Pioneering Neuroscience, 2002, **3**, 37-40

Inhibition of the reverse mode of the sodium/calcium exchanger reduces facilitation of paired excitatory postsynaptic potentials in the crayfish neuromuscular junction.

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ABSTRACT

Changes in neurons account for various types of learning. One way this learning is accomplished on a cellular level is through facilitation of excitatory postsynaptic potentials (EPSPs). Calcium, the ion responsible for neurotransmitter release and the amplitude of EPSPs, is regulated through various cellular mechanisms including the sodium/calcium exchanger. By inhibiting the reverse mode of the sodium/calcium exchanger we examined the contributions of the exchanger to facilitation, measuring both the first EPSP and changes in facilitation. KB-R7943, the exchanger inhibitor, effectively decreased facilitation while leaving the first EPSPs unaffected. Our results suggest that further experiments should be directed towards measuring the effect of KB-R7943 on the first EPSP while inhibiting K channels and thereby increasing the duration of the action potential.

INTRODUCTION

Learning or adaptation is an important mechanism for a species' survival. Simple or involuntary learning, such as habituation and classical conditioning, occurs without the organism's awareness of behavioral change (Bloom & Lazerson, 1988). Hawkins et al, (1982), studied classical conditioning on a cellular level and suggested that conditioning and habituation result from facilitation of post-synaptic excitatory potentials (EPSPs). They illustrated their theory by measuring facilitation in paired-stimuli EPSPs in the post-synaptic cell and then relating the facilitation to the organism's larger responses. Facilitation is attributed to residual calcium, or calcium that exists in the cell after an action potential occurs before the cell returns to its resting state (Blaustein and Lederer, 1999). That is, due to extra intracellular calcium the EPSP resulting from the second stimuli typically has a higher amplitude than the first EPSP because the pre-synaptic cell emits more neurotransmitters in the presence of extra calcium. Variable levels of intracellular calcium effect facilitation, which results in various behavioral and emotional changes responsible for shaping an organism's development (Blaustein and Lederer, 1999).

Due to its central role in intracellular signaling, calcium concentration inside the cell must be accurately controlled (Blaustein and Lederer, 1999). Both extracellular calcium imported through voltage-gated and receptor-gated channels, and intracellular stores of calcium in the endoplasmic reticulum and mitochondria probably contribute to residual calcium. The influence of each of these mechanisms on calcium and calcium dependent processes during neurotransmitter release is unclear, but clarification of these points is necessary to gain a better understanding of adaptation and learning mechanisms.

The coupled exchange of sodium and calcium was discovered in 1960 (Blaustein and Lederer, 1999). The mechanism responsible for this exchange, the sodium/calcium exchanger, can be found in very similar forms in both vertebrates and invertebrates. The exchanger operates in both directions based on the net electrochemical gradient. That is, depending on concentration gradients and voltage across the membrane the exchanger may import calcium and export sodium, or the reverse in a ratio of three sodium ions per one calcium ion (Blaustein and Lederer, 1999). During the cell's resting state the exchanger imports calcium while expelling sodium. As the cell depolarizes the exchangers reverse direction, importing calcium in exchange for sodium (Blaustein et al.,1969).

It is unlikely that the exchanger is responsible for the calcium influx that triggers transmitter release due to its relatively distant location from neurotransmitter release (Table 1). The average variscosity length is 4.26 μm (Florey and Cahill, 1982). That is, due to the brief duration of action potentials the calcium would not have enough time to diffuse to neurotransmitter release sites thereby influencing release (Fogelson and Zucker, 1985). The exchanger has been shown to be influential during paired-stimuli experiments because the delay between stimuli allows the calcium enough time to

diffuse to release sites (Atwood, H., Charlton, M., Thompson, C., 1983). However, the degree of the exchangers' influence in this capacity is unknown, and it is to this question that we direct our experiment.

	Tip of the Action Potential	Paired-stimuli Delay
Time	200 μs	10 ms
Estimated Calcium Diffusion	0.05 μm	2.5 μm

Table 1. Comparison of calcium diffusion time and distance.

Using KB-R7943 we inhibited the action of the sodium/calcium exchanger in the reverse mode (Chadwick et. al, 2001). We measured EPSPs in fast extensor muscle bundles in crayfish because the nerves and muscle bundles of the crayfish are large. Therefore a microelectrode placed inside the fast extensor muscle bundle readily produces EPSP readings when the motor nerve is stimulated (Stevens, 2000). Mean EPSPs, mean amplitudes, and index facilitations allowed us to examine different degrees of facilitation resulting from inhibition of the sodium/calcium exchanger in the reverse mode (Blaustein and Lederer, 1999).

We hypothesized that inhibiting the exchanger would not effect the amplitude of the first EPSP due to the location of the exchanger in the membrane and the length of the cell's action potential (Blaustein et al.,1969). However, we believed that the amplitude of the second EPSP would diminish in comparison to control EPSPs. We hypothesized that this would happen because the influx of calcium from the sodium/calcium exchanger could contribute to the amount of residual calcium within the cell near the transmitter release site, thus influencing transmitter release and EPSPs in the post-synaptic cell.

METHODS

Solutions

We mixed standard crayfish solution consisting of 5.4 mM KCl, 205 mM NaCl, 2.6mM $MgCl_26H_20$, 2.3 mM $NaHCO_3$, and 2.0 mM Dextrose. We then diluted the solution until it was 5 mL short of the desired volume. Using HCL and NaOH we brought the solution to a pH of 7.4, completed the dilution, rechecked the pH level, and added 13.5 mM $CaCl_22H_20$ last to prevent precipitation.

To create the experimental solution we first made a stock solution of KB-R7943 with a concentration of 2.5 mM. We vortexed the stock solution before every use in the experimental condition and mixed 200 μL of the KB-R7943 stock solution with 100 mL of standard crayfish solution. The resulting concentration of the experimental solution was 5 μM.

Dissection

We dissected the crayfish, removing all extraneous tissue (Stevens, 2000). This procedure left the four fast extensor muscles of the crayfish tail easily accessible for measurements. After dissection, we placed the tail in standard crayfish solution to slow muscle degradation.

Microelectrode Preparation

We pulled microelectrodes from 1.2 mm x .68 mm x 4" capillary tubes. We then filled both the microelectrodes and microelectrode holders with 3.0 M KCl via a syringe. Before each set of readings, we tested the microelectrode for appropriate resistance 5-10 MΩ.

Experiments

We varied between control and experimental conditions over seven testing periods to control for changes in experimental procedures due to our skill levels. During control conditions we placed the crayfish in a dissection dish containing standard crayfish solution (Stevens, 2000). We then placed the stimulator on the caudal margin lateral to the electrode. Using the microscope and micromanipulator, we then placed the microelectrode inside a muscle bundle on the same side of the spine as the stimulator, superior to the stimulator.

Using Scope and MacLab, we measured a membrane potential and began to stimulate the nerve using twin-pulses. To attain clear readings of EPSPs we varied frequency, duration, and voltage of pulses, as well as delay between pulses. We saved measurements on Scope for later measurements of amplitudes, durations, and half-widths of EPSPs. The experimental condition was identical to the control except that the crayfish was placed in the experimental solution containing KB-R7943 instead of the standard crayfish solution during testing.

RESULTS

During the experiments we saved data visually on Scope and later measured the amplitudes of EPSPs, the delay between the first EPSP and the second EPSP, and the half-width of the EPSPs (Figure 1).

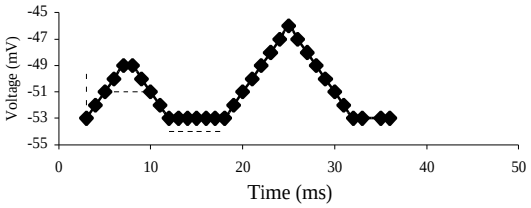

Figure 1. Prototype of paired-stimuli EPSP amplitudes. Amplitude, half-width, and delay (shown left to right with dotted lines) were measured to compare EPSP's

To ascertain whether there was a difference between the first EPSPs in the control condition and the first EPSPs in the second condition we ran two tests, one for sensitivity of effect, and one to judge general effect. The first test, used for its sensitivity, only considered data in which the amplitudes of the first EPSPs ranged from ten millivolts to twelve millivolts. We used these confines to limit possible variability due to changes in the voltage of the stimulus and thus were able to examine possible changes in the EPSPs based on differences in the half-widths of the EPSPs. We chose one-sided t-tests because we hypothesized before testing the location of the means of the experimental data relative to the control data means. We ran a t-test, assuming equal variance, on the confined data sets from the control and experimental conditions. The t-test showed that there was no significant difference ($p<0.341$) between the two sets of data.

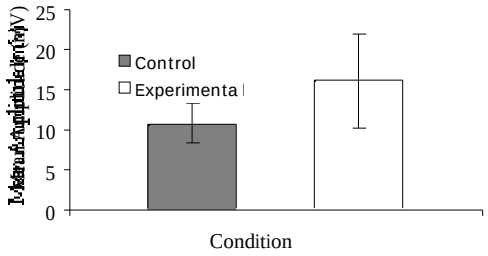

Figure 2. Comparison of control and experimental first EPSP amplitude means ($p<.33$).

For the more general assessment of the first EPSP amplitudes we also ran a one-sided t-test assuming unequal variances on the complete data sets of amplitudes from the first EPSPs in each condition. We assumed unequal variance because of the large difference in standard deviations between the two conditions. This second t-test showed no significant differences ($p<.33$) between the two conditions (Figure 2). These results supported our first hypothesis in which we predicted that KB-R7943 would not affect the first EPSPs in paired stimuli.

In order to examine the effect of KB-R7943 on the degree of facilitation we calculated the facilitation indices for the control and experimental conditions (Lindgren, 2001). By allowing us to assess the changes in the degree of facilitation between the two conditions we could then examine the exchangers' contribution to the amount of residual calcium in the synapse after an action potential (Figure 3). A t-test between the indices for both conditions indicated a statistically significant difference ($p<0.001$). We also calculated the facilitation difference based on the facilitation means of the EPSPs in the separate conditions. This calculation displayed 23% facilitation decrease in the experimental condition. These findings support our hypothesis, indicating the influence of the sodium/calcium exchanger on facilitation.

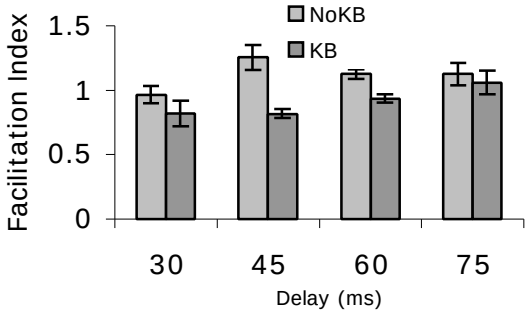

Figure 3. Means of facilitation indices grouped by delay, ±5 ms. Significant differences exist in Delay 45 and Delay 60.

During observations we also discovered that paired EPSPs in the experimental condition are less obviously similar during the beginning of the experiment, than at the end when the inhibitor is more fully effecting the exchangers. No data recordings of this observation were made.

DISCUSSION

Inhibition of the reverse-mode of the sodium/calcium exchanger reduced facilitation of paired-stimuli excitatory postsynaptic potentials. However, as hypothesized, the inhibitor did not influence the amplitude or half-width of the first EPSPs. Our findings support previous work in the area and confirm both of our hypotheses.

We attribute the stability of the first EPSP readings to the length of the action potential and the position of the sodium/calcium exchangers (Blaustein and Lederer, 1999). That is, as the sodium/calcium exchangers are located, for the most part, away from

the neurotransmitter release sites, the action potential is too brief for the calcium introduced by the sodium/calcium exchangers to arrive at the release sites before the end of the action potentials. However, when the reverse mode of the exchanger is inhibited during paired-stimuli conditions the influence of the exchanger can be seen more readily. The influence is apparent in the facilitation of EPSPs because inhibition of the exchanger decreases the amount of calcium that is brought into the cell after depolarization. Thus, the amount of residual calcium is lowered, and facilitation is reduced.

In order to further examine the role of the sodium/calcium exchanger in facilitation, we suggest further studies directed at the action potential duration. Altering action potential duration by inhibiting potassium channels with TEA or 4-AP would allow further understanding of exchanger influence in the reverse mode. That is, our results and the interpretation of our results suggest that KB-R7943 might have a measurable effect on an initial EPSP if the duration of the action potential were increased to ten milliseconds. Due to the lengthened action potential calcium would have adequate time to diffuse to neurotransmitter release sites. This information may be important to assess learning patterns that are effected by presynaptic action potential duration and allow production of more specific pharmaceuticals.

ACKNOWLEDGEMENTS

We would like to thank Dr. Lindgren and Dr. Rempel-Clower for their guidance in this experiment and Sue Colby for her equipment assistance and patience. Additionally, peer reviewers, Embelli Mudoh, Nihad Heto, and Judd Swanson, are greatly appreciated.

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