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Changes in Synaptic Delay and EPSP Amplitude Induced by TEA with 3,4-DAP, Temperature and Post-Tetanic Potentiation.

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ABSTRACT

We tested the current model of calcium influx and neurotransmitter release mechanisms by measuring changes in synaptic delay and amplitude of excitatory post-synaptic potentials (EPSPs). Changes in calcium influx and neurotransmitter release were generated following the application of tetraethylammonium bromide (TEA) with 3,4-Diaminopyridine (DAP), post-tetanic potentiation (PTP), and temperature variation at the crayfish neuromuscular junction. Previous research has shown that TEA with 3,4-DAP broadens the pre-synaptic action potential, which increases synaptic delay and EPSP amplitude. PTP, however, has been found to increase the rate of calcium influx, thereby decreasing delay and increasing EPSP amplitude. Other research demonstrated that an increase in temperature narrowed pre-synaptic action potentials, which should decrease delay and decrease EPSP amplitude. We hypothesized that these independent variables would have the predicted effects based on the model for both dependent variables: TEA and decreased temperature would increase delay and EPSP amplitude, while PTP would decrease delay and increase EPSP amplitude. Using intracellular recording, we began and ended each trial under normal conditions to establish a baseline and to test the reversibility of each variable. Our results supported the current model that TEA and temperature would increase synaptic delay, and that TEA and PTP would increase EPSP amplitude. However, we found that a decrease in temperature decreased EPSP amplitude and PTP did not significantly decrease delay, signifying that these variables must affect different mechanisms involved in synaptic transmission.

INTRODUCTION

Depolarization in the pre-synaptic terminal induced by the arrival of an action potential causes voltage-dependant calcium channels to open, allowing calcium to enter the pre-synaptic cell. The calcium ions trigger synaptic vesicles to fuse with the pre-synaptic cell membrane and release neurotransmitters into the synaptic cleft (Hille 1984). If this proposed model of synaptic transmission is correct, then the electrochemical driving force of calcium and the permeability of the membrane to calcium should affect the rate of calcium influx, given by the equation $I_{Ca} = g_{Ca}(E_m - E_{Ca})$ (Matthews 1998). Consequently, the concentration and rate of calcium influx should modulate the amount of neurotransmitters released (Matthews 1998).

To test this proposed model that relates calcium influx to neurotransmitter release mechanisms, we can measure two factors that are dependent upon the influx of calcium: synaptic delay and excitatory post-synaptic potential (EPSP) amplitude (Lin and Faber 2002). Synaptic delay is defined as the period between the arrival of a pre-synaptic action potential at the nerve terminal and the start of the post-synaptic response. Changes in delay can reflect variation in any of the processes from calcium influx to subsequent depolarization of the post-synaptic cell. EPSP amplitude can also indicate changes in the amount of neurotransmitters released (Lin and Faber 2002).

To examine changes in calcium influx and neurotransmitter release, we manipulated three independent variables to test their effects on synaptic delay and EPSP amplitude. These three variables were: broadening of pre-synaptic action potentials using potassium channel blockers, post-tetanic potentiation (PTP) and variation of temperature.

Tetraethylammonium Bromide and 3,4-Diaminopyridine

Tetraethylammonium bromide (TEA) and 3,4-Diaminopyridine (DAP) block delayed rectifier and leak potassium channels, respectively. The block of these channels slows potassium efflux during the repolarization phase of the pre-synaptic action potential, prolonging its duration. Based upon the previously mentioned model, the prolonged depolarization should keep the voltage-dependent calcium channels open for a longer time, which should raise internal calcium concentration. However, since the voltage gated calcium channels open later, calcium influx will be delayed (Kumamoto and Kuba 1985). These changes should be reflected in the post-synaptic response in the form

of a broadened and delayed EPSP. The research of Vyshedskiy et al. (2000) supports this prediction as they found that TEA enhanced synaptic delay during unfacilitated release as the action potential duration increased. Furthermore, Wachman et al. (2004) found that 3,4-DAP broadened the pre-synaptic action potential thus enhancing calcium influx and neurotransmitter release. Since these potassium channel blockers increase neurotransmitter release, they have been used to treat various neuromuscular diseases such as the Lambert-Eaton Myasthenic syndrome (Wachman et al. 2004).

Post-tetanic Potentiation (PTP)

Post-tetanic potentiation is a form of synaptic plasticity that influences calcium influx and enhances neurotransmitter release (Koyano et al.1985). According to the calcium influx model, the application of high frequency stimulation should enhnace the rate of increasing internal calcium concentration in the pre-synaptic terminal. Synaptic delay should then decrease due to faster vesicle fusion triggered by the additive effect of both residual calcium and subsequent influx of calcium during stimulation. This buildup of calcium should increase the amount of neurotransmitters released. This change would be observed by an increase in the EPSP amplitude. Vyshedskiy et al. (2000) supported this hypothesis when they found that paired-pulse stimulation reduced delay because the conversion process of calcium channels to high-affinity binding sites for calcium ions that trigger vesicular release had already occurred during facilitation. The enhanced rate of neurotransmitter release, induced by PTP, has been shown to affect both memory and learning (Koyano et al.1985).

Temperature

Temperature affects the rates of conformational changes of ion channels, but has relatively little effect on the diffusion rate of ions (Matthews 1998). Temperature-sensitive processes in the pre-synaptic terminal have been shown to influence calcium influx during synaptic transmission (Barrett et al. 1978). Increasing the temperature caused shorter pre-synaptic action potentials with a higher peak, which reduced calcium currents and shortened the delay of calcium influx (Barrett et al. 1978). In contrast, a decrease in temperature would decrease the rate of the opening and closing of ion channels, delay and prolong calcium influx, which would then increase neurotransmitter release. Pierau et al. (1976) found that a lower temperature produced an increase in both EPSP amplitude and synaptic delay, supporting the model's hypothesis. Since many physiological changes and processes depend upon temperature, further study of the effects of temperature on synaptic transmission might explain how temperature-sensitive mechanisms would influence the release of neurotransmitters.

Hypothesis

While the effects of all three independent variables combined could demonstrate how the variables would modulate synaptic transmission, we proposed to test whether each separately upheld the model of calcium influx and neurotransmitter release on the crayfish neuromuscular junction. We compared the results of the application of TEA with 3,4-DAP, tetanic stimulation, and lower temperature on delay and EPSP amplitude. These findings could be used to better understand if these variables primarily affect calcium influx as proposed by the model, or influence other mechanisms involved in synaptic transmission. The crayfish is an ideal preparation for studying changes in synaptic transmission (Vyshedskiy et al. 2000) and its excitatory synapses are similar to mammalian central nervous system synapses. While the effects of TEA and 3,4-DAP on calcium influx are generally well known in inhibitory and excitatory synapses of other organisms, we retested the effects of TEA with 3,4-DAP on the excitatory crayfish neuromuscular junction. We also examined if the model would accurately predict changes in calcium influx induced by the application of PTP and lowered temperature. Based on previous research, we hypothesized that both TEA and a lower temperature would increase synaptic delay and EPSP amplitude, while tetanic stimulation would decrease delay and increase EPSP amplitude.

All three independent variables had varying effects on both dependent variables. With the application of TEA with 3,4-DAP, we found that both synaptic delay and EPSP amplitude increased, supporting the proposed model. However, a lowered temperature increased synaptic delay, while decreasing EPSP amplitude, indicating that temperature does not exert is effects on synaptic transmission solely via an affect calcium influx. Finally, we found that PTP did not significantly decrease synaptic delay, but increased EPSP amplitude, both of which are trends that support the model.

MATERIALS AND METHODS

Basic Preparation

We dissected crayfish to expose innervating nerves lining the lateral and medial tail extensor muscles. The dissected tail was bathed in standard crayfish saline, changed every 15 minutes, with a composition of: 5.4mM KCl, 205mM NaCl, 9.35 mM

$MgCl_26H_2O$, 2.3mM $NaHCO_3$, 2.0mM Dextrose, and 6.75mM $CaCl_22H_2O$ at a pH of 7.4. To stimulate the innervating nerve and measure an EPSP, we used a suction electrode to suck up a nerve and a micro-electrode to record cell membrane potential. Capillary microelectrodes of 1.2 mm diameter were made using a Pul-1 microelectrode maker, and filled with 3M KCl. We maintained electrode resistance at 7-10 MΩ for every trial so that recordings were accurate, and kept frequency const ant for each experiment once an EPSP was generated. We eliminated junction potential using reference electrodes placed in the crayfish saline before penetrating the cells.

Basic Trial Procedures

For each trial, we used a normal-experiment-wash sequence. We began testing with normal crayfish saline at room temperature in order to establish a baseline for comparison to experimental data. After applying our variable and recording experimental data, we attempted to create a wash, and revert to normal conditions. This process both demonstrated whether our variables were in fact reversible, and ensured that time was not a factor, showing that conditions remained constant with the exception of those due specifically to the change in experimental conditions. If a variable proved irreversible, a second crayfish was prepared and placed directly in experimental saline. By comparing these results using the second crayfish to the normal conditions, time was eliminated as a factor.

Measurement of Synaptic Delay and EPSP Amplitude with TEA with 3,4-DAP

We used 1 mL of 10 mM TEA and 1 mM 3,4-DAP combined, added to 99 mL of normal Ringer solution. A stock solution of aqueous 1M TEA and 0.1M 3,4-DAP was frozen in 1 mL aliquots. We first conducted an experiment under normal conditions to find the synaptic delay and EPSP amplitude at room temperature in normal crayfish saline by taking readings of eight average sweeps of stimulation from five separate cells. We then switched to TEA with 3,4-DAP saline and let the specimen bathe for approximately 10 minutes prior to experimentation to ensure that the chemical inhibited most of the potassium channels and diffused through the solution. We then repeated the procedure described above to find synaptic delay and EPSP amplitude. Finally, we washed our specimen and recorded EPSPs once again using normal Ringer solution.

Measurement of Synaptic delay and EPSP Amplitude with Varying Temperature

We again began with a normal saline solution and measured EPSPs. To lower temperature, we added saline ice cubes until the temperature of the Ringer solution was approximately 10°C. Temperature was monitored using a digital thermometer probe, which was placed within 0.5 -1 cm of the nerve. Ice cubes were composed of saline solution so that chemical concentration did not become diluted, and were added to maintain conditions throughout the experiment as needed. Once EPSPs were generated and recorded, we returned the saline to room temperature and repeated the measurement.

Measurement of Synaptic Delay and EPSP Amplitude with Tetanic Stimulation

After establishing baseline recordings under normal conditions, we applied tetanic stimulation by changing the frequency of stimulation from 0.25 PPS to 25 PPS for a five second period. Immediately following the stimulation, we rapidly recorded the EPSPs generated until the amplitude returned to a normal level. This entire procedure was repeated four times in four separate cells. During stimulation, the threshold potential increased because of the limited refractory period, thus making it more difficult to obtain an EPSP. To counter this effect, we found the threshold potential by adjusting the voltage knob until an EPSP was first generated. We then increased the voltage four times beyond threshold to ensure an EPSP was being generated every time the nerve was stimulated.

Data Analysis

Data were collected, analyzed and interpreted using the Scope program and Excel. MacLab and a SD9 stimulator were used to amplify and record data. Synaptic delay was measured from the beginning of the stimulus artifact to the start of the EPSP. We recorded EPSP amplitude as the difference from the beginning of the EPSP to its maximum peak. We compared mean synaptic delay from our raw experimental data for each trial to normal conditions in order to analyze the effects of delay on calcium influx. EPSP amplitudes for each experiment were compared by measuring the percent change in EPSP amplitude using Equation 1.

Equation 1: Percent Change

$$\frac{[\text{Experimental amplitude} - \text{normal amplitude}]}{\text{Normal amplitude}} \times 100$$

To calculate the significance of our data, we used a p-value <0.05 for t-tests with both equal and unequal variances at a 95% confidence level. We collected mean values and standard deviation as well.

RESULTS

In order to test the current model relating calcium influx to neurotransmitter release, we investigated the influence of broadening of action potentials, post-tetanic potentiation and varying temperature at the neuromuscular junction of the crayfish by measuring changes in synaptic delay and EPSP amplitude. To measure synaptic delay and EPSP amplitude, we used intracellular recording.

Tetraethylammonium Bromide and 3,4-Diaminopyridine

TEA and 3,4-DAP increased average synaptic delay. In normal saline, average synaptic delay was 2.27 ms. However, the addition of the 10 mM TEA and 1mM 3,4-DAP solution caused synaptic delay to increase by an average 0.480 ms to 2.75 ms (Figure 1a). Based on an equal-variance t-test, this increase was significant (p=0.0061). Although a wash was conducted, we could not successfully generate EPSPs as the membrane potential increased with prolonged exposure to the TEA with 3,4-DAP solution. Therefore, the wash results were not included in our analysis. Standard deviation (SD) was 0.057 ms for normal and 0.29 ms for TEA with 3,4-DAP, demonstrating relatively low variability in our data (n= 5, n=5 respectively).

We also found that EPSP amplitude increased with the addition of TEA and 3,4-DAP. The mean amplitude increased by 73% after applying our experimental solution (Figure 1b). Again, using statistical analysis, we determined that our results were significant for an increase in EPSP amplitude (p= 0.0014). In calculation of significance, we eliminated one outlying data point. This outlier was our final EPSP recording under experimental conditions, and we hypothesize that the discrepancy was due to prolonged exposure. As for synaptic delay, we could not determine whether TEA with 3,4-DAP was reversible for EPSP amplitude because our wash was unsuccessful. Standard deviation for amplitude for normal conditions was 2.30 mV and 2.83 mV for experimental (n= 5, n=4 respectively).

These findings support our original hypothesis that TEA with 3,4-DAP broadens pre-synaptic action potentials, shown by an increase in synaptic delay and EPSP amplitude. Furthermore, the prolonged repolarization of the pre-synaptic nerve terminal due to the blockage of potassium channels caused an increase in calcium influx. This is shown by the broadened and elevated EPSPs of the post-synaptic response, as illustrated in Figure 1c.

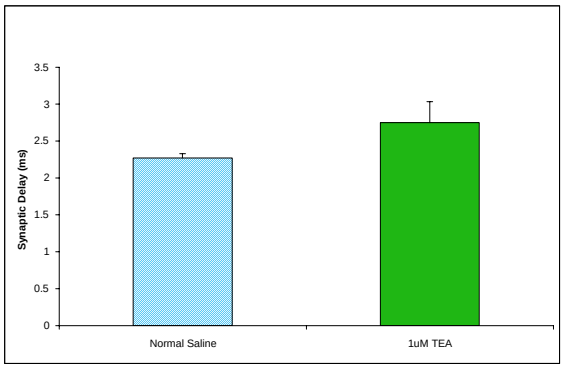

Figure 1a. TEA and 3,4-DAP solution increased synaptic delay. The two bars (n=5 for both) demonstrate the average synaptic delay recorded before and after the application of the TEA with 3,4-DAP solution. The error bars signify standard deviation from the mean.

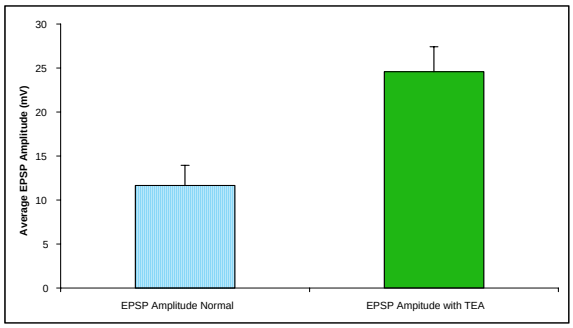

Figure 1b. TEA and 3,4-DAP solution increased EPSP amplitude. Each bar (n=5 for normal, n=4 for experimental) shows the average EPSP amplitude recorded in mV both before and after TEA with 3,4-DAP solution addition. The error bars represent the standard deviation from the mean.

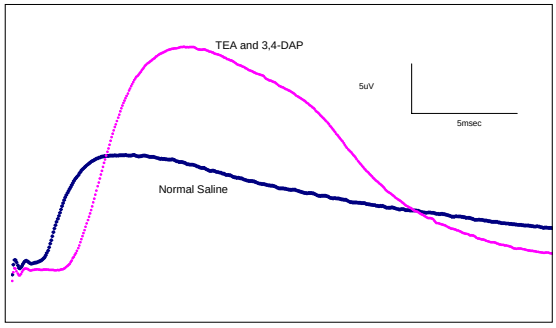

Figure 1c. Representative traces of TEA and 3,4-DAP solution show broadened EPSP duration. The line graphs show two EPSP recordings, before and after the application of the 10 mM TEA and 1 mM 3,4-DAP solution. They measure membrane potential over time.

Decrease in Temperature

To test the effect of a decrease in temperature on synaptic delay and EPSP amplitude, we reduced normal crayfish saline temperature from 18°C to 10°C by adding saline ice chips. Decreasing saline temperature caused an increase in mean synaptic delay (Figure 2a). This difference of 0.89 ms was significant (p=0.022) and standard deviation was

relatively low (SD normal= 0.60 ms, SD experimental=0.66 ms, SD wash= 0.16 ms, n= 5 for all). Following a return to 18°C, synaptic delay returned to its original values indicating reversibility.

However, a decrease in temperature decreased EPSP amplitude. Our mean normal EPSP amplitude was 6.46 mV, while the amplitude at 10°C was 2.38 mV and 4.00 mV for our wash. For easier comparison, we established our mean normal results at 100% and found percent change in amplitude for a lower temperature and wash (Figure 2b). The results showing that amplitude decreased were significant (p=0.019 at 10°C). Standard deviation was 12% for experimental and 21% for wash, which showed a consistency in our data. Although in Figure 2b the EPSP amplitude for the wash appeared to remain significantly lower than baseline amplitude, statistical analysis suggested that there was no significant correlation between the wash and the normal. This implied that a decrease in temperature had a reversible effect on EPSP amplitude. Figure 2c effectively shows the similarity between normal and wash data.

Our hypothesis for the effects of decreased temperature on post-synaptic response was only partially supported by our results. As we predicted, synaptic delay decreased as temperature was lowered, but EPSP amplitude also decreased, contrary to our hypothesis. Our results indicated that temperature might have affected other mechanisms in synaptic transmission, as opposed to primarily calcium influx and neurotransmitter release.

Post-Tetanic Potentiation

We applied tetanic stimulation for a five second period by increasing the frequency of stimulation to 25 PPS. Approximately three normal readings were obtained before application of the tetanic stimulation, and then readings were continuously recorded until amplitude returned to the normal level, which therefore meant the number of recordings varied between trial sets. For the purposes of data analysis, we used the first four recordings following tetanic stimulation and the last four recordings as EPSP amplitude returned to baseline.

Synaptic delay decreased with the application of PTP, but this decrease was not significant (at a 95% confidence level) when comparing normal readings to the sets of either immediate or delayed readings after tetanic stimulation was applied (p= 0.25 and p= 0.99 respectively). The lack of significance between the normal and delayed readings illustrated that the two conditions were similar. However, a comparison of the immediate readings and delayed readings was significant (p= 0.044). A larger time gap between immediate and delayed recordings might have

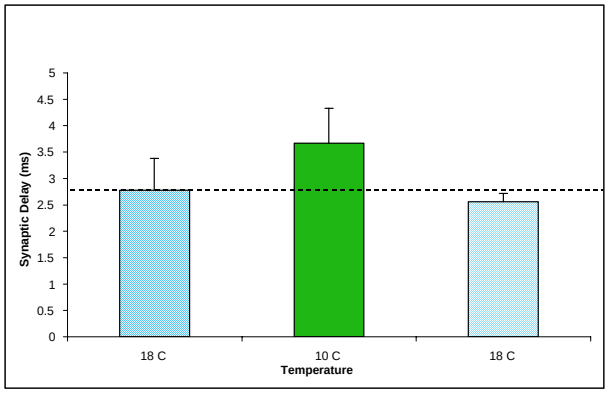

Figure 2a. Decreased temperature increased synaptic delay. The three bars represent the three trials conducted to measure the effect of lowered temperature on synaptic delay: normal (18°C), experimental (10°C) and wash (18°C). Each bar shows the average synaptic delay recorded (n=5 for each). The error bars represent the standard deviation from the mean synaptic delay.

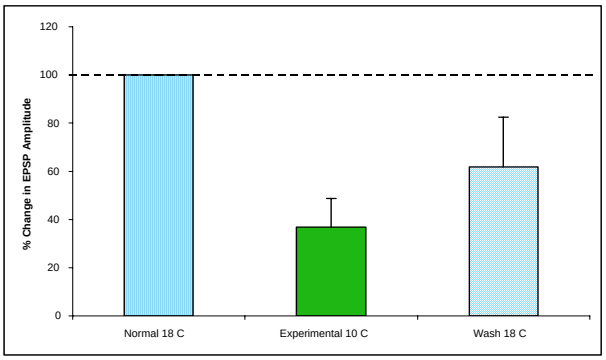

Figure 2b. Decreased temperature decreased EPSP amplitude. The three bars represent the percent change of EPSP amplitude. Three trials were conducted to measure the effect of lowered temperature on synaptic delay: normal (18°C), experimental (10°C) and wash (18°C). The normal bar is represented as 100% because the mean of the normal amplitude was used as a baseline comparison for experimental and wash data (n=5 for experimental and wash). The error bars show standard percent deviation.

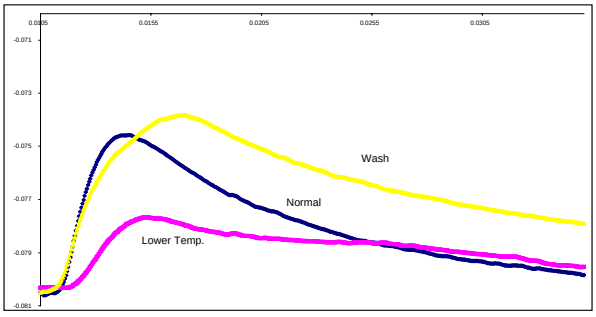

Figure 2c. Representative traces of EPSPs generated under normal and experimental conditions. The line graphs represent change in membrane potential as a function of time. Each line shows data collected from an EPSP induced in one cell, all taken from the same crayfish.

enabled PTP to have a significant effect. As shown in Figure 3a, the recorded data was consistent, with small standard deviations for all three trial sets (normal SD = 0.28, immediate readings SD = 0.27, delayed readings = 0.30, n=4, n=16, n=16 respectively).

The percent change in EPSP amplitude increased with the application of tetanic stimulation. The increase in amplitude immediately after stimulation was significant (Figure 3b, p=0.00036). In contrast, as shown in Figure 3c, the EPSP amplitude decreased below normal amplitude over time. While the delayed EPSP amplitude was lower than the normal amplitude, there was no significant correlation at 95% confidence level between the two, signifying that the delayed amplitude was close to normal (p= 0.068). The standard deviation was higher than our other results, as we took recordings from different cells and applied different volts to obtain an EPSP, which could have altered the percent increase in amplitude (SD immediate= 62%, SD delayed=53%, n=16, n=16 respectively).

Synaptic delay, immediately following PTP application, did not significantly decrease and thus did not support our hypothesis. However, the significant increase in EPSP amplitude, immediately after tetanic stimulation, supported our hypothesis that PTP would enhance EPSP amplitude through augmented neurotransmitter release.

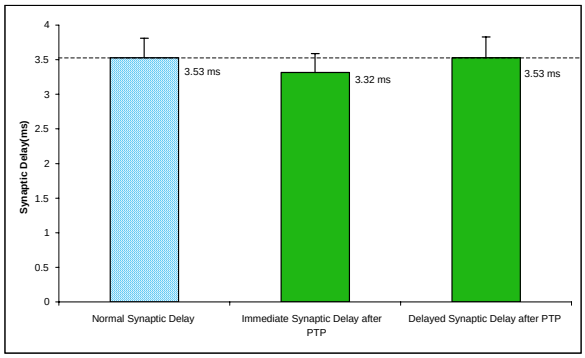

Figure 3a. Synaptic delay decreases immediately after PTP and returns to normal after 30 seconds. The three bars represent the average synaptic delay taken from four different cells (n=4, n=16, n=16 respectively). The error bars represent the standard deviation from the mean.

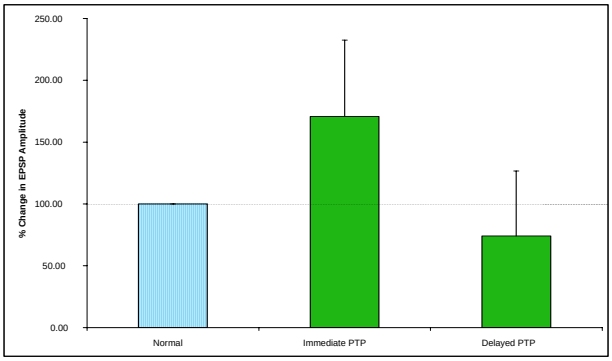

Figure 3b. Application of PTP increases EPSP amplitude immediately and decreases amplitude after approximately 30 seconds. The three bars represent the percent change in EPSP amplitude, with normal at 100% as baseline (n=16 for immediate and delayed). The error bars represent the percent deviation from the mean.

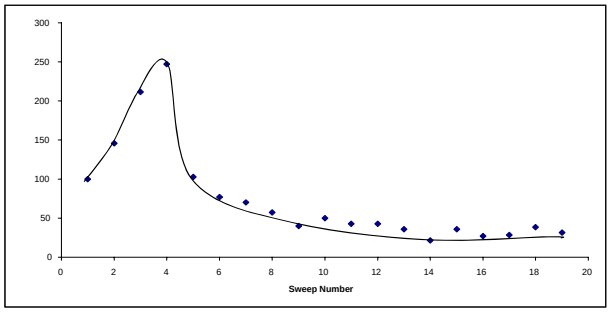

Figure 3c. Illustration of percent change in EPSP amplitude with the application of PTP over the duration of 30 seconds. The first data point represents the mean normal EPSP amplitude (100%) once again used as a baseline. The other data points represent the percent change in amplitude over several sweeps. The line traces the general trend of the EPSP amplitude.

DISCUSSION

Tetraethylammonium Bromide and 3,4-Diaminopyridine

The application of 10mM TEA and 1mM 3,4-DAP increased both synaptic delay and EPSP amplitude. Although we were not able to obtain results from our wash due to overexposure and could not determine if TEA with 3,4-DAP had a reversible effect, our results confirmed the findings of Kumamoto and Kuba (1985) and Vyshedskiy et al. (2000) that TEA increases synaptic delay and EPSP amplitude. Our results from TEA with 3,4-DAP supported our hypothesis of the proposed calcium influx model: TEA with 3,4-DAP blocks potassium channels, which prolongs the repolarization phase of the pre-synaptic action potential and keeps the voltage-dependent calcium channels open for a longer time period. The prolonged repolarization reduces the driving force of calcium entry, which delays calcium influx. The period between vesicle fusion to subsequent neurotransmitter release then increases. Furthermore, the increase in calcium influx leads to an enhanced release of neurotransmitters, shown by a larger EPSP amplitude.

Post-Tetanic Potentiation

Immediately after the application of PTP, we found a significant increase in EPSP amplitude, but did not find a significant decrease in synaptic delay and thus, we could not support our hypothesis that synaptic delay would decrease due to a buildup of internal calcium in the pre-synaptic cell. Vyshedskiy et al. (2000) postulated that there would be no detectable change in synaptic delay if stimulation only raised internal calcium concentration enough to trigger release of vesicles in the facilitated state. This might explain why we found that PTP significantly decreased synaptic delay after delayed recordings. However, our results indicated a significant increase in EPSP amplitude. This finding supported our hypothesis and confirmed the results of Koyano et al. (1985) that an increase in EPSP amplitude was due to an increase in neurotransmitter release.

Because the calcium influx model suggests a rise in calcium in the pre-synaptic cell, supported by the increase in EPSP amplitude that we found, this increased calcium concentration should also have caused faster vesicle fusion, and subsequently a decreased synaptic delay. Since our findings demonstrated that PTP caused an insignificant decrease in synaptic delay, we propose that tetanic stimulation might affect different mechanisms that control calcium concentration in the pre-synaptic cell.

Temperature

A decrease in temperature significantly increased synaptic delay, but decreased EPSP amplitude. The increase in synaptic delay supported our hypothesis and confirmed the findings of Pierau et al. (1976). They found that a lower temperature produced an increase in synaptic delay because temperature-sensitive processes in the pre-synaptic terminal delay calcium influx. A cooler temperature would then slow the rate of conformational changes of ion channels, which in turn would delay and prolong calcium influx. However, we found a significant decrease in EPSP amplitude, which disproved our hypothesis. Our results for EPSP amplitude were different from the findings of Pierau et al. (1976) who conducted their research on cat spinal motoneurones. This discrepancy might be explained due to the use of a mammalian organism as opposed to a crustacean. Another possible explanation for our findings is that a decrease in temperature affects post-synaptic mechanisms as opposed to pre-synaptic processes. Temperature might have changed the conformation of neurotransmitter receptors, or inhibited the reuptake process of neurotransmitters, shown by the research of Vizi et al. (2003) who found that at a lower temperature inhibited the release of dopamine in the olfactory bulb in the presence of cocaine.

Future Research

The three independent variables, TEA with 3,4-DAP, temperature and PTP do not primarily affect the same mechanisms in synaptic transmission, as shown by the varying results of each variable on synaptic delay and EPSP amplitude. The effects of TEA with 3,4-DAP on calcium influx and neurotransmitter release are generally understood and were also supported by our results. However, since temperature did not affect EPSP amplitude as predicted by the model, further investigation of the exact mechanisms influenced by temperature would be useful. In addition, investigation of how PTP affects pre-synaptic processes during tetanic stimulation would help explain how other mechanisms might be involved in increasing neurotransmitter release and affecting synaptic delay. Given that each variable caused varying responses for synaptic delay and EPSP amplitude, future experiments could investigate the effects of all three variables combined. Such future examinations would help understand how differing variables affect each other and synaptic transmission overall

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