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Effect of Extracellular Calcium Concentration on Facilitation in the Crayfish Neuromuscular Junction.

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ABSTRACT

The influx of calcium into the presynaptic cell is the primary mechanism by which the release of neurotransmitter into the synaptic cleft is achieved. This release of neurotransmitter is essential in the transmission of action potentials between neurons. The concentration of calcium outside the cell influences the amount of calcium that passes into the presynaptic cell, which subsequently influences the amount of neurotransmitter released and the intensity of excitatory postsynaptic potentials. Residual calcium from recently fired EPSPs also serves to increase the amount of calcium in the presynaptic cell and facilitate neurotransmitter release. We measured the level of facilitation between a first and second EPSP, triggered by paired pulse stimulation, in varying concentrations of extracellular calcium, to determine the role of calcium concentration in cooperativity and facilitation. The results demonstrated differences in the level of facilitation across concentrations, with the highest facilitation occurring between 3.38 mM Ca and 6.75 mM Ca outside the cell. This supports previous research on the nature of cooperativity in calcium dependence.

INTRODUCTION

Calcium is essential to synaptic transmission. Calcium influx into the presynaptic cell triggers synaptic vesicles already docked at the cell membrane to release neurotransmitter into the synaptic cleft (Andreu & Barrett, 1979; Mulkey & Zucker, 1991). When the extracellular concentration of calcium is raised, the concentration gradient across the cell membrane is higher, causing a greater influx of calcium into the cell than occurs at lower concentrations, causing more neurotransmitter to be released (Dodge & Rahaminoff, 1967). A greater concentration of neurotransmitter in the synaptic cleft causes more neurotransmitter gated ion channels to open in the postsynaptic cell, increasing the amplitude of the potential.

At very low levels of calcium, facilitation is low because the movement of calcium across the membrane is negligible. Conversely, when the concentration of calcium is high, a saturation effect occurs, most likely at the site at which calcium binds to an intracellular protein rather than at the actual calcium channel in the cell membrane, producing a diminished rate of return. This causes a ceiling effect on the speed and intensity of excitatory postsynaptic potentials (Dodge & Rahaminoff, 1967). In the middle range, facilitation occurs dramatically in a phenomenon that is known as cooperativity (Parnas, Parnas & Segel, 1986). This phenomenon has been studied in many different preparations, showing that rates of cooperativity vary somewhat across species (Parnas, Parnas & Segel, 1986; Dodge & Rahaminoff, 1967), although it has not been specifically investigated in the crayfish neuromuscular junction, to the best of our knowledge. We hypothesize that facilitation in the paired pulse stimulation will be most prominent at a 50% calcium concentration (6.75 mM), with much lower facilitation at normal concentration (13.5mM) and somewhat decreased facilitation in 25% and 75% concentrations. This pattern of facilitation is expected to follow a nonlinear curve (Dodge & Rahaminoff, 1967).

We manipulated the extracellular concentration of calcium from 3.38 mM to 13.5 mM in the crayfish neuromuscular junction to find the optimal level of calcium to facilitate neurotransmitter release and facilitation in paired pulse stimulation at a delay of approximately 50 ms. We found that, when facilitation was measured in terms of the difference between the amplitude of the second and the first EPSP, facilitation was significantly higher at 6.75 mM Ca, whereas an analysis of the ratio between the two amplitudes indicated that facilitation was highest at 3.38 mM Ca concentration outside of the cell. An additional analysis performed by dividing the difference between the two EPSPs by the amplitude of the first EPSP showed comparable results to those found in the simple ratio manipulation.

MATERIALS AND METHODS

Preparation

We conducted this experiment on the lateral extensor muscle found in the tail of the crayfish. To reach this muscle we cut longitudinally along the edges of the shell, from the base of the tail to the tip. We peeled back the outer middle shell and discarded the excess muscle to expose the muscles we were interested

in. While the excitatory post synaptic potentials were being measured, we kept the crayfish in a solution of saline. This saline kept the crayfish cells from dying while we were researching. We manipulated the calcium concentration in this saline to perform our experiment.

Setup

To get data we used two microelectrodes, one to record the resting potential in the cell and the other to deliver a stimulating pulse to the muscle. The recording electrode was filled with 3.0 M KCl solution and kept at a high level of resistance in order to more accurately measure the voltage across the membrane. This resistance level was generally restricted to between 15mV and 150mV to maximize accuracy. The other electrode had a larger opening and was used to hold a nerve bundle through which a current was passed. This current ranged from approximately 10V to approximately 100V and was increased as necessary in order to elicit EPSPs. Generally, voltage was increased as time wore on, indicating that gradual cell death was a significant factor in the amount of current necessary to elicit cell response. This electrode was filled with saline solution. Also in the solution were the two reference electrodes that grounded the micro-electrodes. The information we collected through these electrodes was routed to a computer that recorded the data for us. The standard saline that we based our experimental calcium concentrations was

205mM NaCl
5.4 mM KCl
13.5mM $CaCl_2$
2.67mM $MgCl_2$
10.0 mM Tris Buffer (pH 7.4)

Calcium concentrations

We used three different calcium concentrations and a control to do our experiments. The control was made up of the same concentrations of ions as the mixture above. Our three test concentrations were 25% of the normal calcium, 50% of the normal calcium, and 75% of the normal calcium. To obtain the different concentrations, we mixed the normal saline solution with a zero calcium solution of the following composition:

205 mM NaCl
5.4 mM KCl
0 mM $CaCl_2$
16.1 mM $MgCl_2$
10mM Tris buffer (pH 7.4)

The concentration of $MgCl_2$ in the buffer was manipulated to maintain consistent pH in the solution, to control for pH as a confounding variable. We changed the saline solution every fifteen minutes, unless we were in the middle of a measurement. In that case we changed at the next possible moment. This procedure was performed to prevent cell death.

Measurements

Facilitation was measured in terms of the amplitude of each of the two EPSPs produced by a paired pulse electrical stimulus set at a delay of 50ms. Amplitude of EPSPs was measured from the resting potential to the peak of the depolarization in the EPSP.

RESULTS

Our results indicate that facilitation does occur during paired pulse stimulation at a delay of 50 ms with all extracellular calcium concentrations ranging from 3.35 mM to the standard solution of 13.5 mM. When the data is studied in terms of the mean differences between the amplitude of the second and first EPSPs, this facilitation appears to be greatest at a concentration of 6.75 mM Ca (50% of normal), during which the mean difference in amplitude between the first and second EPSPs is approximately 2.35 mV. Figure 1 shows the mean difference between the EPSPs at the four different concentrations of extracellular calcium.

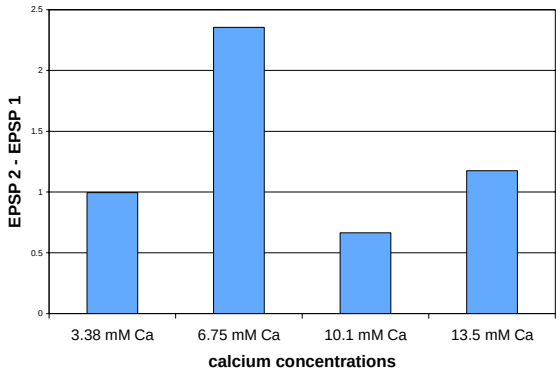

Figure 1. Differences in mean EPSP amplitude at ascending concentrations of extracellular calcium, demonstrating facilitation. Level of facilitation is highest at 6.75 mM Ca. For 3.38 mM Ca, n=14; for 6.75 mM Ca, n=12; for 10.1 mM Ca, n=13; and for 13.5 mM Ca, n=12.

T-tests conducted between the mean amplitudes of the second and first EPSPs showed that the difference between the two amplitudes was statistically significant at a level of $p = .05$ for each of the four concentrations of calcium. This indicates that the difference between the first and second pulses was due to facilitation rather than to any random error in measurement.

T-tests on the mean differences between EPSPs also demonstrated a statistically significant

difference between the level of facilitation in the 13.5 mM Ca saline solution and the 6.75 mM Ca solution ($p = .042$). Significant differences were also found between the 6.75 mM Ca solution and the 10.1 mM Ca solution ($p = .005$), as well as between the 3.35 mM Ca solution and the 6.75 mM Ca solution ($p = .014$).

Statistically significant differences in facilitation (as measured by mean differences in amplitude) at the level of $p = .05$ were not found between the 3.35 mM Ca and 10.1 mM Ca solutions ($p = .149$), the 3.35 mM Ca and 13.5 mM Ca ($p = .291$), or the 10.1 mM Ca and 13.5 mM Ca solutions.

Figure 2 shows the ratio of the amplitude of the second EPSP over the first EPSP at each of the four concentrations.

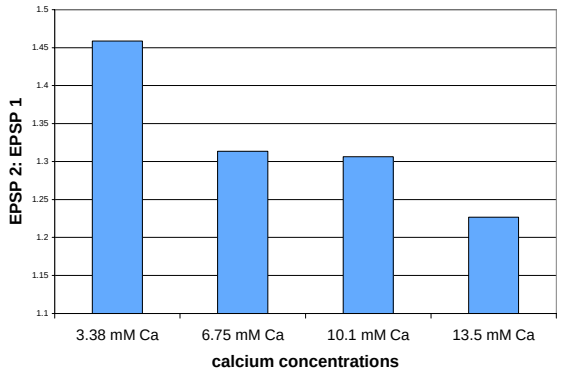

Figure 2. The ratio of second EPSP amplitude over first EPSP amplitude at increasing levels of extracellular calcium concentration. When viewed as ratio data, facilitation appears to be highest at 3.38 mM Ca. For 3.38 mM Ca, n=14; for 6.75 mM Ca, n=12; for 10.1 mM Ca, n=13; and for 13.5 mM Ca, n=12.

When facilitation was measured by the ratio of the second EPSP over the first rather than as the difference in amplitude, it was found to be greatest at a concentration of 3.38 mM Ca. The difference in facilitation between 6.75 mM Ca and 10.1 mM Ca was not found to be statistically significant ($p=.912$), nor was the difference between 3.38 mM Ca and 6.75 mM Ca. However, the difference in facilitation between 13.5 mM Ca and 3.38 mM Ca was found to be significant ($p=.048$). suggesting a significantly higher level of facilitation in 3.38 mM Ca solution than in normal saline.

Figure 3 shows an additional data manipulation that measured facilitation in terms of the difference in amplitude between the second and first EPSPs divided by the amplitude of the first EPSP. This manipulation produced similar results to the ratio measurement, with facilitation being highest at the 3.38 mM Ca concentration, lowest at 13.5 mM Ca (standard solution), and approximately equal in the 6.75 mM Ca and 10.1 mM Ca concentrations. A t-test between the 13.5 mM Ca solution and the 3.38 mM Ca solution shows a statistically significant difference in facilitation ($p=.024$). The difference between the 3.38 mM Ca solution and the 6.75 mM Ca solution was not found to be statistically significant ($p=.063$), which lends support to the idea that facilitation may be highest at some level in between the 25% and 50% conditions. This would help explain why our results appear different depending on the manipulation used to analyze the data.

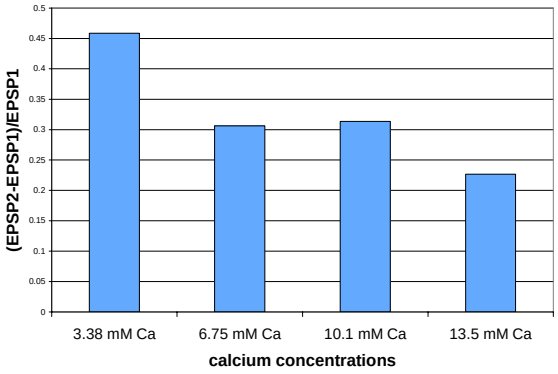

Figure 3. The difference in amplitude between second and first EPSPs divided by the amplitude of the first EPSP. This manipulation mirrors the results of the ratio analysis, with facilitation being highest at 3.38 mM Ca and lowest at 13.5 mM Ca (standard saline solution). For 3.38 mM Ca, n=14; for 6.75 mM Ca, n=12; for 10.1 mM Ca, n=13; and for 13.5 mM Ca, n=12.

DISCUSSION

In this experiment, we attempted to determine the effect that different levels of calcium have on facilitation during synaptic transmission. To test this we manipulated the amount of calcium in the saline that the crayfish preparation was bathed in. When the crayfish preparation had been in the modified saline for 5 minutes or more (in order to limit the effect of residual calcium from saline with a higher calcium concentration), we measured the EPSPs of the crayfish in each concentration and compared their amplitudes. We used a normal saline solution (13.5 mM Ca), a 50% calcium solution (6.75 mM Ca), a 25% calcium solution (3.38 mM Ca) and a 75% calcium solution (10.1 mM Ca). We hypothesized that the peak amount of facilitation would occur at 50% calcium level, with less at 75% percent and 25%, and the least facilitation at normal calcium levels.

Contrary to our hypothesis, several of our manipulations indicated that facilitation is greatest at 25% calcium, with the amount of facilitation decreasing as the calcium concentration increases. However, our hypothesis does seem to be confirmed when facilitation is measured simply as mean differences in amplitude between the EPSPs. This difference between results with regard to our manipulations could have been due

to several factors with regard to our difficulty in comparing EPSPs. The biggest reason was that we found a large variation in amplitude of EPSPs even within the same calcium concentration and on the same crayfish. Also, we feel that we may not have gathered enough data to strongly support or not support our hypothesis, due to the difficulty in getting the experiment to work correctly.

Our results did, however, indicate that there is a significant difference in the level of facilitation between a normal saline solution (13.5 mM Ca) and a 25% solution (3.38 mM Ca), with facilitation being higher in low levels of calcium. Overall, our results seem to indicate that facilitation is highest somewhere around the 3.38 mM Ca and 6.75 mM Ca levels. This is significant because it shows that the level of calcium in normal saline, which is designed to mimic the conditions inside of the living crayfish tail, is not ideal for synaptic transmission. Perhaps this serves as a natural adaptation in the animal to avoid over-stimulation of nerve cells.

To continue the investigation into effects of calcium concentration on the crayfish neuromuscular junction, we feel that first it would be important to get a stronger base of data from this type of experiment, and to possibly experiment with changing settings such as the delay setting on the stimulator. We initially chose our delay setting of 50 ms because of preliminary studies that showed a reasonable amount of facilitation (the difference in amplitude was clearly visible to the naked eye) at this setting in normal saline solution. Using a lower setting of delay may produce greater amounts of facilitation, as previous research has shown that rapid stimulus tends to increase the level of facilitation (Mukhamedyarov, Zefirov & Palotás, 2006; Hess & Kuhnt, 1992). Changing these settings may also make this experiment work more or less well. Future research could also study the relationship between stimulus trains and residual calcium levels inside the presynaptic cell to determine optimum levels of delay for facilitation (Brain & Bennett, 1995).

After increasing the amount of data, it would be interesting to try and find exactly how much calcium was needed to create an EPSP. This could be done by gradually increasing the calcium concentration in the saline until an EPSP was recorded. This would give information relating to our experiment and possible give way to another hypothesis that could be supported by this type of experiment.

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