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CALP2 Calcium/Calmodulin Agonist does not affect End-Plate Potential Amplitudes at the Crayfish Neuromuscular Junction

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ABSTRACT

Ca^{2+}/calmodulin (CaM) is a protein that can bind to and activate a variety of target proteins within the pre- and postsynaptic cells of the neuromuscular junction (NMJ). An important presynaptic CaM target is CaM-kinase II (CaMKII). Upon activation, CaMKII has been shown to increase neurotransmitter release from the vertebrate presynaptic terminal. This study addressed the effects of CALP2, a CaM agonist, at the crayfish NMJ. No previous research has studied the effect of CaM agonists on end-plate potential (EPP) amplitudes at the crayfish NMJ, hence the novelty and importance of our experiments. Using single and paired-pulse stimulation of the motor nerve, we measured EPPs in the absence and presence of CALP2. Our results indicate that the application of CALP2 had no significant effect on EPPs. However, this preliminary research highlights the need for a more in-depth investigation to circumvent concerns of small sample sizes and possible CALP2 degradation.

INTRODUCTION

Calcium (Ca^{2+}) ions present at the neuromuscular junction (NMJ) are critical for modulating an array of neuronal functions, such as neurotransmitter release (Katz and Miledi, 1965) and experience-dependent plasticity (Lisman *et al*., 2002). Calcium/Calmodulin (CaM) is a highly conserved, cytoplasmic-calcium binding protein that can bind to and regulate a variety of protein targets within the presynaptic and postsynaptic cells of the NMJ. CaM has a high affinity for calcium and it undergoes a conformational change once Ca^{2+} ions are bound to all four EF-hand motif sites, thus activating the protein (Gifford *et al.,* 2007). Once activated, CaM acts as an intracellular signaling molecule that modulates many presynaptic and postsynaptic functions. In the presynaptic neuron of the NMJ, CaM may function as a Ca^{2+} sensor that modulates release of neurotransmitter (Chen *et al.*, 1999) and regulates the refilling of fast-releasing synaptic vesicles of the ready-releasable pool (Sakaba and Neher, 2001).

One of CaM's presynaptic target proteins is calmodulin-dependent kinase-II (CaMKII). In the presynaptic terminal, CaMKII regulates the mobilization of vesicles to be docked at the membrane for release (Greengard *et al.*, 1993). In the mouse, Pang *et al* (2010) were able to inhibit the expression of CaM with an RNAi knock down (KD) technique, and as result, found that neurotransmitter release was impaired. However, in a mutant, when CaMKII was overexpressed and CaM was also knocked down, neurotransmitter release increased relative to CaM KD alone. The authors suggested that when CaM expression is reduced, but CaMKII is overexpressed, residual CaM is more likely to locate and bind CaMKII, thereby recovering neurotransmitter release. Their results provide clear evidence that CaM binds to CaMKII in the mouse presynaptic neuron, and in doing so, promotes neurotransmitter release (Pang *et al*., 2010).

The activation state of CaMKII varies depending on the degree of the Ca^{2+} signal and may function as a detector for calcium oscillations and a synaptic activity sensor. Weak Ca^{2+} signals caused by low frequency stimulation can lead to CaMKII activation that is short lived. Brief CaMKII activation occurs because $[Ca^{2+}]_i$ quickly declines post-stimulation, which only allows for a brief period of CaM activation. Once the local Ca^{2+} signal decays and CaM becomes inactivated; CaM dissociates from the kinase, which also inactivates. If the period and magnitude of the $[Ca^{2+}]_i$ signal is greater, such as during high frequency and/or paired-pulse stimulation, steady-state CaM activation is increased. Under these conditions, two CaM molecules can bind CaMKII, leading to kinase autophosphorylation. Here, CaMKII remains active even after $[Ca^{2+}]_i$ returns to basal levels. This property allows the kinase to alter its activities depending on the frequency and duration of a stimulus (Lisman *et al*., 2002).

To our knowledge, no one has investigated the effects of Calcium/Calmodulin agonists on EPP amplitudes at the crayfish NMJ. Our research sought to characterize the effect of calcium-like peptide (CALP) 2 application on EPPs at the crayfish NMJ. CALP2 is a cell-permeable CaM agonist that competitively binds to the EF-hand Ca^{2+}-binding sites and activates CaM in the absence of

Ca^{2+} influx or release from intracellular endoplasmic reticulum stores. A few recent studies have utilized calcium-like peptides to investigate various physiological functions of CaM (Broeke *et al*., 2003), but none have focused on the effect of CALPs on EPP amplitude at the NMJ. We hypothesized that CALP2 application would increase the EPP amplitudes at the crayfish NMJ relative to the control condition. Because CaM has been shown to promote neurotransmitter release at the vertebrate mouse NMJ via activation of CaMKII (Pang *et al*., 2010), we expected to see a similar increase in EPP amplitudes at the crayfish NMJ. By applying CALP2 and stimulating the crayfish motor nerve, we speculate that the combined contribution of Ca^{2+} influx and CaM agonist application would increase the steady-state activation of CaM within the presynaptic nerve, relative to control treatment without CALP2. Consequently, increased neurotransmitter release and greater EPP amplitudes at the crayfish NMJ should result when in the presence of CALP2.

In our study, we stimulated the motor nerve of a crayfish NMJ preparation and made electro-physiological measurements of postsynaptic cell end-plate potential (EPP) amplitudes. First, EPPs were recorded by bathing the prep in normal crayfish Ringer's solution (control condition). Second, the solution was removed, replaced with Ringer's containing CALP2 (experimental condition), and EPP recordings then followed. Results indicate that CALP2 application did not significantly increase the amplitude of EPPs relative to the control.

MATERIALS AND METHODS

Dissection. In the crayfish abdomen, the ventral exoskeleton and flexor muscles were removed, thus exposing the abdominal extensors. Extensor muscles and their innervating motor nerves were used for all experiments. A single abdominal segment was isolated by cutting just proximal and distal to that segment. The segment was cut down the midline in order to separate it into two sections. One of these sections was placed in a dish where we performed electrophysiological recordings.

Treatment. Normal Ringer's solution contained (mM): 5.4 KCl, 196 NaCl, 2.6 MgCl, 13.5 $CaCl_2$, 10 Hepes (pH 7.4). For the control treatment one segment was placed in a dish with 2.75 mL of normal Ringer's solution and recordings were taken. For the experimental treatment, the normal Ringer's solution was replaced with Ringer's plus 100 µM CALP2 (Tocris Bioscience; Ellisville, Missouri), which sat for ten minutes before recordings were taken. A total of three indepentent trial runs (experimental replicates), following the experimental progression outlined above, were carried out using abdominal sections excised from three individual crayfish. For clarification, we chose to call experimental replicates, "trials" or "trial runs."

Electrophysiology. The presynaptic motor nerve was drawn into a glass suction electrode filled with crayfish Ringer's solution or the Ringer's solution with CALP2 for control and experimental procedures, respectively. The motor nerve was stimulated by applying paired-pulses at approximately 2-3 V, for a duration of 0.6 ms with a delay of 50 ms. A micro-electrode, filled with 3M KCl and connected to a conventional amplifier, was used to record postsynaptic EPP amplitudes following nervous stimulation. Resistance of all electrodes was 5-20 MΩ. EPP amplitudes were measured as the immediate voltage change following stimulation to the peak of EPP response. Three separate EPP amplitude measurements (sample replicates) were taken from the same muscle cell, in one crayfish section, for both the control and CALP2 treatment conditions. For convenience, we will refer to the sample replicates as "samples." As a final clarification, three samples were recorded during both the control and CALP2 treatments, for all three independent trial runs.

Data Analysis. The first of the paired-pulses was used for analysis of single pulse EPP amplitudes. The three EPP sample measurements, recorded from control and CALP2 treatments, were averaged for each of the three trial runs. A percent change was calculated for each trial run using the formula: ((avg EPP CALP2) / (avg EPP cont)). A total % change was calculated by averaging the percent changes from the three trials, which is defined as the change in EPP amplitudes during CALP2 treatment relative to the control. For analysis of paired-pulse EPP amplitudes, we calculated a ratio of the second pulse (P_2) relative to the first (P_1) using the formula $((P_2 - P_1) / P_1) * 100$. This resulted in a ratio for each sample, under both control and experimental conditions. Ratios were averaged for each trial and again averaged to yield the total % change in ratios. A paired t-test was also used to test for a difference between control and experimental condition in EPP amplitudes. The null hypothesis is that EPP amplitudes under experimental condition are the same as control condition. We rejected the null hypothesis if $p \leq 0.05$.

RESULTS

CaM undergoes a conformational change and becomes activated when Ca^{2+} has bound to all four of its EF-hand sites. The activated CaM protein can act on CaMKII in the presynaptic terminal to promote neurotransmitter release. We hypothesized that application of CALP2, a calcium-like peptide CaM agonist, would promote neurotransmitter release and thereby increase EPP amplitudes at the crayfish NMJ. To study the effects of CALP2 on EPPs at the crayfish NMJ, we applied single and paired-pulse stimulation to the motor nerve and recorded EPP amplitude. Experiments were performed in the presence of CALP2 (100 µM) and its absence.

Effects of CALP2 on single pulse stimulation EPP amplitudes.

Percent changes were calculated for each trial run as a measure of the increase or decrease (with respect to 100%) in EPP amplitudes with the application of CALP2 relative to the control. In the absence of CALP2, the percent change in EPP amplitudes were (A) 294%, (B) 91%, and (C) 84% for the three trial runs, respectively. The cumulative percent change was 157±119%. Overall, there was no significant change in EPP amplitude when CALP2 was applied. However, the first trial run did result in a large, significant increase in EPPs with CALP2 application (Fig. 1A).

CALP2 did not affect paired-pulse facilitation

We calculated a ratio of the second pulse EPP amplitude relative to the first, for both the control and CALP2 conditions. The percent change in paired-pulse facilitation (PPF) is a comparative measure of an observed increase or decrease in EPP ratios with the application of CALP2 relative to the control. The percent changes in paired-pulse EPP ratios for each of the three trial runs were (A) -37%, (B) 96%, and (C) 45%, respectively. The percent change in the cumulative paired-pulse EPP ratio was 35±67%. Therefore, the application of CALP2 did not significantly alter PPF.

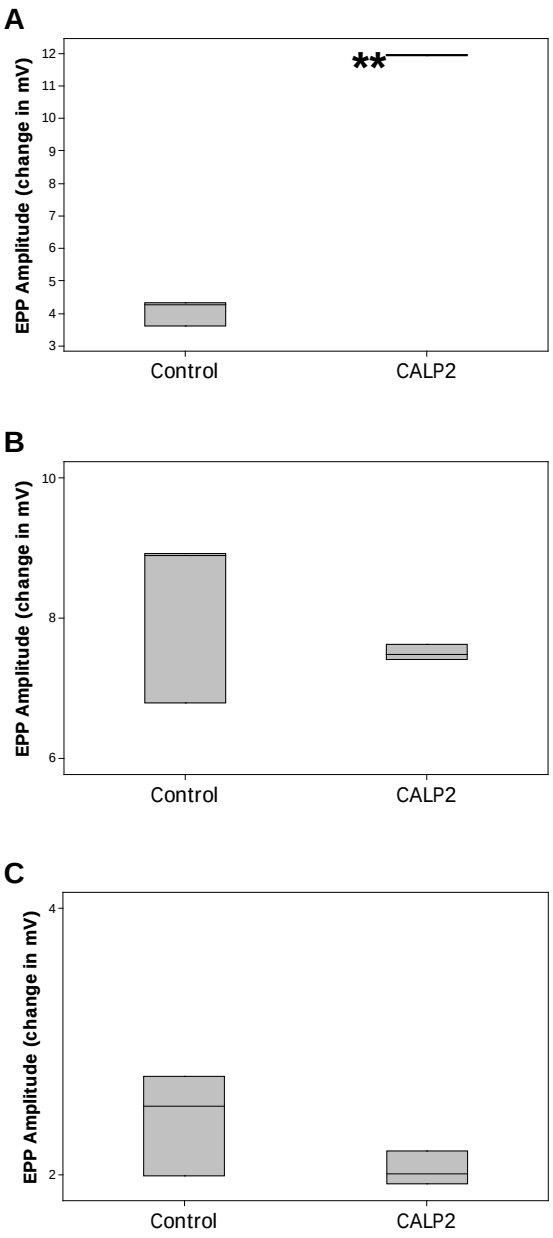

Figure 1. Box plots of single pulse stimulation EPP amplitudes for three trial runs. EPP amplitude represents the measured voltage change immediately following stimulation to the peak of EPP response. Boxes correspond to measurements from three samples for each of control and CALP2 treatments. Each graph shows results of one of three trial runs. (**A**) Application of CALP2 significantly increased EPP amplitude in the first trial run (**P<0.001). (**B** and **C**) However, CALP2 application had no effect (P>0.05) on EPP amplitude in successive trial runs.

DISCUSSION

In this research, we sought to characterize the effect of a CaM agonist, CALP2, on EPP amplitudes at the crayfish NMJ. Previous research has shown that activated CaM can interact with CaMKII in the presynaptic terminal. CaMKII has been shown to play a role in modulating the quantity of ready-releasable vesicles and regulating vesicle release probability at the NMJ presynaptic membrane (Pang *et al.*, 2010). Because activated CaMKII may increase neurotransmitter release probability, we predicted that during application of CALP2, steady-state CaM activation would increase and act through the presynaptic CaM-CaMKII pathway to promote neurotransmitter release. Therefore, we expected to see an increase in EPP amplitudes with CALP2 treatment relative to the control. However, the results inadequately support our hypothesis. Overall, CALP2 did not significantly increase EPP amplitudes at the crayfish NMJ, but we suspect this may be due to small sample sizes and possible CALP2 degradation.

In the three trial runs of our experiment, we witnessed one trial in support and two trials in contradiction to our hypothesis. In the first, trial A, EPP amplitude significantly increased with the application of CALP2, as predicted (Fig. 1A). Also, it is noteworthy that in this trial the PPF ratio decreased with CALP2. A decrease in PPF ratio is consistent with an increase in CaM activation and a subsequent increase in neurotransmitter release. This result was expected based on our hypothesis that CALP2 would increase steady-state CaM activation, thereby activating CaMKII, which has been shown to have various presynaptic effects such as increased neurotransmission (Pang *et al*., 2010).

In the following trials, B and C, however, the EPP amplitudes displayed no statistically significant change from control to experimental samples (Fig. 1B and C). Had we seen an overall increase in EPP amplitude from control to experimental samples, this would have implicated CaM activation in increased neurotransmitter release. Alternatively, had we observed no change in EPP amplitudes over all three runs, this might have been an indication that CaM may not have an effect on neurotransmitter release, which would contradict previous research, or that CALP2 is not an effective CaM agonist. Additionally, PPF ratios increased 96% and 46% in trial runs B and C, respectively. This result was also inconsistent with trial A and our hypothesis that increased CaM activation, via CALP2, would increase neurotransmission and thereby decrease PPF ratios.

Because trial A showed a significant increase, and the latter two trials showed no change, we considered possible explanations for this. We speculate that the technique used to prepare the CALP2 solution may have been acting as an unrecognized variable in the experiment. On the first day of our experiments, the CALP2 solution was prepared fresh and used immediately for trial A. The remaining solution was aliquoted to 1.5 mL tubes and frozen to -20 ^{o}C for use in trials B and C during successive weeks. The freezing and subsequent thawing of CALP2 may have caused the calcium-like peptide to denature or have simply caused an adverse effect impairing its ability to agonize CaM activation. The difference in results from trial A to the others was not likely due to a difference in crayfish physiology. Because CaM is a highly conserved protein with a variety of functions, it is unlikely that its presence and structure would fluctuate between individual crayfish to cause this drastic difference in our results. Therefore, CALP2 may have denatured or degraded during solution preparation for trials B and C, rendering the peptide ineffective. This is a likely explanation for the result that cumulative EPP amplitude changes and the PPF ratio were not significantly altered with CALP2.

In future research, performing a greater number of trial runs would alleviate a concern for the thoroughness of this study. More trials would allow for a more definitive conclusion as to the effect CALP2 has on the crayfish NMJ. Also, to reduce the possibility that CALP2 was biologically-altered, CALP2 solutions should be prepared fresh for each experimental trial run. Once able to verify that CALP2 has an effect on EPP amplitude at the crayfish NMJ it would additionally be possible to study its effect on PPF.

This research is novel since there has been no previous research looking at the effects of CAPL3 on EPPs using the crayfish NMJ preparation. In the future, further research similar to what we have performed will hopefully fill gaps in knowledge related to the application of CaM agonists, its subsequent effects on EPP amplitudes at the crayfish NMJ, and provide an additional method for studying Ca^{2+}-independent CaM activation.

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