Octopamine Operates Through the cGMP Pathway at the Crayfish Neuromuscular Junction.

PELLE HALL, COLIN BROOKS, JORDAN MARKS, and CHRIS KAISER-NYMAN
Department of Biology, Grinnell College, Grinnell, Iowa

ABSTRACT

We studied the neurohormone octopamine, attempting to determine if the cGMP pathway is involved in the modulation of synaptic transmission by octopamine in *Procambarus clarkii* muscle cells. We applied a soluble guanylyl cyclase inhibitor (ODQ) along with octopamine in different combinations to the prepared crayfish and measured excitatory postsynaptic potential (EPSP) amplitudes of extensor muscles of crayfish. Our results support our hypothesis that cGMP is involved in the octopamine pathway, as application of ODQ decreased the effect of octopamine on EPSP amplitudes.

INTRODUCTION

The nervous system allows animals to send and receive signals both internally and externally. One of the basic units of the nervous system is the neuron. Neurons transmit messages through the movement of an electrical charge. Neurons selectively allow the influx and efflux of charged ions across their membranes to create an electrical current that runs down their axon (Lindgren, C., 2010). When an electrical signal reaches the end of a neuron, it must be transmitted to the next neuron or the signal will not reach the brain (in most cases) and be responded to by the body. This transmission of a signal from one neuron to another occurs at the synapse through the release of neurotransmitters that travel from the presynaptic (sending) cell, to the next neuron, which receives the signal and responds accordingly. A range of responses is possible. A lone, weak signal could elicit no response, and the electrical signal would cease. A strong enough signal, or a series of smaller signals could cause depolarization in the postsynaptic cell and transmission of the signal. An excitatory postsynaptic potential (EPSP) is the response of a postsynaptic cell to a stimulus, so a change in EPSP amplitude signifies that the synapse has changed in some way. The plasticity, or ability to change handling of signals, of synapses is very important in allowing animals to respond dynamically to their environment, and ultimately to think and learn (Lindgren, C., 2010). There are multiple ways of initially triggering this plasticity, including through the addition of modulating chemicals and repeated stimulation of the synapse (Lindgren, C., 2010).

These modulating chemicals, or neuromodulators, can have a variety of effects on the signal, including amplification, blockage, or transmission of the message the signal is sending. Many neuromodulators work through secondary messenger pathways. The neuromodulator stimulates a receptor site on the membrane of the neuron, which releases a molecule inside the cell that then acts on another molecule, which in turn can act on another, and so on (Bear, et al., 2001).

One of the neuromodulators that affects synaptic plasticity via a secondary messenger pathway is octopamine. Octopamine is a neurohormone found in crayfish that affects a variety of biological and behavioral reactions including regulation of heart beat, synaptic transmission at neuromuscular junctions, escape reflexes, and aggressiveness of crayfish (Djokaj, et al., 2001). With octopamine, the effect is varied in different crayfish specimens; in some specimens octopamine increases EPSP amplitudes, while octopamine decreases EPSP amplitudes in other specimens (Djokaj, et al., 2001). These effects are the result of a complex signaling pathway involving secondary messengers.

cAMP and cGMP are two secondary messengers whose pathways often overlap (Badhwar, et al., 2006). Research has shown that octopamine's effects are linked with cAMP pathways (Groome, J. R. & Watson, W. H., 1989). However, past research has not conclusively determined the role of cGMP pathways in the function of octopamine.

We attempted to determine whether octopamine works through cGMP pathways. To investigate this, we applied a soluble guanylyl cyclase inhibitor, ODQ, along with octopamine to crayfish preparations. Guanylyl cyclase is an enzyme that catalyzes the production of cGMP. By adding ODQ and octopamine, we tested whether cGMP pathways play a role in octopamine's effects. We hypothesized that cGMP pathways are used by octopamine and blocking the

cGMP pathway will lower octopamine's effects as a result. Our experimental data supported this hypothesis.

MATERIALS AND METHODS

Preparations

To examine the effects of octopamine and ODQ we used glass and suction microelectrodes to measure changes in the EPSP amplitudes of crayfish extensor muscles. Our crayfish were of the species *Procambarus clarkii*. These crayfish were supplied by Carolina Biological Supply Company (North Carolina, USA) while our octopamine and ODQ were supplied by Sigma-Aldrich (Missouri, USA).

Each crayfish was submerged in ice prior to being used in our experiment. We removed the tail from the body before cutting along the ridge on each side that extends to the telson, removing the uropods in the process. After using a fingernail to push out the muscle bulk still loosely attached to the tail, we had access to the superficial extensor muscles running in bundles along the inside of the shell. We performed our experiment at room temperature.

Solutions

Our crayfish dissections were each exposed to a series of solutions. Our standard solution used to collect baseline readings was a saline solution of 5.4mM KCL, 196mM NaCl, 7.1mM $MgCl_2 \bullet 6H_2O$, 10mM Sodium Hepes buffer, and 6mM $CaCl_2 \bullet 2H_2O$, with a pH of 7.4. Each tail section was exposed to the saline solution before any other, and each subsequent solution was created by the addition of chemicals to the same base saline solution. We changed solutions every 15-20 minutes by removing the solution bathing the crayfish with a syringe, removing as much solution as practical, before pouring the next solution into the dissection plate.

After collecting baseline readings we switched to a saline solution containing a 100µM concentration of ODQ. After collecting readings in the ODQ solution, we changed solutions for one containing both 100µM ODQ and 1µM octopamine. Following the collection of more data the solution was changed to one containing only 1µM octopamine. Lastly, the dissection was returned to the saline solution.

Equipment

We pinned each tail to a silicone elastomer in the bottom of a glass bowl. The bowl was filled with enough solution to cover the entire tail. To measure EPSPs in the crayfish muscle junction we used two micromanipulators, one holding a glass microelectrode and the other a plastic suction electrode. The suction electrode was attached to a Grass Instruments SD9 Stimulator so we could electrically stimulate the motor nerve, while the glass microelectrode was attached to an ADInstruments PowerLab 4/25 and an ADInstruments MacLab Bridge Amp connected to a Macintosh Mac Mini computer running Scope, allowing us to record voltage change inside muscle cells.

The glass microelectrodes were formed by heating and pulling apart glass tubes with a diameter of 1.2mm, leaving two shorter tubes with very fine points. We filled every microelectrode with a solution of 3M KCL, before inserting it into an electrode holder containing the same solution. The glass microelectrodes had resistances between 5 and 20 mega ohms.

Measurements

To record an EPSP we used a dissection microscope to find nerves with loose ends on the lateral areas of the interior of the tail. The number and ease of finding these nerves varied greatly from dissection to dissection. Once we found a nerve, with saline already drawn into the suction electrode tip to complete the electrical circuit, we pulled back on a syringe attached to the suction electrode to gather part of the nerve into its plastic tip.

With a nerve in the suction electrode, we placed the tip of the glass microelectrode into the solution and zeroed the readings using the Scope software's "zeroing" function before inserting the tip into nearby muscle bundles, typically anterior and medial to the nerve being stimulated. Following each insertion we stimulated the nerve with anywhere from 5 to 10 volts, and observed the voltage being recorded by the microelectrode on the computer. If we observed an EPSP in Scope we recorded the data, before attempting to insert the microelectrode into other muscle cells near the nerve. After every attempted recording we zeroed our equipment.

RESULTS

Our experiment tested if cGMP pathways play a role in octopamine's effect on EPSP amplitudes in crayfish. We bathed prepared crayfish muscles with solutions of ODQ and octopamine to see if blocking the synthesis of cGMP affected octopamine's effect on EPSP amplitudes. We predicted that blocking the cGMP pathway with ODQ would inhibit octopamine's effect and that washing out the ODQ would bring the EPSP amplitude back to the amplitudes observed before any chemicals were applied.

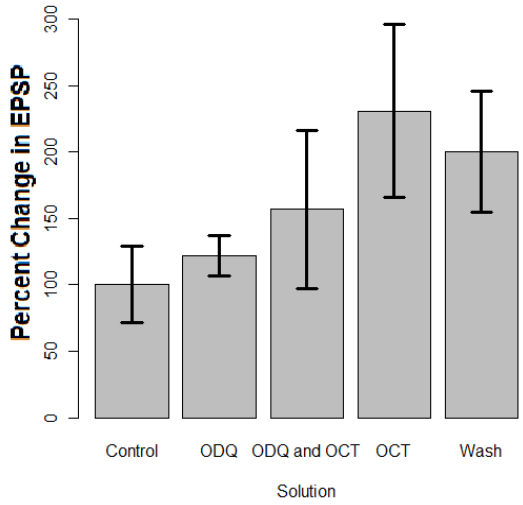

Figure 1. The effects of ODQ and octopamine on EPSP amplitudes. The bars represent the percent change in the averages of EPSP amplitudes of 4, 6, 8, 6, and 5 measurements, respectively. The error bars represent one standard error of the mean for each average. The measurements were taken sequentially on one preparation, so first saline was applied, then ODQ, then ODQ and octopamine together, then octopamine alone, then finally saline again.

Figure 1 shows the average percent change in EPSP amplitudes when various solutions were applied to the crayfish. The solutions were applied sequentially to the same preparation, so first saline was applied, then ODQ, then ODQ and octopamine together, then octopamine alone, then finally saline again.

These results show that cGMP did play a role in the effects of octopamine. Using Student's t-test, there was not a significant difference between the EPSP amplitudes of the control and the ODQ solution ($p = .20$) or between control and the ODQ and octopamine mixture ($p = .13$). There was a significant difference between EPSP amplitudes of control and octopamine ($p = .01$) as well as between control and the wash after solutions were applied ($p = .01$).

These differences show that octopamine significantly increases EPSP amplitudes in crayfish. This effect, however, was shown to be inhibited by ODQ, a cGMP inhibitor. This result indicates that octopamine operates at least partially through the cGMP pathway.

DISCUSSION

We experimented with a *Procambuss clarkii* crayfish to determine if cGMP pathways function with octopamine. To test our hypothesis that cGMP does, in fact, play a role in octopamine's effects on EPSP amplitudes, we used ODQ, a soluble guanylyl cyclase inhibitor which prevents the synthesis of cGMP. By observing the effects of solutions of ODQ and octopamine on EPSP amplitudes, we hoped to be able to tell if octopamine functions through cGMP pathways.

Our results indicate that cGMP pathways are involved in octopamine's functioning. The significant change in EPSP amplitude between the ODQ + octopamine solution and the octopamine-only solution show that octopamine's effects on EPSP amplitude were reduced by the presence of ODQ. However, after applying a wash, the resulting amplitude was not reduced to the degree we expected. The two possible explanations for this are that the chemicals were not fully washed out or the chemicals have an effect that cannot be reversed by the removal of the chemicals. The washing procedure we used may not have been effective due to the use of only one rinse with saline. Future work with more thorough washing techniques may reveal which of these possibilities is the case.

A major problem with our experiment is a lack of data, as all our data came from one preparation. This poses the problem of the crayfish potentially not being representative of the whole population, which may be quite important as octopamine is know to have different effects on different preparations (Djokaj, et al., 2001). Also, we have a limited amount of data, meaning that further sampling could reduce the standard error of the data and might mean that the ODQ, or ODQ + octopamine solutions could show significant changes in EPSP amplitudes.

Our study indicates that octopamine does work through cGMP pathways, but we did not determine whether it is working presynaptically or postsynaptically. Determining where later steps of cGMP pathways are and how they function could be important to many areas of research, as cGMP, and the related cAMP, are important second messengers in many signaling pathways.

Many questions arise from the results of our experiment. Our results support the conclusions of Badhwar et al. that cAMP and cGMP have overlapping pathways, as inhibiting the synthesis of cGMP did not completely negate the affects of octopamine. This is in contrast to the findings of another experiment, where it was determined that only cAMP and not cGMP was involved in the effects of 5-HT and octopamine (Araki, et al., 2005). Further experimentation to more definitively determine if octopamine utilizes the cGMP pathway is warranted, especially in light of these contradicting results.

It would also be worth exploring experimentation inhibiting both cGMP and cAMP pathways to

determine if they are the only pathways that octopamine uses or if there are other important secondary messengers involved that have yet to be fully investigated. Octopamine's effects also appear be linked to the secondary messenger IP_3, which could serve as the basis for another experiment (Farooqui, T., 2007). Future research might try to find the exact role of each secondary messenger octopamine utilizes, and to what degree each secondary messenger plays in creating octopamine's observable effects.

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