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P/Q-type Calcium Channels Contribute to the Release of Neurotransmitters when N-type Calcium Channels are Inhibited in the Crayfish Neuromuscular Junction.

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ABSTRACT

Neurotransmitter release is caused by an influx of calcium through voltage gated calcium channels in the presynaptic terminal. To learn more about voltage gated calcium channels, we applied Omega-Conotoxin GVIA solution to the crayfish neuromuscular junction. With this toxin, we were able to block one type of voltage-dependant calcium channel, the N-type calcium channel. We predicted that the excitatory post synaptic potential (EPSP) amplitude would decrease, but not disappear completely, because of the compensation of calcium ion influx by the P/Q-type calcium channels. We found that the EPSP amplitude decreased significantly, recovered slightly, and then gradually decreased again following the application of Omega-Conotoxin GVIA solution.

INTRODUCTION

There are several different types of voltage gated calcium channels. The six main types are: L, N, P, Q, R, and T, although the predominant types used for neurotransmitter release are N, P, and Q; N-type channels are responsible for most of the excitatory transmitter release before maturity of the organism, whereas P/Q type channels are responsible for most of the excitatory transmitter release later in life (Reid, Bekkers, & Clements, 2003).

There have been many experiments done to determine the functions of calcium channels. In one experiment, it was found that, with the elimination of P/Q type channels, N type channels were able to make up for the loss of the P/Q type channels (Urbano, Piedras-Renteria, Jun, Shin, Uchitel, and Tsien, 2003). This was found by first genetically altering mice to be born without P/Q-type channels. Next, a chemical was used to inhibit the N-type calcium channels. When combining the two calcium influxes, they found that the N-type calcium channels were doing more than 100% of their normal amount of work, indicating that the N-type channels were compensating for the missing P/Q-type calcium channels. By inhibiting the N-type channel with Omega-Conotoxin GVIA, we were testing to see if the P/Q-type channels were able to make up for the function normally carried out by the blocked N-type channels.

We collected several measurements of EPSP amplitudes and used this information to determine whether or not the same amount of neurotransmitters is released in the absence of N-type channels. We hypothesized that the EPSP would decrease but not disappear entirely when N-type calcium channels were inhibited because P/Q-type would still be functioning properly. Our results indicate there was, in fact, a decrease in EPSP after the application of Omega-Conotoxin GVIA solution.

MATERIALS AND METHODS

Preparation

The crayfish was kept in an ice tub for approximately 20 minutes to slow its metabolism and reduce its reaction time when the tail was removed. Holding the crayfish tail, with the ventral side facing up, we cut along the ridge on each side of the tail and removed the outer layer of the ventral side exposing the inner organs. Next, we pushed out the extra tissue, leaving only the dorsal shell and superficial extensor muscles connected to nerves which we secured with pins in a Sylgard-coated chamber containing standard Ringer solution. This standard Ringer solution consisted of 205.0 mM NaCl, 5.4 mM KCl, 13.5 mM $CaCl_2$, 2.6 mM $MgCl_2$ and 10.0 mM Tris (pH 7.4) and was

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replaced every fifteen minutes to maintain a normal functioning preparation.

Preparing the Electrodes

We used microelectrodes to measure postsynaptic activity and suction electrodes to stimulate the nerves. These two electrodes were made using an electrode pullers PUL-1 (a World Precision Instrument) to pull a glass capillary tube apart. The first microelectrode was filled with 3 M KCl and attached to a microelectrode holder, which was also filled with 3 M KCl. Both were then placed on a micromanipulator, which was connected to MacLab. The other wire, connected to the data collecting system, was immersed in the solution to ground the microelectrode setup. The suction electrode was made by starting with a microelectrode and giving it a blunt point by sanding the tip. This suction electrode was attached to a voltage stimulator.

Establishing a Control

We began the experiment by using the microscope to find a nerve. The suction electrode was used to first intake some of the Ringer solution and then the nerve. The microelectrode was then inserted into the solution and zeroed to avoid the junction potential and allow for easier data analysis. The resistance was checked to ensure that the microelectrode was working properly. The resistance was supposed to be between 4 MΩ and 20 MΩ. The tip of the microelectrode was inserted into a superficial extensor muscle cell in the same segment of the crayfish tail from which the nerve had been drawn. We stimulated the nerve at a frequency of 0.2 Hz, increasing the voltage until the nerve reached threshold. We then used the Scope program to take repetitive samples measuring the amplitude of the EPSP. The average found was used as the control EPSP amplitude for the preparation.

Applying the Toxin

Once the control had been established, we used a pipette to remove the standard Ringer solution and replaced it with Omega-Conotoxin GVIA solution obtained through Research Biochemicals International. The 0.01 µM Omega-Conotoxin GVIA solution was applied to the preparation. We again began stimulating the nerve and increased the voltage to reach threshold. We used Scope to record repetitive samples and find the average amplitude. We stimulated the nerve and took readings every five minutes (changing the solution every fifteen minutes) until the EPSP amplitude started decreasing steadily, indicating the nerve measured was exhausted.

RESULTS

We found EPSPs were impacted by application of the Omega-Conotoxin GVIA solution. As seen in Figure 1, the toxin caused a significant drop in EPSP ($p<0.05$) amplitude from the control to the smallest amplitude of each crayfish. After the initial decrease, the EPSP amplitude began to recover at a marginally significant rate ($p=0.099$). The EPSP amplitude then began to decrease again.

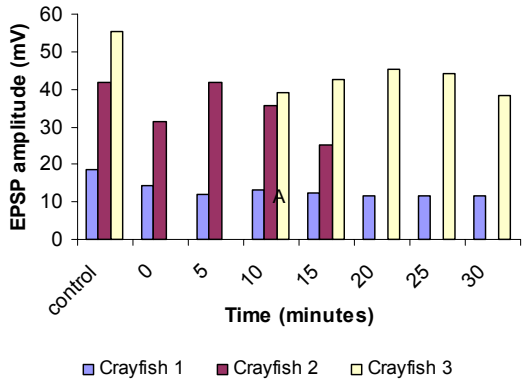

Figure 1. There was a clear drop in EPSP amplitude after initial application of Omega-Conotoxin GVIA. The EPSP amplitude slowly began to recover before decreasing again as the nerve became exhausted. Any times that all three data bars are not shown should not be interpreted as an EPSP amplitude of zero. We do not have zero and five minute recordings for the third crayfish due to difficult of collecting data at that time; that is, the lack of data for those times (as well as for the second crayfish after 15 minutes) does not indicate the EPSP amplitude was zero, just that the data was not collected. We chose not to use the means of each time for the three crayfish because we did not collect readings for each time.

As time progressed, the duration of the EPSP increased, as Figure 2 shows. The EPSP with the toxin immediately applied was 1.82 msec longer than the control EPSP (4.05 msec). The duration continued to increase over time.

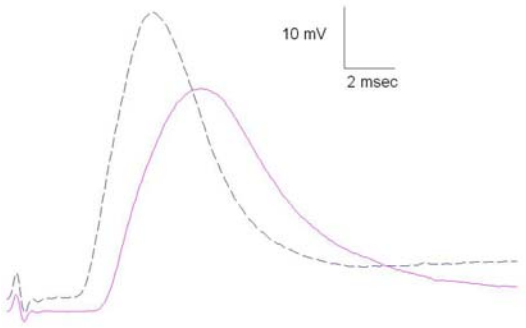

Figure 2. In the data found, there was a visible increase in duration of the EPSP measurements. Also, the reaction for the same stimulus was delayed over time. The dotted line is an EPSP measurement for the control and the solid line is an EPSP measurement immediately after the application of the Omega-Conotoxin GVIA solution.

As Figure 3 exhibits, the initial amount of neurotransmitters released dropped slightly as determined by finding the integral of the EPSPs ($p=0.17$). The trend of neurotransmitter release reflected that of EPSP amplitude; after the initial drop, it increased and then deceased.

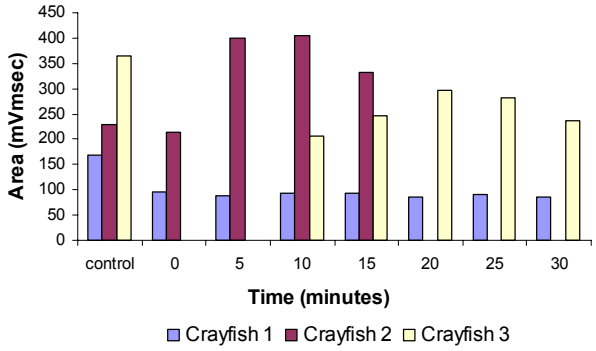

Figure 3. We used the integral to find the area enclosed by each EPSP and the resting membrane potential. This reflects how much neurotransmitter was released. The larger the area, the more neurotransmitter was released. Once again, any times that all three data bars are not shown should not be interpreted as an EPSP amplitude of zero. Once again, we did not combine the results for each crayfish to find the mean because we did not collect data for each time.

DISCUSSION

Our results indicate that our hypothesis was correct; when Omega-Conotoxin GVIA solution was applied to the crayfish, blocking the N-type calcium channels, the EPSP decreased. At the end of each experiment, the EPSP began to decline steadily. However, we do not believe it was due to the reaction to the chemical, but rather to the fact that the crayfish had been dissected for more than an hour at that point, and was probably exhausted. We were unable to test if the lack of EPSP was because of an exhausted nerve in the crayfish neuromuscular system because the chemical, Omega-Conotoxin GVIA is irreversible. For future experiments, this confound could be eliminated by having a second preparation at the same time and measuring the EPSP over time and then comparing the rate of decline of EPSP amplitudes.

In Figure 1, we observed that when the chemical was applied, the amplitude of the EPSP progressively decreased. After the preparation was in the Omega-Conotoxin GVIA solution for ten minutes, the amplitude of the EPSP dropped 29.1%. After the initial drop in EPSP amplitude, it gradually began to return to its control EPSP amplitude. However, the amplitude was only able to increase to within 18.6% of the control amplitude before the crayfish nerve wore out, causing the EPSP amplitude to gradually decrease. There are a few possible explanations for why this slight increase in amplitude occurred. The first is that the stored calcium in the presynaptic neuron could have been making up for the loss of calcium influx caused by the blocked N-type channel. Second, the P/Q-type channels may have become more active to compensate for the absence of the N-type channel. In a previous study, it was shown that when P/Q-type channels were absent, N-type channels were able to make up for a small part of the loss of work done by the P/Q-type channels (Urbano et al., 2003). The reverse of this may have occurred in our experiment. However, the compensation of a missing channel both in the study mentioned and in our experiment only affected a small percentage of the amplitude. In one set of data, the EPSP initially dropped by 25.2%. However, the EPSP amplitude recovered after five minutes to within 0.8% of the control. It would be interesting to see if in future experiments, this extreme recovery occurs again.

Additionally, the Omega-Conotoxin GVIA solution may not have completely blocked all N-type calcium channels. If this were the case, our results would reflect only partial blockage of N-type channels, indicating P/Q-type channels were not the only calcium channels functioning. To ensure that these results accurately showed the effect of a system with completely blocked N-type channels, higher concentrations of Omega-Conotoxin GVIA

solution as well as other chemicals that target only N-type calcium channels could be applied.

Figure 2 showed us that duration of EPSP increased over time. A possible reason for this is the toxin blocked the N-type channels, meaning the P/Q-type channels were responsible for most of the neurotransmitter release. The toxin may have caused the P/Q-type channels to close late. This could be tested by a simple patch-clamp experiment measuring how long each channel remained open.

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