Pioneering Neuroscience, 2010, **11**, 1-4

2-APB Negates the Enhancement of EPSP Amplitudes Induced by 5-HT in the *Procambaris clarkii* Neuromuscular Junction.

ROSEMARY RAST, GRACE RYAN, and KATHERINE SITTIG
Department of Biology, Grinnell College, Grinnell, Iowa

ABSTRACT

5-HT is known to increase the amplitude of excitatory postsynaptic potentials (EPSPs) in the crayfish neuromuscular junction, but it is not known how 5-HT does this. It is possible that 5-HT acts through IP_3-induced intracellular Ca^{2+} release. To test this, we bathed a crayfish neuromuscular junction in a solution of 5-HT plus the IP_3 inhibitor 2-APB and measured EPSP amplitudes. We compared these with EPSP measurements taken in a solution with 5-HT alone. We found that the application of the IP_3 inhibitor counteracts the amplitude-increasing effect of 5-HT. This supports our hypothesis that 5-HT works through IP_3-induced Ca^{2+} increase to increase the amplitudes of EPSPs in the crayfish neuromuscular junction.

INTRODUCTION

The knowledge of how nerves function is imperative to understand bodily functions in any animal. Many chemicals are involved in the nervous system and most of them have multiple effects. For example, 5-HT, one of the most abundant neurotransmitters in the nervous system, has been shown to increase excitatory postsynaptic potential (EPSP) amplitudes in the crayfish neuromuscular junction (Dropic et al. 2005 and Etzkorn et al. 2006). The exact mechanism behind this is unknown, but the increase in EPSP amplitudes could be due to one of two intracellular calcium release receptors—that of either IP_3 or ryanodine (Mattson et al. 2000). Past research has also suggested a link between the effects of IP_3 inhibitors and 5-HT on EPSP amplitudes (Dropic et al. 2005). 2-APB is known to be an IP_3 inhibitor but may affect EPSP amplitudes through mechanisms other than an IP_3-induced Ca^{2+} release (Dropic et al. 2005). Through application of the IP_3 inhibitor 2-APB, we aim to determine whether 5-HT affects EPSP amplitudes through IP_3-induced Ca^{2+} release and if 2-APB and 5-HT have an unknown combined effect on EPSPs. This research is important because understanding the effects of 5-HT and the mechanisms through which it affects EPSPs will lead to a greater understanding of many neurological functions and how they occur.

We hypothesized that the application of an IP_3 inhibitor, 2-APB, will negate the effects of 5-HT on EPSP amplitudes. We already know that 5-HT causes an increase in EPSP amplitude (Etzkorn et. al. 2006), however, we wanted to know the combined effects of 5-HT and 2-APB. Our data supported our hypotheses that 5-HT works through an IP_3-induced Ca^{2+} release and that 2-APB decreases EPSP amplitudes when 5-HT has previously been applied to the preparation.

MATERIALS AND METHODS

To test the effects of 5-HT and 2-APB, we took EPSP measurements in 5-HT alone and in 5-HT plus 2-APB. We then washed the specimen in a high Ca^{2+} saline solution and took EPSP measurements to eliminate the possibility that any effects we observed were due to uncontrollable variables, such as time.

Organism Preparation.

We acquired our crayfish from Carolina Biological Supply (North Carolina, USA), and we used the *Procambaris clarkii (P. clarkii)* species of crayfish. The tails of the *P. clarkii* specimens were dissected to access the nerves and muscle cells of the tail. The specimens were put over ice, the tails were removed, and everything down to the superficial dorsal exterior muscle was removed from each specimen by cutting along the lateral ridges of the specimen's tail. The cuts were made close to the anterior surface so as to leave as much of the nerves intact as possible. The ventral half of the exoskeleton was then removed, along with the muscle tissue, and the dorsal half of the *P. clarkii* exoskeleton was then pinned into a dissection dish.

Electrophysiology.

Microelectrodes were used to measure EPSPs. They were created using a World Precision Instruments PUL-1 microelectrode puller. Heat was applied to the 1.2mm borosilicate glass tubes and the tubes were pulled apart in approximately seven to eight seconds to create

microscopic ends that could enter a muscle cell without damaging it. Once pulled, the microelectrodes were filled with 3M KCl and attached to a microelectrode holder, also filled with 3M KCl. The microelectrode holder was then placed on a microelectrode manipulator that was used, along with a Leica ZOOM 2000 microscope, to insert the microelectrode into a cell. Microelectrodes were replaced once the resistance was determined to be less than 20MΩ.

We used a suction electrode to hold a selected nerve in place and stimulate it with a GRASS SD9 stimulator. The selected nerve was always located in the same muscle segment as the cell from which we were taking our measurements.

Solutions.

The standard crayfish saline we used for our control was composed of 5.4mM concentration of KCl, 196mM NaCl, 2.6mM $MgCl_2 \cdot 2H_2O$, 10mM Na Hepes Buffer, and 13.5mM $CaCl_2 \cdot 2H_2O$, and had a pH of 7.4. We put 5-HT into the saline solution by diluting 100µL of 10mM 5-HT into 100mL of standard crayfish saline solution to get a final concentration of 10µM. We put 2-APB into the saline solution by diluting 200µL of 50mM 2-APB into 100mL of standard crayfish saline solution to get a final concentration of 100µM. These concentrations were chosen in order to have the greatest possible effect on the crayfish.

Measurements.

The experiment was performed on the superficial dorsal extensor tail muscle cells of the crayfish. Initially, we took seven EPSP measurements using the 10µM 5-HT saline solution, and we took fourteen more EPSP measurements after replacing the solution with fresh 5-HT saline solution of the same concentration. We then replaced the 5-HT saline solution with saline solution containing both 10µM concentration of 5-HT and 100uM concentration of 2-APB and took thirty-five EPSP measurements. Finally, we washed the preparation twice with standard crayfish saline solution and took twenty EPSP measurements.

RESULTS

The purpose of this experiment was to determine whether 5-HT increases EPSP amplitudes in the *P. clarkii* neuromuscular junction through an IP_3-triggered intracellular release of Ca^{2+}. Baseline EPSP measurements in the cells were taken in the 5-HT solution. The solution was then replaced with saline containing both 5-HT and 2-APB and EPSP amplitude measurements were taken in this solution and then in a standard crayfish saline wash solution.

The average EPSP amplitudes in the crayfish neuromuscular junction soaked in 5-HT, 5-HT+2-APB, and wash solutions were found to be 7.5mV, 6.8mV, and 5.3mV, respectively. However, the time span over which this data was taken was found to be very important to the experimental results. The first four measurements in 5-HT solution were taken less then five minutes after the application of the experimental solution, and were found to be significantly lower than the those measurement taken in the following five minutes. The mean value in the first five minutes was 4.4mV as compared to the mean value of 8.4mV in the latter data. This same trend can be seen in data taken less than ten minutes after the application of the 5-HT+2-APB solution as compared to that taken in the following fifteen minutes. These trends can be seen in the scatter plot in Figure 1, and suggest that these chemicals take differing amounts of time to take effect in the cell. The difference between the time taken for 5-HT and 2-APB to take effect can be explained by the roles that these two chemicals play in the cell. 5-HT acts as a neurotransmitter that takes effect by binding to receptors on the outside of the muscle cell, while 2-APB acts a second messenger inside of the cell. This would account for the increased amount of time that it took for 2-APB to take effect on the cell.

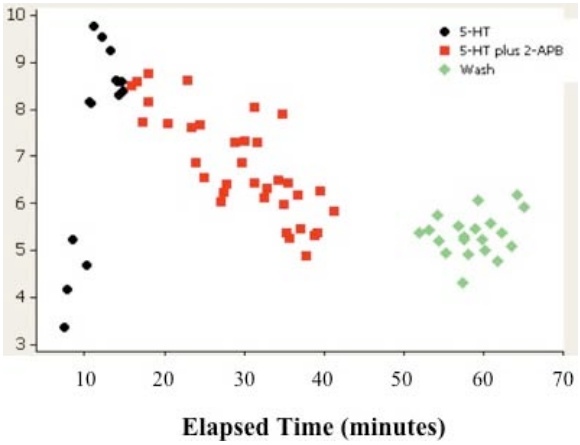

Figure 1. EPSP amplitudes in 5-HT, 5-HT plus 2-APB, and standard saline wash solutions over time. 5-HT was applied at 5 minutes, 5-HT plus 2-APB at 15 minutes, and the wash solution at 50 minutes.

We do not, therefore, consider data taken within the first five minutes of 5-HT application and the first ten minutes of 5-HT plus 2-APB application to be representative of the effects of these chemicals. In order to create a more accurate representation of the effects of the chemicals, this data was disregarded and new mean EPSP amplitudes for 5-HT, 5-HT plus 2-APB, and wash solutions were found to be 8.4mV, 6.3mV, and 5.3mV, respectively, as is illustrated in Figure 2. A t-test performed on these mean

EPSP values for the 5-HT and 5-HT+2-APB solutions produces a p-value of <0.001, thus disproving the null hypothesis and suggesting that the difference found between EPSP amplitudes in the two solutions is statistically significant.

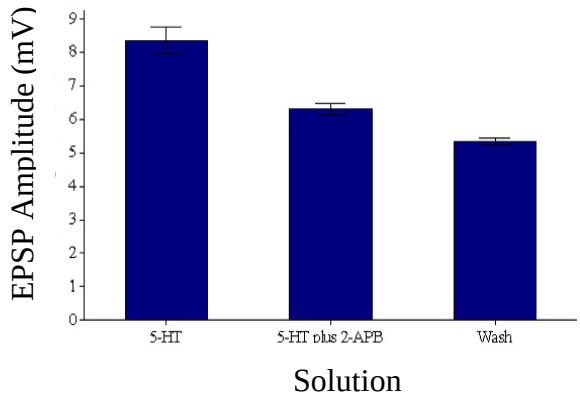

Figure 2. Average EPSP amplitudes in *P. clarkii* muscle cells in 5-HT, 5-HT plus 2-APB, and standard saline wash solutions at room temperature. Bars represent mean EPSP amplitudes for each solution where n=11 for the 5-HT solution, n=25 for the 5-HT plus 2-APB solution, and n=20 for the wash solution. Error bars represent one standard error of the mean. Data was excluded from the first five minutes of 5-HT application and first ten minutes of 2-APB+5-HT application. The data shown was taken over a subsequent five minute interval for 5-HT, a subsequent fifteen minute interval for 2-APB plus 5-HT and a ten minute interval for the wash solution. The p-value for 5-HT and 5-HT plus 2-APB is <0.001.

EPSP amplitudes varied somewhat around the mean, as can be seen in Figure 2, but this does not impact the observed results of the experiment.

As the mean values found for EPSP amplitudes in the 5-HT saline and the 5-HT+2-APB saline demonstrate, 5-HT lowers the average EPSP to a level near that of the average EPSP when the preparation is in a wash solution. Our data indicates that 2-APB counteracts most of 5-HT's effect on EPSP amplitude.

DISCUSSION

These experimental results support our hypothesis that the IP_3 blocker 2-APB negates the enhancement of EPSP amplitudes in the crayfish neuromuscular junction caused by 5-HT, and that 5-HT therefore has its effect on the synapse through IP_3-induced intracellular calcium release.

Effect of 5-HT and 2-APB on EPSP amplitudes.

5-HT is known to increase EPSPs in the neuromuscular junction (Dropic et al. 2005), and our data supports such findings. On average, the EPSP measurements taken over five minutes after the application of 5-HT were 3.0mV higher than those EPSP measurements taken in the wash solution, and this indicates to us that 5-HT has an EPSP-increasing effect.

On average, the EPSP measurements taken over ten minutes after the application of 5-HT+2-APB were 2.1mV lower than the EPSP measurements taken over five minutes after the application of 5-HT alone. This indicates to us that 2-APB counteracts most of the effect 5-HT has on EPSP amplitudes. Since 2-APB is a known IP_3 inhibitor (Peppiatt et al. 2003), we have therefore concluded that 5-HT works through IP_3-induced Ca^{2+} release. Our results support our hypothesis that 2-APB mostly negates the effects of 5-HT on EPSP amplitude, as well as supporting the hypothesis that 5-HT affects EPSP amplitude through IP_3-induced Ca^{2+} release.

We saw a small decrease in average EPSP amplitude between the measurements taken in the 5-HT+2-APB solution and those taken in the wash solution. By looking at Figure 1 we observed that there was a continual decrease over time in average EPSP amplitude during the application of the 5-HT+2-APB solution. We believe that this decrease is a result of 2-APB taking time to exhibit its full effects because of its role as a second messenger, and we believe that the average EPSP amplitudes would have leveled out if the preparation were left in the 5-HT+2-APB solution for a longer period of time.

Unexplained increase in EPSP amplitudes after a second application of 5-HT solution.

Our first seven measurements of EPSPs taken in 10μM 5-HT saline solution were noticeably lower than the fourteen taken after we replaced the 5-HT saline solution with fresh solution of the same concentration. The EPSP amplitudes taken in the first batch of saline solution were close to those taken when the specimen was washed with standard crayfish saline solution. By the time we obtained data in the first batch of solution, however, the specimen had been sitting in the solution for nearly an hour, and we have concluded that the unexpectedly low EPSP amplitudes taken in the first batch of 5-HT could have been due to the length of time the specimen was bathed in a batch of solution without the solution being changed. There is evidence that 5-HT receptors can become desensitized (Corradi et al. 2009), but desensitization of 5-HT receptors would most likely be a long term-effect, and therefore would have continued to affect EPSP amplitudes long after we replaced the 5-HT saline solution. It is more likely that the 5-HT was deactivated during the first hour due to its suggested instability in solution (Huang and Kissinger 1996).

Possibilities for further experimentation.

Past research has suggested that 5-HT could have its affect on EPSP amplitudes through a different intracellular Ca^{2+} release mechanism governed by ryanodine (Dropic et al. 2005**)**. Although our experimental results suggest that the effects of 5-HT on EPSP amplitudes are governed by IP_3, it is possible that both chemicals play a role. Further experimentation could help determine whether or not ryanodine affects 5-HT's, and, if so, how IP_3 and ryanodine work together in this role.

Past research has also suggested a link between the effects of caffeine and 5-HT on EPSP amplitudes in the neuromuscular junction (Dropic et al. 2005). Caffeine, like 2-APB, is an IP_3 inhibitor and could therefore affect 5-HT's impact on EPSP amplitudes in a manner very similar to those created by 2-APB and observed in this experiment (Peterson and Cancela 1999). Further experimentation could be done both to confirm this role of caffeine in the cell and its link to 5-HT and to explore other possible effects of caffeine on EPSP amplitudes that have been suggested by past research (Celenza et al. 2007).

ACKNOWLEDGEMENTS

We thank Clark Lindgren, our professor, Sue Kolbe, our lab assistant, and Grace Hazeltine Bartman and Molly Wingfield, our mentors, for assisting us in performing our experiments and for their patience and support.

REFERENCES

Celenza, K.M., E. Shugert, and S.J. Vélez. 2007. Depressing effect of caffeine at crayfish neuromuscular synapses II. Initial search for possible sites of action. *Cellular and Molecular Neurobiology* 27 (3):381-93.

Corradi J., F. Gumilar, and C. Bouzat. 2009. Single-channel kinetic analysis for activation and desensitization of homomeric 5-HT(3)A receptors. *Biophysical Journal* 97 (5):1335-45. {abstract only}

Dropic, A.J., E. Brailoiu, and R.L. Cooper. 2005. Presynaptic mechanism of action induced by 5-HT in nerve terminals: Possible involvement of ryanodine and IP3 sensitive Ca stores. *Comparative Biochemistry and Physiology, Part A* 142: 355-361.

Etzkorn, L., A. Griffith and A. Guetzko. 2006. Serotonin enhances EPSP amplitudes via the release of calcium stores at IP3 receptor sites and 2-APB inhibits these receptors and decreases EPSP amplitudes at the crayfish neuromuscular junction. *Pioneering Neuroscience* 2006 7: 5-8.

Huang, T. and P. T. Kissinger. 1996. Liquid chromatographic determination of serotonin in homogenized dog intestine and rat brain tissue using a 2 mm i.d. PEEK column. *Current Separations* 14 (3/4): 114-119.

Mattson, M.P., F.M. LaFerla, S.L. Chan, M.A. Leissring, P.N. Shepel, and J.D. Geiger. 2000. Calcium signaling in the ER: its role in neuronal plasticity and neurodegenerative disorders. *Trends in Neuroscience* 23: 222-229.

Peppiatt, C.M., T. J. Collins, L. Mackenzie, S.J. Conway, A.B. Holmes, M.D. Bootman, M.J. Berridge, J.T. Seo, and H.L. Roderick. 2003. 2-Aminoethoxydiphenyl borate (2-APB) antagonises inositol 1,4,5-trisphosphate-induced calcium release, inhibits calcium pumps and has a use-dependent and slowly reversible action on store-operated calcium entry channels. *Cell Calcium* 34(1): 97-108.

Peterson, O.H. and J.M. Cancela. 1999. New Ca^{2+}-releasing messengers: are they important in the nervous system? *Trends in Neuroscience* 22: 488-495.