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The Effect of Muscarine on the Sodium Calcium Exchanger and Excitatory Postsynaptic Potentials in the Neuromuscular Junction of Crayfish Neurons

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ABSTRACT

Muscarine is a chemical that has been found to continually activate the sodium calcium exchanger of neurons. This experiment tested whether muscarine affected the mechanisms of the sodium calcium exchanger in the neuromuscular junction of crayfish. Using paired pulse facilitation, EPSPs were recorded from crayfish muscles after stimulation at 15 and 20ms delays. Concentrations of 10 and 20μM muscarine solutions were added to the crayfish bath to see if muscarine had an affect on the amplitude of the second EPSP of the paired pulse set relative to the first EPSP. Muscarine was known to continually activate the sodium calcium exchanger in rat brain cells and frog muscle cells, reducing the overall amount of residual calcium in the cell, and therefore reducing the amount of neurotransmitters released affecting the size of the second EPSP of the paired pulse facilitation (less is known about muscarine in crayfish muscle cells). After inconsistent results were obtained, we concluded that the muscarine had a minimal affect on the sodium calcium exchanger, as the amplitude differences were not as drastic as hypothesized. However, muscarine did alter the shape and overall amplitude of the EPSPs, revealing that on some level muscarine does affect the manifestation of EPSPs.

INTRODUCTION

This experiment investigated how the sodium calcium exchanger in the crayfish neuromuscular junction is affected by the neurotoxic chemical muscarine. The role of the sodium calcium exchanger in cells is to exchange one calcium ion for two sodium ions, ensuring that calcium is removed from the cell after its initial influx during synaptic transmission (Blaustein and Lederer, 1999). The regulatory function of the sodium calcium exchanger makes certain that calcium concentrations do not build up, as high concentrations of calcium can prove lethal to neuron cells (Bondarenko, 2004). Muscarine is a chemical that has been found to continually activate the sodium calcium exchanger in both the presynaptic and postsynaptic neuron cells of rat brains (Xu et al, 2006). Muscarine also has been found to increase or decrease depolarization levels of the cell based on the amounts of calcium leaving the cell through the sodium calcium exchanger, which then alters the amount of acetylcholine neurotransmitters released into the synaptic gap (Slutsky et al, 1999).

Based on previous research of the effect of Muscarine on the sodium calcium exchanger, this experiment tested whether muscarine affects the sodium calcium exchanger in the crayfish neuromuscular junction. Furthermore, this experiment aimed to test how continually activating the sodium calcium exchanger with muscarine affected the manifestation of EPSPs in the postsynaptic cell. Paired pulse facilitation was used to compare the initial EPSP, which is caused by an initial calcium influx, to the second pulse of the pair, which is often larger than the first pulse due to residual calcium left in the cell from the initial calcium intake (Wu et al, 1994). The presence of this residual calcium in the cell causes more neurotransmitters to be released into the synaptic gap, which then causes a higher EPSP (Dittan et al, 2000).

We hypothesized that the application of muscarine in the crayfish cell would continually activate the sodium calcium exchanger, which would then remove the residual calcium left in the cell from the initial calcium influx, leaving the second EPSP generated from paired pulse facilitation smaller in amplitude than the first EPSP. The application of muscarine in 10μM and 20μM concentrations revealed that although muscarine does have an affect on the sodium calcium exchanger, as the EPSPs generated under the 10μM and 20μM were altered in amplitude, the muscarine did not have as drastic an affect on the EPSPs as originally thought.

MATERIALS AND METHODS

Dissection/Prep

To prepare the crayfish, we first removed the head portion of a cold-anesthetized specimen and cutting along the sides of the shell, near the ventral surface. We then dissected the ventral muscle mass of the specimen to expose the dorsal muscles and nerve endings. After securing the specimen to an experiment dish, we initially submerged it in crayfish Ringer solution (196mM NaCl, 5.4mM KCl, 13.5mM CaCl2, 2.6mM MgCl2) to preserve normal neural activity. We used a separate crayfish for each set of trials at either a different concentration of muscarine, or a different delay time.

Intracellular data recording

Intracellular recording was used to gather data on EPSPs at the neuromuscular junctions. To achieve this we used intracellular recording electrodes filled with 3M KCl, pulled using an electrode maker, attached to an electrode holder. We removed air bubbles to reduce resistance. The resistance of our intracellular electrode consistently leveled around 12 MΩ.

Extracellular nerve stimulation

To apply voltage to the nerve endings, we used extracellular electrodes also pulled with an electrode maker, and then blunted with sandpaper. These were then attached to another electrode holder, and used to backfill solution into the electrode and the holder (with a suction syringe) until it touched the wire inside the electrode, and then the nerve ending.

Paired Pulse Facilitation

In our experiment, we used paired pulse facilitation to measure the EPSPs at the neuromuscular junction due to voltage applied through the extracellular electrode. By doing this, we were able to measure activity occurring presynaptically; however, we cannot disprove that activity may be occurring postsynaptically. Our resting potential usually fluctuated between about -55mV to -70mV. If changes are happening at the presynaptic level, then changes in EPSP amplitude size can be seen at the postsynaptic level, if there is nothing occurring at the presynaptic level, no change will be seen at the postsynaptic level. We applied the pulses with the electronic stimulator box on these settings: frequency of 2 Hertz; duration of 7.5 ms. We initially had the delay set at 20 ms, but also tested 15ms as well to test different variables of time, and began recording a separate set of trials, at 15 ms delay, after collecting data for 20 ms delay under control, 10 μM muscarine, and 20 μM muscarine circumstances.

Control tests

To establish a baseline with which to compare our experimental findings, a control test was performed first before each experimental set (between 5 and 10 trials each time). This involved stimulating the crayfish using voltage applied to a nerve ending by extracellular electrode, and measuring it with an intracellular electrode in the muscle. With the paired pulse stimulation, we expected to find higher amplitudes in the second EPSP recorded in each stimulation. This is due to the residual calcium that remains after the first EPSP, and is then released during the second, causing the second EPSP to be higher under normal control circumstances (Zucker and Landau, 2004).

Chemical dilution/application

To gauge the effects of increasing concentrations of the muscarine, we elected to use 10 μM and 20 μM concentrations. To achieve this, we calculated that we needed 50 μL and 100 μL of muscarine per 100 mL of prepared Ringer solution, respectively, basing our concentrations off of ones used by Yukihiro et al (2003). Once this was prepared, we removed the normal Ringer solution, which was used to record baseline readings, with a suction syringe, and then applied the muscarine solution bath. We then allowed the solution to percolate for five minutes before taking experimental readings. To prevent data from being skewed by different concentrations of muscarine, we used new crayfish when switching between concentrations.

RESULTS

Our research aimed to test how muscarine affected the sodium calcium exchanger in the crayfish neuromuscular junction and how prolonging the activation of the exchanger affected the amplitude of the second EPSP of a paired pulse facilitation set of simulations. Using different concentrations of muscarine in a normal saline bath, paired pulse facilitation was used to produce EPSPs in the muscle cells of crayfish. The existence of residual calcium in the cell normally would cause the second EPSP of the set to have a higher amplitude than the first (See Figure 1), but if the sodium calcium exchanger is continually activated during synaptic transmission, and therefore calcium is continually being taken out of the cell in exchange for sodium, there will be less, or no, residual calcium. If there is no residual calcium, then the amplitude of the second EPSP of the paired pulse set will be less than that of the first amplitude.

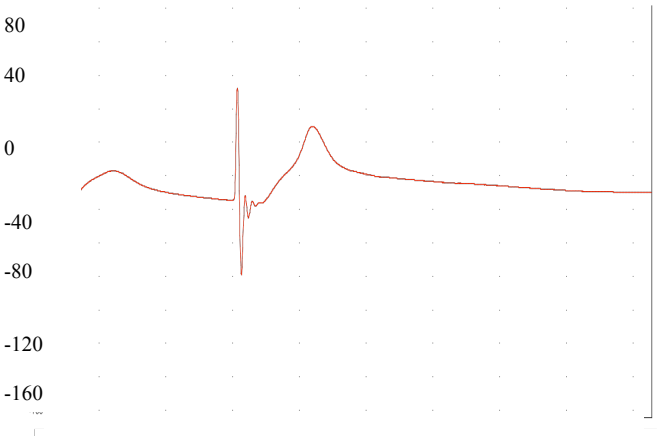

Figure 1.: EPSPs obtained from control trials of experiment. This figure depicts what a normal paired pulse result looks like. The second EPSP is larger than the first because of the residual calcium causing a release of more neurotransmitters into the synaptic gap.

Using the 10µM muscarine solution (50µL of muscarine in 100 mL of solution) at the 15 millisecond delay a normalized average of .42 mV amplitude difference was calculated, meaning on average, the amplitude of the second EPSP was .42 mV higher than that of the first. The average amplitude difference for the control test for 15 ms was .41 mV, only slightly less than the 10µM concentration trails. The 20µM concentration had an average .05 mV difference in amplitude size, bringing the first and second EPSPs much closer in size than in the control and in the 10µM concentration (see Figure 2 for comparison).

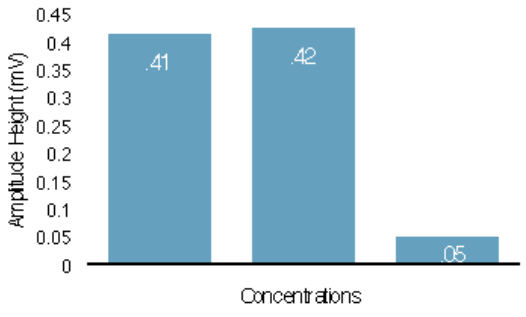

Figure 2: The Average Normalized Amplitude Difference for 15ms paired pulse trials. This graph depicts the comparative average amplitude differences for EPSPs when muscarine was applied in 10 and 20µM concentrations.

In the 20ms paired pulse trials there was an average amplitude difference of -.06 mV for the control data, $10x3^{-3}$ mV for 10µM data, and .09 mV for the 20µM concentration (see Figure 3). The results obtained from the trials done at 15 and 20ms the 10 and 20µM concentrations of muscarine show that our hypothesis can only be partially supported due to inconsistencies in the sets of data. The data collected for 15ms lent much more evidence in support of our hypothesis than the data for 20ms, which was much more inconsistent. Generally though muscarine clearly had some effect on the cells, even if our limited number of tests didn't reliably confirm how extreme it was, merely the application of muscarine caused EPSPs to take longer to return to the resting potential and caused fluctuation in the shape of the EPSP (See Figures 4 and 5).

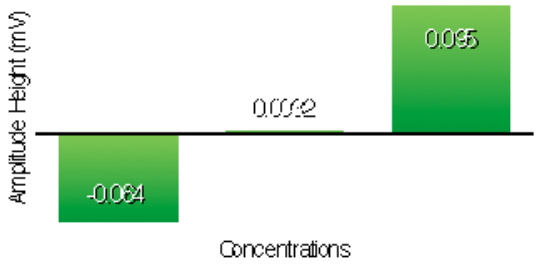

Figure 3: Average Normalized Amplitude Difference at 20ms paired pulse trials. This graph shows the average EPSP amplitude differences calculated for each concentration of muscarine applied to cells compared to the average amplitude difference of the control trials.

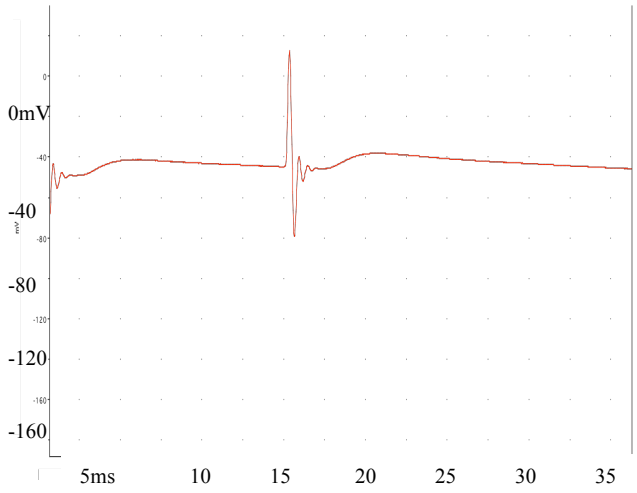

Figure 4: Example of EPSPs collected from 20µM trial. Muscarine was found to elongate the EPSPs as well as decrease the size of the amplitudes overall compared to the amplitude size of the control EPSPs.

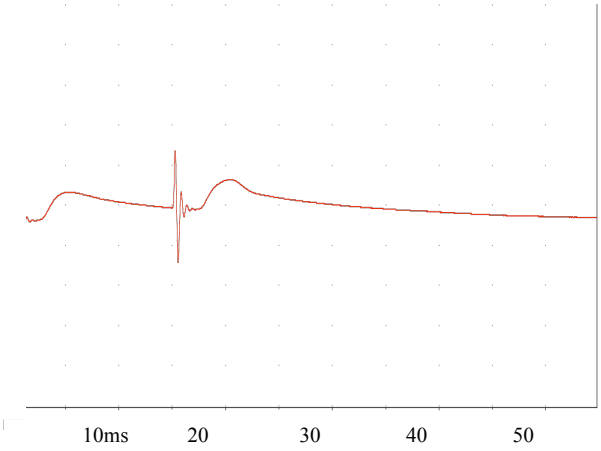

Figure 5: Example of EPSPs collected from 10μM trial at 20ms. As compared to the control trials the EPSPs are less in amplitude and longer lasting, though not quite as affected as the EPSPs of the 20μM trial.

DISCUSSION

We hypothesized that because muscarine continually activates the sodium calcium exchanger, and thereby removes residual calcium that remains in a neuron after the initial stimulation in PPF, the second EPSP of the pair would be relatively smaller than the first. While at both 15 and 20 ms delays, 10 μM concentration tests showed little significant average difference, the average difference between the first and second EPSP showed results inconsistent with what we expected due to prior research done on muscarine in other organisms, with the average differences actually increasing slightly, but significantly.

However, in the 15 ms delay trials, the 20 μM tests showed a significant difference in the direction we had expected: it showed a decrease from the average difference of the control, at .41 mV, to the muscarine trial, at .05 mV, versus the slight increase at 10 μM to .42 mV. (This compared to the results of average differences at 20 μM: -.06 mV at control, $10x3.2^{-4}$ mV at 10 μM, and 0.1 mV at 20 μM). Thus, our hypothesis is partially supported, more so at 15 ms delay than at 20 ms. The relatively small standard errors calculated for the average differences points to the general reliability and consistency of the data gathered (the major exceptions to this rule are the control at 15 ms at .42 SE and the 20 μM at 20 ms at .28 SE, which could be caused by a limited number of trials or by a handful of data present that constitute outliers).

The results we found may have been somewhat smaller than what we expected because muscarine may also act on the sodium calcium exchanger in the reverse mode, causing calcium to enter the cell as sodium is brought out (Xu et al, 2006). This causes calcium to stay in the cell at relatively the same concentrations as initially produced, which may explain why we saw relatively small changes in average differences between the first EPSP and the second during PPF, the residual calcium wasn't leaving the cell. To test whether this is in fact the case, the sodium calcium exchanger inhibitor KBR-7943, which inhibits the sodium calcium exchanger from working in reverse mode, could be used to see the degree to which muscarine actually affects the amount of residual calcium left in the cell (Flores-Soto et al, 2012). If the ability to work in reverse mode is removed, the full mechanism of muscarine's effects on the sodium calcium exchanger could be tested. Muscarine's effectiveness as a neural treatment for neural disorders and degenerative diseases, as discussed in Xu et al (2006), could be very interesting for future research, and could explore the role of muscarine and the sodium calcium exchanger in this regard.

Thus our work, while affected by some outliers, can at least partially confirm that muscarine does indeed cause some decrease in the second EPSP, although less intensely than we expected. Given the opportunity to repeat this experiment, we would definitely repeat all of our trials, with considerably more tests per concentration, and with a greater variety of concentrations, and perhaps experiment with other delay times. Our experimentation on crayfish also asserts the fact that acetylcholine receptors must be present in crayfish neuromuscular junctions, which has previously gone largely unaddressed in scientific literature, as most experiments have been conducted on rat brains (Xu et al, 2006).

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