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Inhibition of the reverse mode of the Na^+/Ca^{2+} exchanger does not affect EPSP amplitudes in the crayfish neuromuscular junction following periods of brief, high-frequency stimulation

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ABSTRACT

Calcium plays a major role in synaptic transmission in which it triggers the release of neurotransmitters into the synaptic cleft. The cell maintains low Ca^{2+} intracellular concentrations by various mechanisms including the Na^+/ Ca^{2+} exchanger. This exchanger pumps out 1 Ca^{2+} ion in exchange for 3 Na^+ ions. In some instances however the Na^+/ Ca^{2+} is capable of functioning in the reverse mode, in which the exchanger pumps out 3 Na^+ ions in exchange for 1 Ca^{2+} ion. We investigated the influence of exchanger's reverse mode in synaptic facilitation. We inhibited the reverse mode of the Na^+/ Ca^{2+} exchanger with the chemical KB-R7943. We induced synaptic facilitation in the presyanptic cell with a five second period of high-frequency stimulation of 20 Hz. However, we found no statistical difference in EPSP amplitude when the reverse mode of the Na^+/ Ca^{2+} was inhibited compared to EPSP amplitudes without KB-R7943.

INTRODUCTION

Calcium plays a critical role in synaptic transmission of a cell. At resting potential, the cell maintains extremely low intracellular Ca^{2+} concentrations. The electrical gradient and the concentration gradient are both oriented to cause a massive influx of Ca^{2+} into the presynaptic cell if a pathway exists for Ca^{2+} in the cell membrane. The influx of Ca^{2+} triggers the release of neurotransmitters into the synaptic cleft where they bind to postsynaptic receptors. Like a key-and-lock analogy, this specific neurotransmitter binding allows ions into the postsynaptic cell which causes a local depolarization called an EPSP.

Excitatory postsynaptic potentials (EPSPs) are local depolarizations that push a postsynaptic cell towards threshold. The number of vesicles released from the presynaptic cell determines the amplitude of an EPSP. EPSPs vary in amplitude depending on the amount of calcium that is available inside the presynaptic cell to trigger the fusion of neurotransmitter vesicles to the membrane of the cell. In addition, the influx of calcium into the cell typically depends on the opening of voltage-gated calcium channels of the presynaptic cell.

There are various mechanisms with which the cell removes Ca^{2+} from the presynaptic cell – one being the sodium-calcium exchanger. The Na^+/Ca^{2+} exchanger is considered one of the most important cellular mechanisms for removing Ca^{2+} (Dipolo and Beauge, 2006). It uses energy stored in the electrochemical gradient of sodium by allowing 3 Na^+ molecules to flow down their gradient into the presynaptic cell in exchange for 1 Ca^{2+}. However, since the transport is electrogenic, meaning that it alters the membrane potential, with enough depolarization of the membrane the direction of the exchanger can be reversed and brings in 1 Ca^{2+} in exchange for 3 Na^+ (Tin-Kyan Lin et al, 2002). The Na^+/Ca^{2+} exchanger may operate in both forward and reverse directions simultaneously in different areas of the cell, depending on the combined effects of Na^+ and Ca^{2+} gradients (Yu & Choi, 1997).

Facilitation is the process by which repeated stimuli produce greater amplitudes of EPSPs in the postsynaptic cell. When a stimulus arrives at the presynaptic terminal, voltage-gated Ca^{2+} channels are opened in the presynaptic cell allowing an influx of Ca^{2+} into the cell. Facilitation occurs from a presynaptic increase in neurotransmitter release (Zucker and Lara-Estrella, 1979). It is attributed to residual Ca^{2+} in the cell, or calcium that exists in the cell shortly after an action potential occurs but before returning to its resting state (Katz, 1967). We enabled synaptic facilitation with a brief, high frequency stimulation that triggered an excess of Ca^{2+} in the presynaptic cell.

The reverse mode of the sodium-calcium exchanger refills Ca^{2+} stores following agonist-induced calcium mobilization (Hiorta, Pertens, & Janssen, 2006). However, does the reverse mode also contribute to facilitation for synaptic transmission? Though it is known that the sodium/calcium exchanger plays some role in maintaining calcium concentrations within a cell and in influencing facilitation (Blaustein et al., 1969), the extent of its role still remains unclear. Using KB-R7943, we inhibited the action of the Na^+/Ca^{2+} exchanger in the reverse mode in the superficial extensor muscle of the crayfish tail (Chadwick et. al, 2001). We hypothesized

that the reverse mode of the Na^+/Ca^{2+} exchanger would play an obvious role in facilitated EPSP amplitude in which its inhibition would decrease residual Ca^{2+} concentrations for EPSPs. Compared to EPSP readings after high frequency stimulation (without KB-R7943), we expected to see decreased facilitated EPSPs with the presence of KB-R7943 because there would be less residual Ca^{2+} for synaptic facilitation. However, our experiments show no statistical difference in EPSP amplitude when the reverse mode of the Na/Ca^{2+} was inhibited (compared to EPSP amplitudes when the reverse mode was *not* inhibited).

MATERIALS AND METHODS

Dissection A crayfish was put under ice to temporarily paralyze it. After movement stopped, the tail was cut off. Lateral incisions were made in the ventral region of the abdomen of the crayfish tail. Once cut, the outer shell area was removed by pulling it off the ventral surface. The mass of muscle tissue was removed carefully to preserve the nerves by pulling it away to expose the superficial extensor muscles.

Microelectrode Preparation There were two different electrodes used: a glass suction microelectrode with a plastic tip to artificially stimulate an action potential inside the crayfish tail and a glass microelectrode to measure membrane potential inside the postsynaptic cell. We pulled microelectrodes from 1.2 mm x .68 mm x 4' capillary tubes (T. Kin, et al, 2002) and filled them with 3-M KCL. Careful not to touch the tip, the microelectrode was rinsed in saline solution to remove any excess KCL that could affect membrane potential inside the cell. The microelectrode was then attached to an intracellular recording device to measure the membrane potential of the crayfish tail. The resistance of the microelectrodes ranged between 5 MΩ and 20 MΩ.

Experiments The dissected crayfish tail was pinned down in a glass dish and put under standard crayfish solution of 5.4 mM KCl, 205 mM NaCl, 2.6 mM $MgCl_26H_20$, 2.3 mM $NaHCO_3$, and 2.0 mM Dextrose. Enough solution was used so that the tail was completely submersed. The suction electrode was positioned on the caudal margin of the crayfish tail in order to stimulate a nerve with an action potential. The microelectrode was subsequently placed inside a muscle bundle on the same side of the suction electrode. The nerve was stimulated with a frequency of 0.2 Hz to obtain a baseline reading of resting membrane potential. In order to mock synaptic facilitation, the frequency was switched to 20 Hz for five seconds, and then back to 0.2 Hz for the remaining readings until it reached the initial resting potential.

After baseline readings were recorded, we looked at EPSPs with KB-R7943. The KB-R794 chemical (obtained from Tocris Crookson Inc.) was used to inhibit the reverse mode of the Na/Ca^{2+} exchanger. The same crayfish tail was thoroughly rinsed with standard crayfish solution for 2 minutes. The dissected crayfish tail was then submersed in 5μM KB-R7943. In addition, the same suction electrode and 3M- KCL glass microelectrode were used to stimulate the nerve and measure the membrane potential of the crayfish tail. The same stimulation procedures were used.

Analysis We analyzed our data by looking at average percent increases of EPSP amplitudes as a way to estimate residual calcium in the presynaptic cell for groups with and without KB-R7943. In addition, we looked at the rate of calcium removal after the high frequency stimulation. We did this by calculating the point at which the amplitude was at its half max and found the corresponding time (*figure 1*).

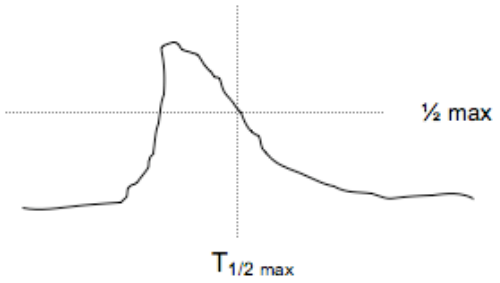

Figure 1

RESULTS

By adding KB-R7943 to the standard crayfish solution, we investigated the effect of the reverse mode of the Na^+/Ca^{2+} exchanger in synaptic facilitation of EPSPs. We looked at the amplitudes of EPSPs under standard crayfish solution and compared it to EPSPs with 5μM KB-R7943 so that the reverse mode of the Na^+/Ca^{2+} exchanger would be blocked.

It was important to get many readings of EPSPs for the baseline group so that there was an adequate basis to compare the experimental KB-R943 EPSPs. *Figure 1* shows a typical EPSP response with a 20 Hz frequency facilitation effect. We started stimulation with a 0.2 Hz frequency. Then there was a sudden 20 Hz frequency for five seconds. The frequency was switched back to 0.2 Hz. With the 20 Hz frequency we built up a calcium concentration which would contribute to a facilitation

effect, which is showed in figure 2 by the sudden spike in all four traces.

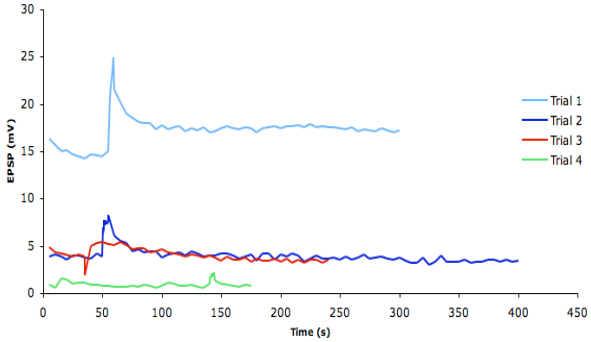

Figure 2. EPSP of baseline group under standard saline solution. (*n=4*) The traces should have a steady baseline membrane potential (frequency=0.2 Hz) followed by the sudden spike (frequency=20 Hz). The frequency returns to 0.2 Hz in which the trace shows a return to the previous resting membrane potential.

We calculated the percent increase of EPSP amplitude to its maximum point (with 20 Hz frequency) from the previous steady baseline with the 0.2 Hz frequency. We found that the average percent increase of EPSP amplitude for control EPSP readings did not differ much from the percent increase of EPSP amplitude for EPSP with KB-R7943 (*figure 3*). The control EPSP readings had an average percent increase of 65% $\pm$ 19% (*n=4*). We are 90% confident that the true mean of the percent increase for the control lies between 46% and 84%. However, our experiments show a 48% increase for EPSP with KB-R7943 (*n=1*), which lie in the interval of the control group. From this data we would speculate that the reverse mode of the Na^+/Ca^{2+} exchanger does not contribute to a difference in EPSP amplitude following a brief high frequency stimulation.

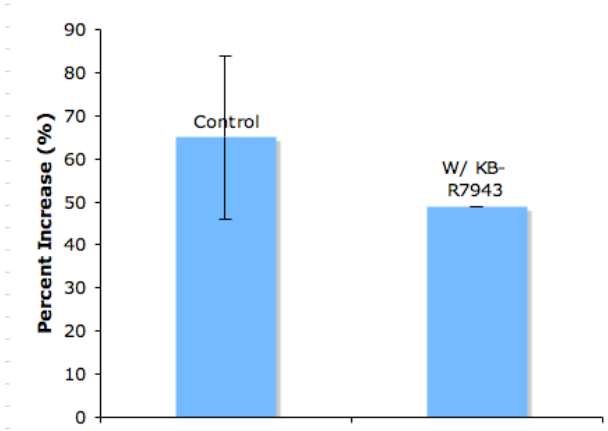

Figure 3. Average percent increase in EPSP amplitude after 20 Hz frequency. Error bar represents $\pm$ 1 S.E. for the control. Sample size: control (*n=4*), with KB-R7943 (*n=1*).

In addition, we looked at the rate of EPSP amplitude decrease by looking at the time of the half-max of the EPSP amplitude before it reached its baseline resting potential by graphical analysis. The control group had an average half max time of 3.0 $\pm$ .57s (*n=4*) and the KB-R7943 group had a half-max of 10s (*n=1*).

DISCUSSION

Our results show that inhibition of the reverse mode of the Na^+/Ca^{2+} exchanger does not exhibit a statistical difference in EPSP amplitude following high frequency stimulation. We hypothesized that blocking the reverse mode, thus limiting Ca^{2+} influx into the cell, would result in lower EPSP percent increase amplitude and a faster rate to resting potential since there would not be as much Ca^{2+} (compared to control EPSPs). However, the percent increase of the KB-R7943 group was 49%, which falls into the 90% confidence interval of the percent increase of the control group (46%-84%). This data contradicts our hypothesis that EPSP amplitudes with KB-R7943 would decrease. Previous studies had showed that inhibition of the reverse mode of the Na^+/Ca^{2+} exchanger reduced facilitation of paired-stimuli EPSPs (T.K., et al, 2002). On the other hand, our data is a bit misleading because our KB-R7943 group only had a sample size of *n*=1. Perhaps this single reading from the KB-R7943 group is an outlier or even a faulty reading. Nonetheless, this small sample size makes it difficult to assess the variability.

In order to make a stronger conclusion, we hope to do more experimentation on crayfish extensor muscle cells with KB-R7943. If this data supports the hypothesis that inhibition of calcium via inhibition of the calcium attributed to the reverse mode of the Na^+/Ca^{2+} exchanger results in lower EPSPs, we would further the study to isolate the influence of a calcium-induced-calcium-release (CICR) mechanism in EPSP amplitudes. CICR uses calcium to promote its own release from intracellular calcium stores such as the mitchochondria and the sacroplasmic reticulum. In order to isolate this CICR possibility, the inhibitor ryanodine can be applied to block the CICR mechanism. If EPSP readings with the presence of ryanodine show a decrease in amplitude, we would be more likely to conclude that the reverse mode of Na^+/Ca^{2+} exchanger contributes to the change of the EPSPs**.**

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