Pioneering Neuroscience, 2011, **12**, 37-40.

Inhibition of Glutamate Carboxypeptidase II Regulates Synaptic Transmission in the Crayfish Neuromuscular Junction

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ABSTRACT

Glutamate carboxypeptidase II (GCP II) is a glial ectoenzyme known to hydrolyze N-Acetylaspartylglutamate (NAAG), one of the most abundant and widely distributed neurotransmitters in the mammalian nervous system. It has been proposed that NAAG is linked to various conditions such as schizophrenia, ALS and diabetic neuropathy. However, the role of NAAG and other glutamatergic peptides at the crayfish neuromuscular junction has not been investigated. By introducing the GCP II inhibitor ZJ-43, we sought to further elucidate the mechanism by which GCPII regulates the synapse. To determine the effects of GCP II, we compared excitatory postsynaptic potential (EPSP) amplitudes of the control experiments and the experiments with the ZJ-43 treatments. We used the method of paired pulse stimulation to determine whether the effect was occurring in the postynapse or presynapse. In addition, we used low frequency and high frequency stimulation. Our results suggest that the inhibition of GCPII decreases the amplitude of the EPSP during both high and low frequency stimulation through mechanisms in either the presynapse or both the presynapse and postsynapse.

INTRODUCTION

N-Acetylaspartylglutamate (NAAG) is one of the most abundant and widely distributed peptide neurotransmitters in the mammalian nervous system (Bergeron, 2005, Urazaev, 2005). NAAG plays an important role in modulating neuronal transmission. NAAG inhibits the release of neurotransmitters, including glutamate, by activating type 3 metabotropic glutamate receptors ($mGluR_3$), a group II mGluR (Flores, 2003, Neale et al., 2005, Yamamoto et al., 2007). In addition, NAAG directly blocks NMDA receptors, reducing excitatory postsynaptic potentials (EPSPs). Furthermore, NAAG activity is also determined by the level of neuronal activity.

In the presence of low levels of stimuli, little or no NAAG is co-released. However, as neuronal activity increases, which can be mimicked by high frequency stimulation, larger quantities of NAAG are released (Neale et al., 2005).

Glutamate carboxypeptidase II (GCP II), a glial ectoenzyme, hydrolyzes N-Acetylaspartyl glutamate (NAAG) to N-acetylaspartate (NAA) and glutamate. The glutamate produced can also activate glutamatergic receptors to modulate synaptic transmission (Flores and Coyle, 2003). Changes in GCP II function have been shown to be critically associated with changes in NMDA receptor activation. Low levels of GCP II activity result in higher extracellular levels of NAAG that lead to NMDA receptor hypofunction by decreasing glutamate release and acting as an antagonist at NMDA receptors in the rat brain (Flores and Coyle, 2003).

Urazaev et al. (2005) has already confirmed that GCP II is present in crayfish medial giant nerve fiber and the hydrolytic activity is activated by glial receptors, primarily $mGLUR_{II}$. However, the GCP II modulatory functions at the crayfish neuromuscular junction have not been investigated. Therefore, we sought to confirm that GCP II plays a role in crayfish neuromuscular transmission-- in addition we aimed to investigate the mechanism by which GCP II regulates the synapse in either the presynapse or postsynapse.

MATERIALS AND METHODS

Crayfish and its preparation

The crayfish was cooled in an ice bath, which served the ethical purpose of reducing the pain the animal would experience when the tail was separated from the thorax. After movement ceased the tail was removed. Two longitudinal cuts were made along the lateral edges of the tail. Ventral portion of the exoskeleton were pulled off and the remaining muscle tissue was gently pushed out. The dorsal exoskeleton containing the superficial extensor muscles and nerves was pinned into a dissection dish. This dish was filled with either 100ml of standard crayfish saline or 100ml of experimental saline.

Drug and Saline Preparation

A GCP II inhibitor ZJ-43 was purchased from the company Tocris. Two groups of experiments- a control

group and a drug group- were conducted. Control group used standard crayfish saline, which consisted of 5.4 mM KCl, 196 mM NaCl, 2.6 mM MgCl2, 13.5 mM CaCl2 and 10 mM HEPES. The Drug group consisted of the standard crayfish saline with ZJ-43 present. The saline with 0.1µM ZJ-43 was made by adding 10 µl of 1mM ZJ-43 into 100ml standard crayfish saline. A lower concentration, .025 µM of ZJ-43, was also used.

Electrophysiology

Electrodes were made by a micropipette puller using 1.2mm glass tubes and filled with 3M KCl. This instrument creates sharp glass tips that provide accurate membrane potential readings via intracellular recording on the computer program called Scope. Microelectrodes were replaced once the resistance was determined to be less than 20MΩ. Nerves were found under the microscope and sucked into an electrode by a syringe. Paired-pulse stimuli were used to test the drugs' effects on the neuromuscular junction through detection on the changes of EPSPs. Two suprathreshold stimuli with a 40ms interpulse interval were given to the neuron with two different frequencies which were once per 2 second and twice per second. The EPSPs were recorded in both the control group and the drug treatment group. EPSPs were recorded, starting with low frequency stimuli for one minute and suddenly changing to high frequency stimuli which were maintain for 10 seconds and then changing back to low frequency.

RESULTS

The aim of our research was to discover the effect of inhibiting of the enzyme GCP II on the excitatory post synaptic responses (EPSPs) in the crayfish neuromuscular junction. To test this, we introduced a saline containing 0.1µM of ZJ-43, a GCP II inhibitor.

Using low (0.2 Hz) and high (2 Hz) frequency stimulation, we uncovered various changes frequency stimulation of the nerve in the standard crayfish saline increased the EPSP by 1.97mV. After the introduction of ZJ-43, the difference in depolarization decreased to 1.57 mV. (fig. 1) Immediately after high frequency stimulation, the return to low frequency caused a slightly greater EPSP amplitude than the EPSP amplitude recorded during the initial low frequency stimulation, a difference of .271 mV. After the addition of ZJ-43, the amplitude of the EPSP was reduced in comparison to the EPSP of the control conditions in both high and low frequency. In contrast to the control conditions, there was a difference between the period directly after 2Hz of stimulation and the initial .2Hz of stimulation. After the introduction of ZJ-43, the EPSP amplitude returned immediately to the initial value at low frequency stimulation.

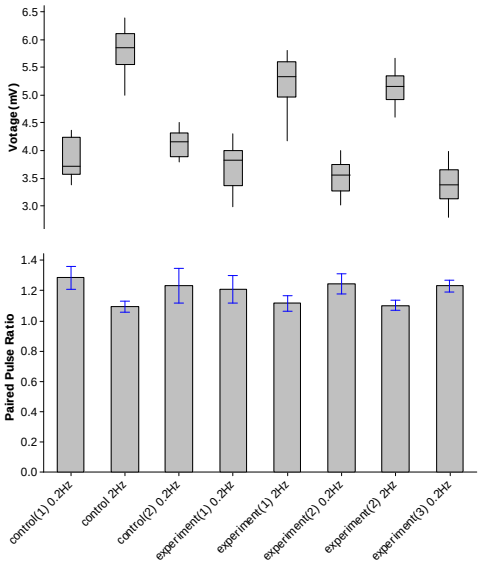

Fig. 1. Control and experiment during low and high frequency and the ratio of the average first EPSP in comparison to the second and median amplitude of EPSPs. The obtained ratio is a result of the second EPSP divided by the first in paired pulse stimulation. Low frequency was at a rate of .2 Hz and high frequency was at a rate of 2 Hz. (Control(1) 0.2Hz, control 2Hz, experiment(1) 2Hz, and experiment(2) 2Hz n=20. Control(2) 0.2Hz and experiment(1) 0.2Hz n=15. Experiment(2) 0.2Hz n=30. Experiment(3) 0.2Hz n=65.)

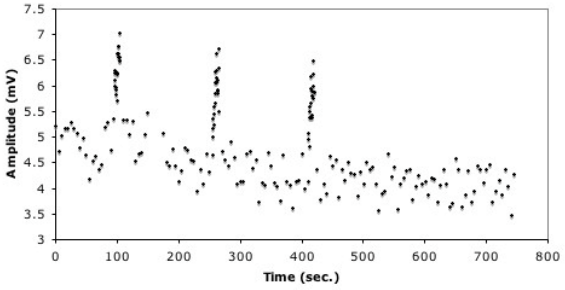

Fig. 2. The effects of ZJ-43 on the amplitude of EPSPs over periods of low frequency and high frequency. High frequency was initiated at three points during this time span at 95 seconds, 255 seconds, and 411 seconds. High frequency stimulation lasted ten seconds each time. Amplitude was recorded every half second during high frequency and every five seconds during low frequency. A total of 208 amplitudes were collected over 745 second time span.

In fig.1, the paired pulse ratios are depicted as they relate to changes in high and low frequency of EPSP amplitude. In the control group, 2Hz stimulation resulted in facilitation. With 2 Hz of stimulation we saw a decrease in the paired pulse ratio of 1.29 to 1.09. After the introduction of ZJ-43, the first 2Hz paired pulse ratio

maintains the facilitation without change. However, the second experiment with 2Hz stimulation decreases in facilitation from 1.31 to 1.07. The decrease of the paired pulse ratio in the second 2Hz stimulation of the experimental group is less than the decrease of the ratio in the 2Hz stimulation of the control group ($p<0.01$; fig. 1).

The effects of ZJ-43 are shown in fig. 2 with EPSP amplitude recorded over a period of 745 seconds. The first 2Hz stimulation in the standard crayfish saline resulted in an EPSP of 5.81mV on average. After the introduction of ZJ-43 at 180 seconds, the 2Hz stimulation resulted in an EPSP of 5.27mV on average. The difference between these two values is 0.54mV ($p<0.001$; fig 1). With the second 2Hz stimulation the amplitude depolarized even less to 5.12 mV, differing from the normal conditions by .69mV ($p<0.001$; fig. 2).

DISCUSSION

The difference in the amplitude of the EPSP during low and high frequency stimulation between the control and experiment groups (fig. 2) suggests that GCP II modifies the crayfish neuromuscular junction. Changes in EPSP amplitudes, when linked to the differences in the ratios of paired-pulse stimulation, suggest that EPSP variations are due to functions in the presynapse.

The reason why we see lower amplitudes of EPSPs in experimental groups compared to control groups is that GCP II can inhibit NAAG's activity (Flores and Coyle, 2003). Therefore as more exogenous NAAG is created from the inhibition of GCPII, more NAAG binds to receptor sites on the presynaptic cell, inhibiting glutamate release or more NAAG directly blocks NMDA receptors and thus lower the amplitude of the EPSP (Neale et al. 2005).

Under the control conditions, we found the changes of paired pulse ratios were related to changes of the frequency of stimulation. When the EPSP amplitude increased from low to high frequency, there was a change in the paired pulse ratio. This ratio change is consistent with Flores and Coyle's hypothesis that glutamate from NAAG hydrolysis can activate glutamatergic receptors in the presynapse to modulate synaptic transmission (2003). We are compelled to conclude that the relationship between the changes of EPSP and paired pulse ratio is due to functions in the pre-synapse because of the ratio change in the paired pulse stimuli. An increase in the first EPSP could be due to the opening of the pre-synaptic voltage gated calcium channels by glutamate release while the second EPSP would not be able to increase as much. Thus, the ratio in the paired pulse stimuli would decrease.

Unlike the control group, upon returning to low frequency stimulation, the experimental group displays a voltage similar to that before the 2Hz stimulation. In the control group, the voltage does not return to the initial recording. Because less NAAG was hydrolyzed and/or less glutamate was able to bind to the receptors on the pre-synapse, the nerve needed less time to return to the initial voltage.

By introducing ZJ-43, we observed a decrease in the EPSP amplitude. Because GCP II enzymatic activity was blocked, higher levels of exogenous NAAG were produced. Because of the difference in paired pulse ratio, similar to the findings in the control conditions, we conclude that the change in EPSP amplitude is due to changes in the presynapse. It is possible that the increased levels of exogenous NAAG are acting on the presynaptic cell to inhibit neurotransmitter release. It is also possible that lowered levels of glutamate facilitate less neurotransmitter release. Neale et al. speculated that the presynaptic receptors activated by NAAG are type II $mGluR_3$ (2000; 2005). Whether the effect is derived from higher levels of exogenous NAAG or lowered levels glutamate cannot be extrapolated from these results. Further experiments are needed to come to a conclusion.

ACKNOWLEDGEMENTS

This work was made possible by the help of Sir Dr. Lindgren (Packer Pride) of Grinnell College. Other vital roles include Sue Kolbe, Ashley Millet and Christopher Kaiser-Nyman.

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