher in the beginning, but it would decrease in the process since the ER would be depleted of Ca^{+2} and could not provide any more. With a small concentration of calcium in the pre-synaptic cell, fewer neurotransmitter molecules would be released, leading to a reduced EPSP recording at the post-synaptic cell (Saliares et al. 2007).

The data in Figure 1 represents the drop of EPSPs in the post-synaptic cell after the crayfish is provided with BHQ depleted in standard saline in comparison with plain standard saline. The results are consistent with those of previous experiments on crayfish (Saliares et al. 2007.) The increase of EPSPs following the wash off of BHQ proves the temporary effect of BHQ on the SERCA pumps. Also, it illustrates the re-intake of Ca^{+2} in the ER. The EPSPs' amplitude will continue to increase and approach that of the initial recording over time. However, we don't know if this is because of the lingering effects of BHQ after it was washed off or if it is because of a calcium rebound into the ER.

Heparin was predicted to actively prevent the release of the calcium from the endoplasmic reticulum by binding to the IP_3 receptor sites and inhibiting the conformational change necessary for the release. In so doing, there would be less calcium in the pre-synaptic cell to bind to synaptotagmin and cause the release of neurotransmitter molecules. Less neurotransmitter molecules would be directly proportional to the observed reduced EPSP reading.

Comparably, to an experiment carried out by Nilsson (1988) with pancreatic B cells where heparin was utilized to investigate the effect of said drug on IP_3-dependent calcium release, it is indicated that IP_3 is not the only substance that will cause the release of ER calcium stores. However, with the inhibition of IP_3 channels a noticeable decrease in EPSP's will be observed.

The data in Figure 1 illustrates that in comparison to a saline solution without heparin EPSP's were at a contrastingly higher level than a solution containing heparin. The increase of EPSPs following the washing off of heparin illustrates further that internal calcium does indeed affect EPSP levels, although due to P-value calculation, this increase cannot conclusively support the observed increase. The fact that the washing off of

heparin does not restore EPSP levels to former levels in depicted results are compounded by multiple variables. These are namely the possibilities that crayfish muscle cells over time die and lose the ability to produce EPSPs of the same levels, heparin may not have been adequately removed from cells, or insufficient time was given to allow IP3 receptors to be able to resume function

In our opinion, further research on the calcium stores of the endoplasmic reticulum is important for the better understanding of how neuromuscular junctions function. The effects of BHQ and heparin on the ER can further be investigated so to get a better understanding of how they work. Also, the combined effect of these drugs should be researched, since our experiment has not fully clarified what happens when SERCA pumps and IP3 receptors are blocked simultaneously. The understanding of how the function of the neuromuscular junction is inhibited is important for contemporary medicine since it can help against maladies that affect the nervous system.

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