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5-HT-activated Ryanodine Receptors Play an Active Role in Increasing Excitatory Post-synaptic Potentials at the Crayfish Neuromuscular Junction.

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ABSTRACT

In this experiment, we investigated the role of serotonin(5-HT)-activated ryanodine receptors in the dorsal superficial extensor muscle cells in the tail of the crayfish (*Procambarous clarkii*). We hypothesized that the 5-HT-activated ryanodine receptors are responsible for some of the Ca^{2+} released in the pre-synaptic cell, and consequently for the heightened amplitudes of the generated Excitatory Post-Synaptic Potentials (EPSPs). To test this hypothesis we measured, using intracellular recording, the amplitudes of EPSPs generated by stimulating individual crayfish nerves under various conditions. We collected baseline data when the crayfish muscle cells were exposed to standard crayfish solution, and compared that with measurements collected when the crayfish cells were exposed to 5-HT, Ruthenium Red, and the two combined. Ruthenium Red blocks ryanodine receptors. Overall, our results support our hypothesis. The presence of 5-HT increased EPSPs, while Ruthenium Red had little effect; when we added Ruthenium Red and 5-HT together, the amplitudes of the EPSPs remained essentially the same as before the addition of 5-HT, signifying that when the ryanodine receptors are blocked, 5-HT has no effect.

INTRODUCTION

The release of neurotransmitter depends on the pre-synaptic increase of calcium, which can originate from ion channels/pumps located on the cell membrane or from calcium stores, released from organelles within the cell (Lindgren 2010). The endoplasmic reticulum, an organelle, releases calcium through ion channels, ryanodine receptors and IP_3 receptors. In our experiment, we focused on the function and significance of the ryanodine receptor (Lindgren 2010).

5-HT (Serotonin) is a neurotransmitter that acts on the neuromuscular junction and enhances EPSPs. Previous research indicates that it acts through 5-HT receptors located on the pre-synaptic membrane, activating a second messenger system, which in turn activates the ryanodine receptors (Ullmer et al., 1996). Opposing research suggests that ryanodine receptors are actually activated by an intracellular rise in calcium released from IP_3 receptors, also located on the endoplasmic reticulum (Zheng et al., 2005). When the ryanodine receptors are activated, they release calcium, thus causing the release of more neurotransmitters into the synaptic cleft, resulting in increased EPSPs (Oxford Reference Online, 2010). We measured the amplitudes of EPSPs in the dorsal extensor muscle of the crayfish under various experimental conditions in order to investigate the role of ryanodine receptors in the enhancement of EPSPs induced by 5-HT.

We measured the amplitudes of EPSPs when the dorsal extensor muscle was bathed in standard crayfish solution, 5-HT, Ruthenium Red, which blocks ryanodine receptors, and a combined solution of Ruthenium Red and 5-HT. We predicted measuring increased amplitudes in the presence of 5-HT. When the muscle cells were exposed to just Ruthenium red, we predicted constant, and lower, amplitudes, because the 5-HT was not present to enhance EPSPs. Lastly, when the crayfish tail was bathed in the combination solution, because the 5-HT was unable to act on the blocked ryanodine receptors, we also predicted constant, and lower amplitudes. The average amplitude was indeed highest with 5-HT, and lower, closer to baseline data, with both Ruthenium Red and Ruthenium Red and 5-HT.

MATERIALS AND METHODS

Tissue Preparation

Before dissection, we submerged crayfish (*Procambarus clarkii),* provided by Carolina Biological Supply, in ice for approximately fifteen minutes, until movement ceased. To isolate the dorsal superficial extensor muscle, we cut the crayfish in half so that only the abdomen and telson remained. We then made two longitudinal cuts, as close to the ventral surface as possible from the top of the abdomen to the telson. We pulled off the swimmerets, and removed all left over muscle gently

with one finger. We pinned down the remaining section--the dorsal exoskeleton, with the superficial dorsal extensor muscle face up, and nerves exposed--in a silicone dissection dish, with a volume of approximately 200 mL.

Solutions/Drugs

We used a standard crayfish saline composed of KCl 5.4 mM, NaCl 196 mM, $MgCl_2.6H_2O$ 2.6 mM, Na Hepes Buffer 10 mM, $CaCl_2.2H_2O$ and 13.5 mM to gather baseline (control) data, and for washes. This solution had a pH of 7.4. We replaced the standard crayfish saline with three other experimental conditions: 5-HT, Ruthenium Red, and 5-HT combined with Ruthenium Red. To create the solution of 10 µM 5-HT we diluted a stock solution of 10 µM in 100 µl aliquots with 100mL of standard crayfish solution 1:1000. We diluted (1:1000) a stock solution of 50 mM Ruthenium Red in 100 µl aliquots with 100 mL of crayfish saline to achieve a final concentration of 50 µM . We simply combined the two solutions for the final experimental condition, making the final concentrations of 5-HT and Ruthenium red half the original.

Microelectrode

We created fine tipped microelectrodes by splitting glass tubes (1.2 mm in diameter—small enough to penetrate individual muscle cells causing minimal damage) using heat. We filled these tubes with 3M KCl, and dipped them in saline solution before attaching them to the micromanipulator. The glass electrodes had resistances of at least 60 mV.

Intracellular Recording

Using a microelectrode attached to a micromanipulator and the computer program Scope, we repeatedly measured the voltage across the cell membrane, under numerous experimental conditions, while stimulating individual nerves (using a suction electrode) with 7 volts, at a frequency of .5 pulses per second. We gathered measurements of the amplitudes of Excitatory Post-Synaptic Potentials (EPSPs) under six conditions, in two sets. First, to test the effect of 5-HT, we measured EPSPs using only standard crayfish solution, added 5-HT, then returned to standard crayfish solution. In order to examine the effect of Ruthenium Red and the relation between Ryanodine receptors and 5-HT, we measured EPSPs of the crayfish exposed first to standard crayfish solution, then Ruthenium Red, then replaced Ruthenium Red with Ruthenium Red and 5-HT, and finally returned to a wash of standard crayfish solution.

RESULTS

We hypothesized that 5-HT-activated ryanodine receptors contribute to the increase in excitatory post synaptic responses (EPSPs) in crayfish (*Procambarus clarkii)* cells. To test this hypothesis, we recorded the amplitudes of EPSPs under six conditions (three washes), separated into two experiments. The results of all the conditions are gathered in Figure 1.

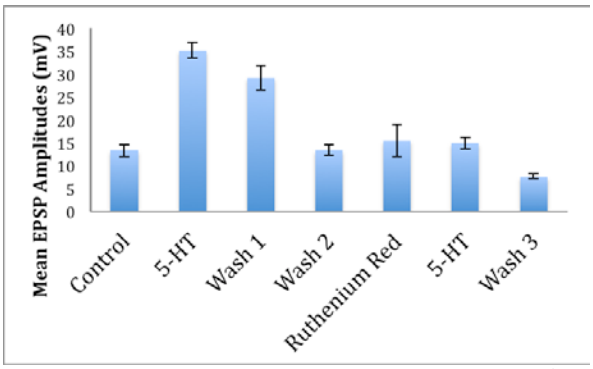

Figure 1. Mean EPSP amplitudes (mV) generated in crayfish muscle cells when exposed to all experimental conditions. For control N=16, for 5-HT N=45, for wash 1 N=23, for wash 2 N=19, for Ruthenium Red N=10, for Ruthenium Red and 5-HT N=98 and for wash 3 N=28. The standard error bars reveal that the enhancement of 5-HT is significant.

The purpose of our first was to show that 5-HT enhances the amplitudes of EPSPs. To accomplish this goal we recorded EPSP amplitudes of a crayfish muscle cell exposed first to standard crayfish solution, then to 5-HT, and finally to a wash of standard crayfish solution again. The results of this experiment are shown in Figure 2. The average EPSP amplitude under the baseline standard crayfish solution was 13 mV. When we added 5-HT, the average EPSP amplitude was 35 mV (a statistically significant difference with a p-value < 0.001). The wash average EPSP amplitude was 29, which, though lower than 5-HT, was still rather high, presumably because of the presence of residual 5-HT.

The second experiment introduced Ruthenium red, a ryanodine inhibitor. We obtained control values, introduced Ruthenium red, expecting no change to the measured EPSP amplitudes, and then added 5-HT, again expecting to observe no difference, because we hypothesized that 5-HT acts through ryanodine receptors, currently blocked by the Ruthenium red. The results to this experiment are depicted in Figure 3.

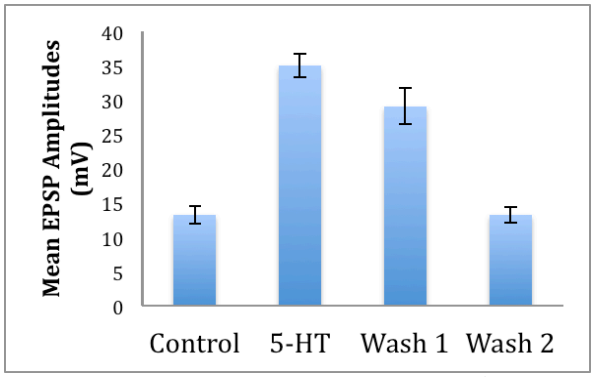

Figure 2. Mean EPSP amplitudes generated in crayfish muscle cells when exposed to control and 5-HT solutions and two washes following the exposure to 5-HT. N values listed in figure 1 description. Standard error bars reveal that the difference between control and 5-HT is significant.

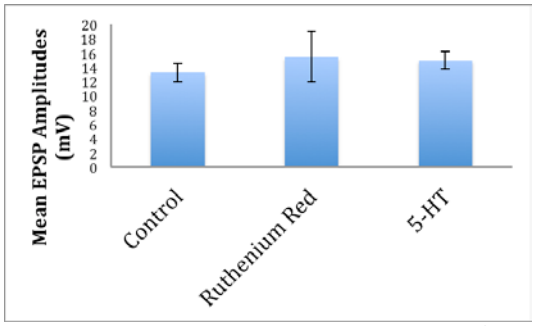

Figure 3. Mean EPSP amplitudes generated in crayfish muscle cells when exposed to standard crayfish solution, ruthenium red, and 5-HT and ruthenium red. N values in figure 1 description. Standard error bars show that there is no real statistical difference between values.

The average EPSP amplitude for Ruthenium red was 15 mV, which is a statistically insignificant difference from the baseline mean of 13 mV, with a p-value of .499 ,

The average amplitude of EPSPs when the crayfish cell was exposed to both Ruthenium red and 5-HT was 14.92 mV, which, when compared with Ruthenium red alone, was also a statistically insignificant, with a p-value of .901.

Comparing the mean amplitude of the 5-HT induced EPSPs with the mean amplitude of the EPSPs produced with the combined solution of 5-HT and Ruthenium red results in a significantly significant conclusion. The p-value of < 0.001 conclusively supports our hypothesis, showing that when Ruthenium red was not blocking the ryanodine receptors, 5-HT was effective in enhancing EPSP amplitudes, but when Ruthenium red was present, the 5-HT had little effect, implying that 5-HT works through ryanodine receptors.

DISCUSSION

Our results support our hypothesis that ryanodine receptors are responsible for a significant portion of the increase in EPSP amplitudes caused by 5-HT. In the presence of 5-HT the average EPSP amplitudes increased by a large portion. This was expected, because 5-HT is a known enhancer of EPSPs, as it causes an increase in intracellular calcium, which in turn causes an increase of neurotransmitter release (Oxford Reference Online 2010). Ruthenium red did not have a significant effect on EPSPs—it did not have effects on the cell that would alter EPSPs apart from blocking the ryanodine receptors. When we added 5-HT to the solution containing ruthenium red, EPSPs decreased to almost the original amount, meaning that blocking ryanodine receptors did affect the enhancement induced by 5-HT.

In this study we examined the role ryanodine receptors play in the enhancement of EPSPs caused by 5-HT. 5-HT activates receptors located on the pre-synaptic cell membrane. Many believe that this in turn activates a second messenger system that activates ryanodine receptors located on the endoplasmic reticulum, releasing intra-cellular calcium stores (Ullmer et al., 1996). Some argue that ryanodine receptors are in fact activated by an increased level of intra-cellular calcium, which is caused by the calcium release from IP3 receptors, also located on the endoplasmic reticulum (Zheng et al., 2005). This would suggest that 5-HT's enhancement does not involve the activation of ryanodine receptors, which would mean that blocking the ryanodine receptors would have no effect on the increased EPSPs. Our results seem to support the theory of a second messenger system activated by 5-HT, seeing as when ruthenium red was added, 5-HT's enhancement was noticeably reduced.

A phenomenon we observed while conducting this experiment was the relation between the proximity of electrode recording device to the suctioned nerve ending and the EPSP. When we stuck the electrode into a cell very close to the nerve, the EPSPs were oftentimes noticeably higher than when the electrode was stuck into cells farther away from the nerve, yet in the same muscle bundle. This may have been simply coincidence, or could suggest weakened or broken nerve connections farther along the muscle bundles.

5-HT is an important hormone found throughout the body, concentrated heavily in the brain and gastrointestinal tract (Oxford Reference Online,

2010). Excessive amounts of 5-HT have been known to cause health issues. One common example is the nausea experienced by chemotherapy patients (Oxford Reference Online, 2010). If ryanodine receptors are responsible for some of the enhancement caused by 5-HT drugs may be targeted towards them in efforts to prevent serotonin toxicity.

To further examine the responsibility of ryanodine receptors in the enhancement induced by 5-HT tests must be performed on the IP3 receptors. It is thought that both of these channels are located on the endoplasmic reticulum membrane and together are responsible for the pre-synaptic intra-cellular calcium release (Ostrovskaya et al., 2007). Studies have shown that these two ion channels function independently yet are both needed if 5-HT is to have it's enhancing qualities (Wilson et al., 2005). By blocking the IP3 receptors, then blocking the ryanodine receptors, all while serotonin is actively enhancing EPSPs, a greater understanding of the responsibility of ryanodine receptors can be discovered.

In conclusion, this study offers evidence that supports the suggestion that ryanodine receptors are responsible for some of the EPSP enhancement induced by 5-HT. This also suggests that a second messenger system exists between the 5-HT receptors on the cell membrane and the ryanodine receptors located on the endoplasmic reticulum.

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