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Serotonin Enhances EPSP Amplitudes via the Release of Calcium Stores at IP_3 Receptor Sites and 2-APB Inhibits these Receptors and Decreases EPSP Amplitudes at the Crayfish Neuromuscular Junction

LAUREN ETZKORN, ABIGAIL GRIFFITH, and AMY GUETZKO
Department of Biology, Grinnell College, Grinnell, Iowa

ABSTRACT

Serotonin induces the release of internal calcium stores in the superficial extensor muscle of the crayfish neuromuscular junction. Inositol 1,4,5-trisphosphate (IP_3) and ryanodine receptor sites have both been implicated as the principal receptor for internal calcium. This study attempted to isolate and interpret the activity at IP_3 receptor sites by enhancing calcium release with serotonin (5-HT) and then inhibiting IP_3 receptors with the chemical 2-Aminoethoxydiphenyl borate (2-APB). We predicted that 5-HT would enhance excitatory postsynaptic potential (EPSP) amplitudes and the addition of 2-APB would mitigate this effect. This would imply that IP_3 receptors are the principal internal calcium receptors when the cell is exposed to 5-HT. Results were significant, mostly supporting our hypothesis. The data indicate that IP_3 receptors are the principal site of serotonin-induced calcium release but other sites and mechanisms are most likely also involved in this process. There is a need for variation in methods and approaches in this type of research to better understand the role of IP_3 receptors in the release of internal calcium stores.

INTRODUCTION

Calcium serves several purposes in synaptic transmission; most importantly it stimulates neurotransmitter release from the presynaptic cell. Calcium release is the process in which internal molecules bind to receptors on organelles within the cell. These organelles contain calcium stores that release calcium when this binding occurs (Locknar et al. 2004). The molecule inositol 1,4,5-trisphosphate (IP_3) has been known to cause calcium release in presynaptic cells (Authi et al. 1987). IP_3 is released when the enzyme phospholipase C (PLC) binds to phosphatidylinositol-4,5-bisphosphate (PIP_2), a membrane phospholipid. IP_3 then binds to receptors on the mitochondria which release calcium stores inside the organelle (Becker et al. 2000). This calcium contributes to the initiation of exocytosis (Dascal et al. 1986).

Serotonin (5-HT) has been shown to enhance neurotransmitter release, specifically glutamate, in various preparations including the crayfish neuromuscular junction (Meller et al. 2002). Meller et al. (2002) found that 5-HT increases production of IP_3, which in turn leads to an enhanced release of calcium from IP_3-sensitive calcium stores on the mitochondria or smooth endoplasmic reticulum. This IP_3 receptor was the focus of our investigation because some research has shown that IP_3 receptors may be the principal source of 5-HT activated calcium release. However, other researchers claim that 5-HT could activate the ryanodine receptors on the smooth endoplasmic reticulum (Dropic, et al. 2005). Other possibilities include unknown processes contributing strongly to enhanced calcium release.

Further research on the mechanism underlying calcium release via IP_3 and ryanodine receptors is important to improve our understanding of all components of neurotransmitter release and calcium dependence. The research done by Dropic et al. (2005) observed the effects of 5-HT on ryanodine and IP_3 sensitive calcium stores and is the basis for our own investigation. They applied 5-HT, adenophostin-A, varying concentrations of ryanodine and caffeine to the crayfish neuromuscular junction (NMJ).

Their study of the presynaptic effects of 5-HT on ryanodine and IP_3 receptors led us to develop an alternative research design to identify the primary receptor involved in calcium release in the crayfish neuromuscular junction. In their experiment a variety of chemicals were applied to the preparation. The excessive number of chemicals applied to the crayfish NMJ limited the researcher's ability to draw firm conclusions about the effects of a single chemical. Due to their complicated methods and design, results were inconclusive and it was difficult to determine causation or correlation from the data. However, despite limitations, the research of Dropic et al. (2005) was well-founded and provides general findings upon which to expand.

We expected to find similar results to those of Dropic et al. (2005) regarding the general effects of serotonin's enhancement of synaptic facilitation. We were interested in learning whether we could find more specific information regarding serotonin's relationship to IP_3 receptors and calcium stores. To address gaps in the previous research, we used a simplified procedure. One of the changes in procedure we made was to combine the serotonin with reduced calcium saline solution. In our design, we applied a single inhibitory chemical, 2-Aminoethoxydiphenyl borate (2-APB), to our preparation. We hoped this would lead to a clearer interpretation and understanding of our results.

We hypothesized that serotonin would bind to its specific receptor, and the activation of this receptor would lead to the increased production of IP_3. IP_3 would then activate calcium release from the mitochondria and the endoplasmic reticulum within the nerve terminal. To test this hypothesis, we compared excitatory postsynaptic potential (EPSP) amplitudes between our three conditions: baseline Ringer's solution, serotonin, and serotonin combined with 2-APB. Our results supported our hypothesis and provided further insight into the specific role of IP_3 receptors.

MATERIALS AND METHODS

Crayfish Preparation

Each crayfish was submerged in an icebed for approximately twenty minutes to slow metabolic processes. We removed the posterior tail area of a crayfish. All extraneous parts were cut away so that only the superficial extensor muscle was exposed. We pinned the sample to the dish filled with a saline solution.

Electrophysiological Measurements

We measured membrane potentials using pulled glass microelectrodes filled with 3.0 M KCl. Additionally, we stimulated nerve endings using a fire-polished suction electrode attached to a Grass SD9 stimulating circuit. We observed the activity of the nerves and muscles with the data collection program Scope v3.6.3, measuring the membrane potential and frequently testing the resistance of the microelectrode (above 10 MΩ was an accepted resistance). When monitoring the amplitude of EPSPs, we documented voltage, duration, frequency and delay of the stimulating circuit. To collect a representative sample, we took measurements from a variety of muscle cells and stimulated multiple nerve endings.

Solutions

We used standard Ringer's solution in our baseline condition and for the two experimental conditions we combined 50mL of regular Ringer's with 50mL of no calcium Ringer's (See Table 1).

Chemical	Concentration (mM)
NaCl	205
KCl	5.4
$CaCl_2$	13.5
$MgCl_2$	2.6
Tris Buffer (pH 7.4)	10

Chemical	Concentration (mM)
NaCl	205
KCl	5.4
$CaCl_2$	0
$MgCl_2$	16.1
Tris Buffer (pH 7.4)	10

Table 1. The compositions of the two Ringer's solutions used in our baseline condition and two experimental conditions. The first Ringer's (left) consists of the standard calcium concentration. The second Ringer's (right) has zero concentration of calcium.

The reduction of extracellular calcium allowed us to ensure that the process we were observing was due to intracellular calcium release as opposed extracellular influx of calcium.

For the serotonin condition we diluted a 1mM 5-HT stock solution to a final concentration of 1 μM. We added 100μL of this 5-HT solution to the half calcium Ringer's solution to establish baseline effects of 5-HT on excitatory postsynaptic potentials (EPSPs).

We used 2-APB, an IP_3 receptor antagonist, in an attempt to identify the specific calcium release mechanism of the crayfish. We made our 2-APB solution by diluting 0.0225 g of 2-APB in 10 mL of DMSO. We added 100 μL of the 2-APB solution to our 100 mL of half calcium Ringer's. In each condition, external solutions were replaced every fifteen to twenty minutes to ensure preservation of the nerves and muscle tissue.

Data Collection

Originally we wanted to expose each crayfish tail to all three conditions, control, 5-HT and 5-HT + 2-APB. Due to time constraints we were unable to do this. We were able to expose each crayfish tail to one or two conditions.

RESULTS

We investigated the enhancing effects of serotonin and the inhibitory effects of 2-APB on the crayfish neuromuscular junction. To test our hypothesis that IP_3 is the primary receptor site involved in the internal release of calcium, we inhibited these receptors with 2-APB and measured the subsequent EPSPs.

To establish a baseline reading we measured EPSPs in a standard Ringer's solution. After adding 5-HT, we waited approximately five minutes before taking initial readings to allow the 5-HT to take full effect. For our second condition we added 5-HT and 2-APB and waited approximately five minutes for the chemicals to become fully active. 2-APB was added to inhibit IP_3 receptors, thus limiting internal calcium release. Examples of two EPSPs for the 5-HT condition and the 5-HT + 2-APB condition can be seen in Figure 1.

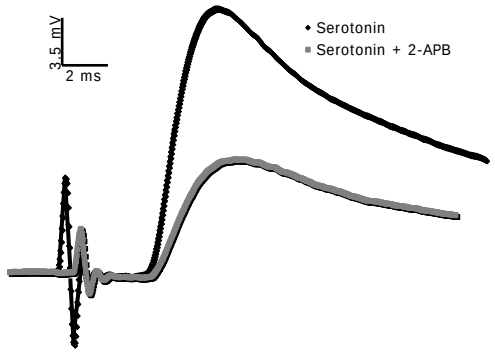

Figure 1. Typical EPSPs for the 5-HT and 5-HT + 2-APB conditions. Depicted here, the EPSP amplitude for 5-HT was 23mV and the EPSP amplitude for 5-HT + 2-APB was 10mV.

We performed a series of t-tests to elucidate the differences between our conditions (Figure 1). As expected, data analysis showed that there was a significant difference between Ringer's and 5-HT (p= 0.001). We also found a significant difference between 5-HT and 5-HT + 2-APB, p= 0.016. This difference signals the expected antagonistic effect of 2-APB on IP_3 receptors. 2-APB successfully hindered the full effect of 5-HT from eliciting larger EPSPs.

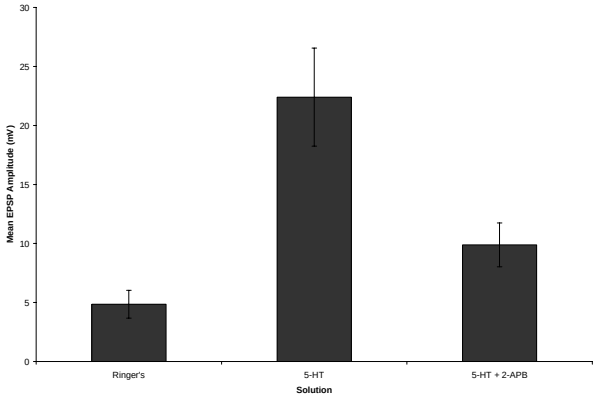

Figure 2. Ringer's, 5-HT and 2-APB conditions. The mean of Ringer's trials was 4.84 mV (n=11). 5-HT enhanced EPSP amplitude as demonstrated by the mean of 22.39 mV (n = 16). 5-HT + 2-APB successfully inhibited IP_3 receptors as shown by the depression of EPSP amplitudes. The mean of 2-APB was 9.88 mV (n=10). Error bars represent the standard error of each condition.

Unexpectedly, there was a significant difference between Ringer's control and 5-HT + 2-APB conditions (p= 0.015). Our hypothesis dictated that we would find either no significance or a slight significance in the opposite direction. We anticipated the EPSPs in the 5-HT + 2-APB condition to be absent or smaller than those elicited in the standard Ringer's condition.

DISCUSSION

Our research demonstrated the enhancing effect of 5-HT on the EPSP amplitude of the crayfish neuromuscular junction, and the inhibitory effect of 2-APB on IP_3 receptors. However, our results suggest that while IP_3 is the primary receptor for serotonin-induced calcium release, it is not the only mechanism by which internal calcium stores are released.

We found three significant differences between our conditions, Ringer's and 5-HT, 5-HT and 5-HT + 2-APB, and Ringer's and 5-HT + 2-APB. The depressed EPSP amplitudes led us to conclude that 2-APB diminishes the amount of internal calcium released through IP_3 receptor activation. This supported our hypothesis that IP_3 is the main mechanism by which internal calcium release occurs. However, there still appeared to be residual calcium triggering small EPSPs, significantly larger in size than those emitted in normal Ringer's solution. This suggests ryanodine receptors play a small but significant role in the release of internal calcium stores and subsequent EPSPs (Dropic et al. 2005). It may also be possible that 2-APB failed to block all IP_3 receptors, leading 5-HT to activate available IP_3 receptors.

Interestingly, we observed frequent action potentials which occasionally followed the observation of EPSPs (roughly 10-12 action potentials throughout both experimental conditions). In addition to observing the action potential with the electrophysiological equipment, we witnessed muscle twitches in our preparation. There are two explanations as to why these action potentials occurred. The addition of an excess amount of 5-HT could have caused a calcium frenzy within the cell, triggering action potentials. The other possibility is that our half calcium solution was still at a high enough concentration to contribute to action potentials.

A limitation to consider was the initial lack of our own raw data for our baseline Ringer's condition. Originally, we were only able to obtain two data points, which could have called into question the internal validity of some of our

conclusions. For example, if the baseline readings were not representative of actual EPSPs in Ringer's all comparisons based on this data could be misleading. However, we were able avoid this problem in internal validity by obtaining data points via personal communication with fellow researchers, Mark Schneider, Nate Kimball and Bethany Prosseda.

Another limitation was time constraints. We were never able to expose a single crayfish to all three conditions. This may limit comparisons because variations may be due to naturally occurring differences in crayfish.

This research is significant because it provides further support for the evidence that was already introduced by Dropic et al. (2005) that IP_3 receptors play a crucial role in serotonin-induced calcium release. We further speculate that ryanodine may contribute to internal calcium release in ways as yet unidentified. Future research should examine more specifically the role ryanodine plays in internal calcium release.

Additional research should address the aforementioned limitations in our study. The use of a half calcium control would be more suitable to compare the experimental conditions to baseline. To reduce the occurrence of action potentials it would be prudent to reduce external calcium even further, as well as reducing the amount of 5-HT.

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