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L-2-Amino–3-phosphonopropionic acid modulates the release of glutamate by blocking presynaptic group II mGlu receptors at neuromuscular junction in *Procambarus clarkii.*

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ABSTRACT

The non-selective antagonist DL-AP3, L-2-Amino-3-phosphonopropionic acid, was used to regulate the response of the Group II metabotroic glutamate receptors (mGlurs) on the presynaptic terminal in the common North American crayfish, *Procambarus clarkii*. The experiment was conducted to better understand the negative feedback mechanism of the Group II mGlurs, thereby furthering current research concerning the adverse effects of amyotrophic lateral sclerosis (ALS) and other degenerative neurological diseases caused by neurotoxicity within the synaptic cleft. Our results confirmed the hypothesis that the addition of the antagonist DL-AP3 to an extracellular fluid containing high concentrations of glutamate precipitated a subsequent increase in the stimulated excitatory postsynaptic potential (EPSP). Likewise, the difference in the addition of DL-AP3 to an extracellular fluid containing a high concentration of glutamate when compared to an extracellular fluid containing a low concentration of glutamate without the addition of DL-AP3 was not statistically significant. The modulation of frequencies between 0.5 Hz and 10 Hz provided further evidence to support our hypothesis by demonstrating that the Group II mGlurs were responsive to increased activity of synaptic transmission.

INTRODUCTION

A high concentration of glutamate within the synaptic cleft produces neurotoxicity. Amyotrophic lateral sclerosis (ALS) has been associated with the slow degeneration of neural receptors (Rothstein J. D., Jin L., Dykes-Hoberg M., and Kuncl R. W., 1993). The process of neurotoxicity has been attributed to the malfunction ofsynaptic transmission. In an experiment conducted by Rothstein et al. (1993), the use of selective ionotropic inhibitors showed the possibility of causing slow neurotoxicity. Rat spinal cord cultures were bathed in increasing concentrations of selective inhibitors that interfered with the transportation of glutamate out of the synaptic cleft. This resulted in excess glutamate being present in the synaptic cleft without a reuptake mechanism. This subsequently caused the degeneration of motor neurons. The extracellular application of DL-AP3 caused similar results to those demonstrated by Rothstein et al. (1993), even though the process of neuorotoxicity was achieved by different means. By initially blocking the metabotropic receptors, the neurons did not recognize the concentration of glutamate, a result similar to that of Rothstein et al. (1993) using ionotropic inhibitors. Our experiment, however, was not focused on neurotoxicity as much as it was focused on the effects of antagonists that may cause neurotoxicity. By studying these processes, we gained a better understanding of regulatory functions of synaptic transmissions.

The ability to manipulate the process of glutamate modulation by the Group II mGlurs will play an important role in developing cures for degenerative neurological disorders. Blocking the negative feedback mechanism of glutamate reuptake has been demonstrated to adversely affect the neurons by causing excitotoxicity. Excitotoxicity is characterized by an excessive concentration of neurotransmitter that can eventually lead to deterioration of the neuron through over-stimulation or degeneration of the cell. The causes of amyotrophic lateral sclerosis, Lou Gehrig's disease, have been associated with slow neurotoxicity caused by an inability to properly regulate glutamate concentration within the brain (Rothstein et al., 1993). Our experimental results provide a better understanding of the mechanisms that affect the modulation of glutamate by the Group II mGlu receptors along the presynaptic terminal.

GABA and glutamate, respectively, function as the major inhibitory and excitatory neurotransmitters in mammals. (Schoepp, 2001). Glutamate receptors can roughly be separated into two distinct categories: ionotropic and metobotropic. A distinguishing characteristic of the ionotropic glutamate receptors is that they are directly associated with the transmission of EPSPs through ligand-gated ion channels. Metobotropic glutamate receptors indirectly affect

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ion channels through the activation of G-proteins. Ionotropic glutamate receptors are primarily located postsynaptically, whereas the metabotropic glutamate receptors have been known to exist at presynaptic, postsynaptic, glial, and heterosynaptic locations (Schoepp, 2001). Another characteristic that distinguishes the ionotropic from the metobotropic receptors is that the process of activating G-proteins and the subsequent transmission of effector proteins by the mGlurs is slower than the ligand-gated channels.

Metabotropic glutamate receptors play an important role in the modulation of the glutamate concentration within the synaptic cleft (Scanziani M., Salin P. A., Vogt K. E., Malenka R. C., and Nicoll R. A., 1997). Metabotropic glutamate receptors have been demonstrated to exist in the crayfish neuromuscular junction even though the structure differs from those found in mammals (Feinstein N., Parnas D., Parnas H., Dudel J., and Parnas I., 1998; Arnone N., Johnston M., and Prekker M., 2000). Crayfish were an ideal model for our study because they exhibit many of the same neural and cellular properties as humans (Blundon & Bittner, 1992), without the complexities that are involved in studying the mammalian nervous system. Metabotropic glutamate receptors modulate the excitability of the central nervous system (Schoepp, 2001). Metabotropic glutamate receptors can be categorized into three distinct subgroups according to the structure and the similarity of their pharmacological receptor sites (Cartmell and Schoepp, 2000). The Group II metabotropic glutamate receptors, specifically the mGlu2 receptor sites, play a fundamental role in the regulation of the concentration of glutamate within the synaptic cleft through a negative feedback mechanism (Scanziani et al., 1997; Cartmell and Schoepp, 2000; Schoepp, 2001). Under normal conditions of low frequency neuronal activity, the concentration of glutamate is not sufficient to warrant a response from the mGlu2 receptors due to its location outside of the synaptic cleft. The binding affinity of glutamate (represented as an EC50 value) to cloned mGlu2 receptor sites was between 0.3 and 20 mM indicating that even a slight concentration of glutamate would cause the mGlu2 receptor sites to activate G-proteins that will decrease the release of glutamate into the synaptic cleft (Cartmell and Schoepp, 2000; Schoepp, 2001). Based upon this knowledge we anticipated that the application of the antagonist DL-AP3 to an extracellular fluid containing high concentrations of glutamate would precipitate a subsequent increase in the stimulated excitatory postsynaptic potential.

MATERIALS AND METHODS

Preparation of Standard Crayfish Saline Solution

To make 1liter of standard saline crayfish saline solution, we mixed 0.4026g of KCl, 12g of NaCl, 0.5288g of MgCl x $6H_2O$, 0.1932g of $NaHCO_3$, and 0.3604g of Dextrose. We added 2.3g $CaCl_2$ x H_2O to prevent precipitation. The target pH for the solution was 7.4. Finally, we added the necessary amount of water to bring the volume of the solution to 1 liter.

Dissection of crayfish

We were given a crayfish tail that was cut from the thorax of a crayfish that had been sitting in ice for approximately 10 minutes. We placed the tail in a 100ml bath of standard saline solution in a Sylgard-lined preparation dish. In order to expose the superficial extensor muscle fibers, we followed the dissection procedure as described by Stephens (1996). We cut longitudinally with dissection scissors through the shell and flexor muscles along the indentations in the abdomen on both sides. Beginning at the anterior, we pulled the two halves of the shell apart and discarded the ventral portion leaving the telson portion attached to the dorsal shell. Using forceps we removed the gut by pulling it away from connective tissue and by holding it to the dorsal side of the tail. The potential difference of the crayfish muscles was recorded from the second segment from the tail of the lateral muscle on the left side.

Preparation of glutamate solution

We calculated the mass of glutamate needed for making a 0.1µM concentration of glutamate solution. We used a digital scale to measure 0.0029g of powdered glutamate. After we measured the desired amount of glutamate we added it to 200ml of standard saline solution. We measured 100ml of the prepared glutamate solution and put it into a different beaker. We then measured 100 µl of the DL-AP3 inhibitor solution and added it to the other 100ml of the glutamate solution.

Preparation of the DL-AP3 glutamate antagonist solution

We were given a stock solution of 10mM DL-AP3 made up in 1M NaOH. This chemical was ordered from the company Tocris Cookson (www.tocris.com). In order to run our experiment we needed a concentration of 10 µM of the DL-AP3 solution. To prepare the solution we used a 20-200µl labpette (brand Labnet) to measure 100µl of the stock solution. Then we measured 100ml of the standard saline solution (5.4mM). Finally, we added the

100µl of the DL-AP3 to the 100ml of the crayfish saline solution

Experiment A
Test 1: Stimulation: 0.5 Hz Chemical(s): none
Test 2: Stimulation: 10 Hz Chemical(s): none
Test 3: Stimulation: 10 Hz Chemical(s): DL-AP3
Experiment B
Test 1: Stimulation: 0.5 Hz Chemical(s): none
Test 2: Stimulation: 0.5 Hz Chemical(s): DL-AP3
Experiment C
Test 1: Stimulation: 0.5 Hz Chemical(s): none
Test 2: Stimulation: 0.5 Hz Chemical(s): glutamate
Test 3: Stimulation: 0.5 Hz Chemical(s): glutamate, DL-AP3

Table 1: Summary of the experiments conducted to test the effects of DL-AP3, metabotropic glutamate antagonist, on the Group II presynaptic glutamate receptors. Each experiment was done with a different crayfish specimen.

Measuring of the EPSPs

We made the electrodes with PUL-1 World Precision Instruments® out of glass capillary tubes with a 1.2 mm x .68 mm dimension and a length of 4 inches. The electrodes were filled with 3M potassium chloride. The resistance of our electrode was in the range between 15 and 20 M_. We viewed the muscle through a Bausch & Lomb microscope (.07x-3x) and the specimen was illuminated with a Fiber-Optic Illuminator (Model 190). We used a recording electrode attached to a micromanipulator in order to record the resting membrane potential. In order to elicit action potentials down the axon we placed the stimulator (*Grass SD9 Stimulator)* on a nerve and placed the recording microelectrode into a cell, which was in the segment directly anterior to the stimulating electrode. Stimulating voltages varied according to each preparation throughout the experiments, and depended on the magnitude of the stimulus threshold. Baseline resting membrane potentials and EPSPs were measured in standard crayfish saline solution prior to adding the solution containing the DL-AP3 metabotropic antagonist. Table 1. briefly summarizes the procedural steps of the three experiments conducted in this research project. The amplitude of EPSPs generated was the recorded variable.

RESULTS

High frequency stimulation with the addition of mGlur antagonist DL-AP3

In this experiment, we compared the initial readings of a cell stimulated at 0.5 Hz to a cell stimulated at 10 Hz. As hypothesized, the amplitude of the EPSPs generated at the low level frequency was higher than those stimulated at the high frequency. At 0.5 Hz, the mean recording was 37.5 mV as opposed to that of 10 Hz, 19.9 mV (Figure 1). This was due to the excess glutamate released by the higher stimulus binding with more Group II presynaptic metabotropic receptors than at the lower stimulus frequency. Statistical analysis provided further evidence that there existed a significant difference between the mean of these two groups. A one-tailed t-test was used in which the alternative hypothesis was that the mean of the 0.5 Hz stimulation without the addition of DL-AP3 would be higher than the 10 Hz stimulation without the addition of DL-AP3. The data analysis suggested that the null hypothesis ought to be rejected (P = 0.002) in favor of the alternative hypothesis.

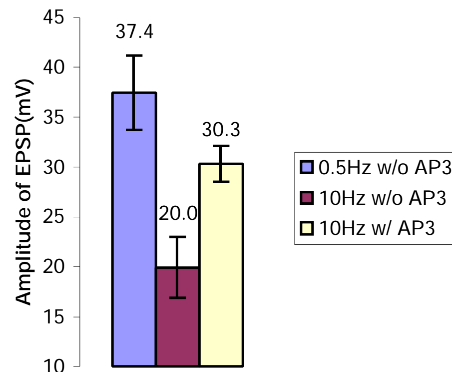

Figure1: An increase in the stimulation level of a presynaptic terminal with no inhibitor demonstrates the normal function of Group II presynaptic receptors, which lowers glutamate release consequently lowering recorded EPSP. However, with the incorporation of DL-AP3 into the environment, the normal function was inhibited, thus lowering the Group II receptors ability to regulate glutamate levels and causing raised EPSPs. The error bars indicate the level of variation within the group mean.

We anticipated the magnitude of the EPSPs generated by the 10 Hz stimulation after DL-AP3 had been added to the extracellular fluid would be similar to those obtained at the DL-AP3 free, 0.5 Hz level of stimulation. In comparing the group mean from 0.5 Hz stimulation without DL-AP3 to the group mean of 10 Hz stimulation with DL-AP3 using a two-tailed t-test no statistically significant difference was found between the means of the two different groups (P = 0.119). A comparison was then made using the group mean of the 10 Hz stimulation without the addition of DL-AP3 to the group mean of the 10 Hz stimulation with the addition of DL-AP3 using a one-tailed t-test. The alternative hypothesis that the group mean for the 10 Hz stimulation without the addition of DL-AP3 would be lower than the group mean for the 10 Hz stimulation with DL-AP3 was supported by data analysis (P = 0.007). This implies that DL-AP3 effectively blocked the natural negative feedback mechanism of the presynaptic Group II mGlurs. The mGlu2 receptors were unable to modulate the release of glutamate into the synaptic cleft even though the neuron was experiencing a period of high activity.

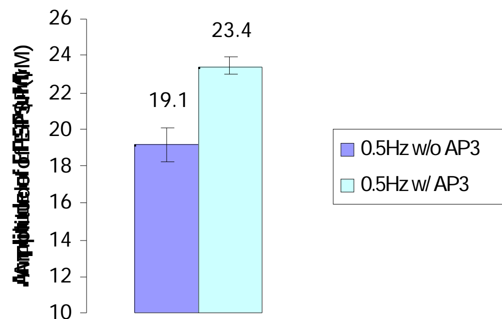

Figure 2: The addition of the Group II presynaptic inhibitor, DL-AP3, increased the mean recorded EPSP when the presynaptic terminal is stimulated at 0.5 Hz. The error bars indicate the level of variation within the group mean.

Low frequency stimulation with the addition of mGlur antagonist DL-AP3

In the case of low frequency stimulation, we expected to see no change in the EPSPs with the addition of DL-AP3 because the mGlu2 receptors would not begin the mechanism of negative feedback since the antagonist would theoretically block the glutamate receptors (Figure 2). We used a two-tailed t-test to demonstrate that there was a statistically significant difference between the group mean of the 0.5 Hz without the addition of DL-AP3 to the group mean of the 0.5 Hz with the extracellular application of DL-AP3 (P = 0.004). When DL-AP3 was added to the solution it was also in the presence of glutamate. Since they both bind to the same receptors, the two can be said to be in competition. Had all receptors been blocked we may have seen an EPSP almost congruent to 0.5Hz. However, the presence of glutamate in the cleft prevented the DL-AP3 from binding with all the receptors. This unexpected result will be further explained in the discussion section.

Modulation of Glutamate Concentration at Low Frequency Stimulation

We added glutamate to the standard ringer solution to further analyze the role it plays in synaptic transmission. A primary reason for this was to compare the results to high frequency stimulation. With the addition of DL-AP3, we observed similar findings as *High frequency stimulation with the addition of mGlur antagonist DL-AP3.* DL-AP3 blocked the metabotropic receptors and the magnitude of EPSPs returned to higher levels. The mean EPSP stimulated at 0.5 Hz without any chemicals was 22.1 mV. With the addition of glutamate the EPSPs decreased to 14.2 mV. With the addition of both glutamate and DL-AP3 we saw only a small increase, as the mean EPSP rose to 16.4 mV (see figure 3). Although the change does not seem very pronounced between the group mean of the 0.5 Hz with the modulated glutamate concentration and the 0.5 Hz with the modulated glutamate concentration and the addition of DL-AP3, the variance within the two groups was 0.402. Thus, the null hypothesis was not supported ($P < 0.001$) even though there was little numerical difference between the group means.

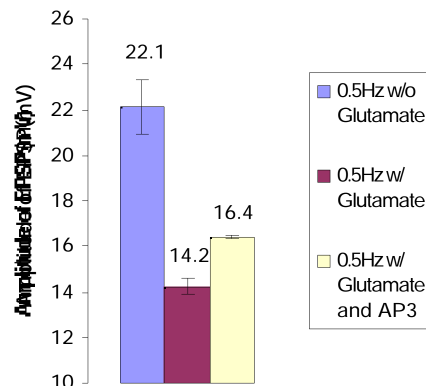

Figure 3: The stimulation of the presynaptic terminal at 0.5 Hz while in an environment with high glutamate concentrations significantly lowers the recorded mean level of EPSPs due to the cell reacting as if stimulated at high levels. The addition of DL-AP3 into the glutamate rich solution raised mean EPSP levels. While being blocked by DL-AP3, the receptors cannot recognize the increased concentration of glutamate and cannot react as normal. The error bars indicate the level of variation within the group mean.

DISCUSSION

DL-AP3 prevented the normal process of homeostasis by blocking the presynaptic mGlurs. This was reflected by the high EPSPs obtained during periods of high activity. The addition of DL-AP3 into the saline solution created a situation in which the cell could not recognize the concentration of glutamate within the synaptic cleft. DL-AP3 binds with auto-receptors without creating a reaction, effectively disabling them. This was confirmed by the amplitudes of the EPSPs that were obtained under conditions of low frequency (0.5 Hz stimulation), high glutamate concentration, and the addition of DL-AP3 (Figure 3). This reaffirmed the present knowledge concerning the nature of Group II mGlurs. The mGlu2 receptors have been demonstrated to play a primary role in the modulation of glutamate concentration (Rothstein et al., 1997; Cartmell and Schoepp, 2000; Schoepp, 2001). The regulatory function of the Group II mGlurs was demonstrated by the experiment conducted by Feinstien et al. (1998) in which the ionotropic glutamate receptors were blocked by the antagonist NMDAR, thus disabling the natural glutamate reuptake process. This caused an excess of glutamate to develop within the synaptic cleft that triggered a response from the presynaptic metabotropic glutamate receptors. This process was then shown to exist in crayfish as well as mammals by the experiments conducted by Arnone et al. (2000) Carmell and Schoepp (2000) developed a comprehensive categorization of the metabotropic glutamate receptors according to function, structure of amino acids, and pharmacological responses.

The effect upon the synaptic transmission of EPSPs in the common North American crayfish by the non-selective metabotropic glutamate antagonist can best be demonstrated by comparing the results found in the *High frequency stimulation with the addition of mGlur antagonist DL-AP3* to the *Modulation of Glutamate Concentration at Low Frequency Stimulation*. The experimental results demonstrated that DL-AP3 effectively blocked the process of the negative feedback mechanism at the presynaptic terminal that modulated the release of glutamate into the synaptic cleft. In an ideal situation, we expected a cell to produce higher EPSPs when generated at low frequencies relative to those generated at high frequencies. This is due to the function of the presynaptic mGlu2 receptors. Under circumstances of high stimulation these Group II mGlurs acted as a negative feedback mechanism that modulated the concentration of glutamate within the synaptic cleft. Low concentrations of glutamate did not stimulate the presynaptic Group II metabotropic glutamate receptors to decrease the flow of glutamate. During periods of high activity (10 Hz stimulation), the neuron responded by decreasing the flow of glutamate into the synaptic cleft.

The results obtained in the *Low frequency stimulation with the addition of mGlur antagonist DL-AP3* deviated from our original hypothesis. The low frequency stimulations were significantly altered by the addition o f DL-AP3. We can attribute this to three possible circumstances. First, the 0.5 Hz stimulation may have been high enough to activate the group II mGlurs. Second, the neuron may have accumulated glutamate within the synaptic cleft due to repetitive stimulation. Lastly, it may be possible that under any stimulation we can expect glutamate to flow out of the synaptic cleft and activate mGlu2s to a certain extent. These possibilities do not undermine our hypothesis that high frequency stimulation propagates a response from the negative feedback mechanism.

Further experiments could include an analysis of the long-term affects of DL-AP3 upon the degeneration of the neuron. This could be compared to the findings of Scanziani et al. (1997) to determine the function of the ionotropic glutamate receptors in the negative feedback mechanism. This would also expand upon the research conducted by Rothstein et al. (1992)by further studying the specific reuptake process.

In conclusion, we have demonstrated the presence of presynaptic Group II metabotropic glutamate receptors. Their relation to the modulation of glutamate has been demonstrated by the varying the concentration of glutamate, varying the stimulation levels, and adding the metabotropic glutamate antagonist to the extracellular fluid.

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