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Ruthenium red does not affect paired-pulse facilitation at the crayfish neuromuscular junction

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ABSTRACT

Paired-pulse facilitation (PPF) of neurotransmitter release occurs when synapses in crayfish and rat hippocampi undergo paired stimuli separated by approximately 50ms. The activation of ryanodine receptors (RyRs) on the endoplasmic reticulum is believed to facilitate PPF by releasing calcium from intracellular stores in response to calcium influx. In order to better understand the role of internal calcium levels in synaptic plasticity, we inhibited the ryanodine receptors with ruthenium red and induced PPF. Our results did not support our dual hypotheses that ruthenium red would decrease excitatory postsynaptic potential (EPSP) amplitude and would increase PPF.

INTRODUCTION

Abnormalities of intracellular calcium concentration caused by dysfunction in synaptic plasticity and cell damage or death are implicated in a wide range of neurological diseases, such as Alzheimer's, Parkinson's, amyotrophic lateral sclerosis, and central nervous system trauma (Pivovarova and Andrews 2010). The ryanodine receptor (RyR) on the endoplasmic reticulum is crucial in regulating intracellular calcium concentration, and contributes to synaptic transmission by responding to calcium influx with calcium release from cellular stores.

Wang and Kelly (1997) demonstrated that RyR inhibitors in rat hippocampal slices increase paired-pulse facilitation (PPF), which is a type of synaptic plasticity. Synaptic plasticity refers to short-term changes in neuronal action in response to certain stimuli, and PPF specifically occurs when a neuron is stimulated twice, about 50 ms apart. In this event, leftover calcium from the first stimulus increases the amplitude of the response to the second stimulus. Ikemoto et al. (2001) studied how ryanodine receptors contribute to the increase of intracellular calcium in response to calcium influx in mouse skeletal muscles.

Because of prior research focusing on the relationship between ryanodine receptors and intracellular calcium in vertebrates, we chose crayfish partly because research is lacking in invertebrates. Further, we studied the neuromuscular junction of crayfish to verify results observed in other sample organisms, and to ascertain that those results represent processes that are highly conserved across animals, and potentially present in humans. We studied the relationship between ryanodine receptors and intracellular calcium by applying ruthenium red, which is an inhibitor that blocks the RyR from detecting calcium, and by extension from releasing calcium from intracellular stores. In this way, we aimed to determine if RyR inhibition would increase or decrease initial EPSP amplitudes, and whether it would increase or decrease the ratio of second EPSPs to first EPSPs in PPF. Furthermore, by better understanding the effects of RyR inhibition on synaptic transmission, and whether RyR inhibition facilitates PPF, we hoped to better understand the mechanisms that regulate intracellular calcium. Research into PPF can reveal more information about the relationship between intracellular calcium and synaptic plasticity, and such understanding could pave the way for new palliative therapies for neurological disorders related to calcium concentration.

Since the activation of RyRs releases intracellular calcium, if we inhibit RyRs we expect that calcium levels would decrease and EPSP amplitudes will decrease. We hypothesized that application of the RyR inhibitor ruthenium red to the crayfish neuromuscular junction will decrease the EPSP amplitudes, and would increase the ratio of the second EPSP to the first one in PPF, meaning that it facilitates PPF. Our results appeared to show that EPSP amplitudes decreased and PPF increased, which would support both hypotheses, but statistical analysis revealed that the results were not conclusive in either case, meaning the hypotheses was not supported.

MATERIALS AND METHODS

Sample Preparation

The crayfish samples, of the genus *Orconectes* were anesthetized before dissection by submersion in an

ice water bath until thoroughly sedated. The abdomen was clipped off of the cephalothorax, and we removed the ventral section of the abdominal exoskeleton. Next, we clipped along the ventral edge of the curved lateral exoskeleton, and manually removed the intestines and deep phasic flexors to expose the deep phasic extensor muscle. Following dissection, the sample was pinned down to a petri dish partially filled with Sylgard.

Drug Application

The control saline solution was designed to mimic conditions in the crayfish extracellular matrix: 5.4mM KCl, 196mM NaCl, 10.1mM $MgCl_2$, 10mM HEPES, and 6 mM $CaCl_2$, all dissolved in deionized water, with a pH of 7.4. We used a stock solution of 5 mM Ruthenium red in 750 µl aliquots (Tocris Bioscience), which was diluted 1:1000 with 75 ml of crayfish saline to achieve a final ruthenium red concentration of 50µM. Using a syringe, we replaced the solution every thirty minutes for both the control and ruthenium red conditions, to prevent tissue death from interfering with results.

Equipment

We pulled microelectrodes using a WPI PUL-1 automated electrode puller. We stimulated the nerve with an A-M Systems Incorporated Model 3000 AC/DC differential amplifier and a Grass SD9 stimulator. We used a Leica ZOOM 2000 light microscope, paired with micromanipulators, to observe the samples. For data observation and recording purposes we used Labchart version 8.0.5 on a MacBook Pro, and an ADInstruments PowerLab 4/25.

Stimulation Procedure & Data Collection

For electrophysiological purposes, half of the electrodes were filled with 3M KCI solution to function as recording electrodes, while half were partially sanded to function as suction electrodes. We frequently performed resistance tests on our electrodes when they were not inside the muscle tissue to verify their functionality. A resistance range of 10-20 Ω is necessary as per Silverman-Gavrila et al. (2005). For both conditions, we carefully inserted the recording microelectrode into the muscle fiber, and suctioned an associated loose nerve fiber with the suction microelectrode to stimulate it. The delay between paired pulses was 50 ms.

Data Analysis

In each sample organism, we took a series of measurements of the control condition before taking a serious of measurements of the ruthenium red condition. Since the electrodes were not moved between the control condition and the ruthenium red condition, we can consider the data sets related. The values in each series of measurements were averaged, and we applied paired t-tests to compare the means of three trials in each condition.

RESULTS

In order to determine whether RyRs contribute to synaptic transmission and paired-pulse facilitation at the crayfish muscular junction, we measured the EPSPs in both the ruthenium red (50µM) and the control solution (crayfish saline) using electrophysiology techniques.

Synaptic Transmission

We expected that inhibiting the crayfish RyR would decrease the strength of synaptic transmission by reducing the amount of available calcium. To examine this possibility, we applied ruthenium red to the neuromuscular junction and measured the resulting EPSPs through electrophysiological techniques, evoking PPF once every minute, with a delay between paired stimuli of 50ms for fifteen minutes. While the results were statistically insignificant after application of a paired t-test, the results showed a possible decrease in EPSP amplitude after application of the drug relative to the control (n=3, p = 0.16) (Fig 1).

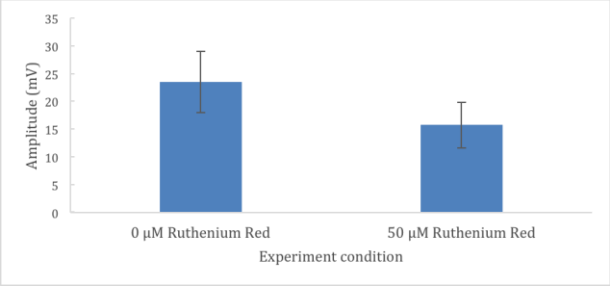

Figure 1. The effect of 50µM ruthenium red on average initial EPSP amplitude (mV). The 50µM ruthenium red solution appeared to cause relatively smaller EPSPs. The results are not statistically significant (n = 3, p > 0.05). Error bars represent standard error of the mean. N = 3 in all cases.

Paired-Pulse Facilitation

We hypothesized that the presence of ruthenium red would increase the ratio between the magnitude of the second EPSP to the magnitude of the first EPSP in PPF. In the control group of our experiment, we applied paired pulse stimulation with an interval of 50 ms between stimulations to the crayfish every minute, and we measured the ratio between the magnitude of the second EPSP and the magnitude of the first EPSP in PPF. Then, we applied ruthenium red to the solution, stimulated the sample, and recorded the ratio every minute. The

application of the drug increased the ratio and facilitated PPF, but a paired t-test showed that the difference between the ratios was not significant (n = 3, p = 0.19) (Fig. 2).

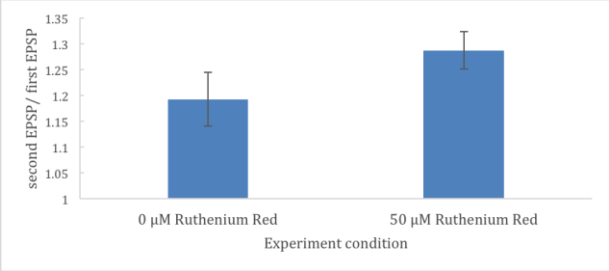

Figure 2. The effect of ruthenium red on the ratio of the magnitude of the second EPSP to the magnitude of the first EPSP in PPF. The ratio increased after the drug application, but the difference between two groups is not significant (n = 3, p > 0.05). Error bar represents the standard error of means. N = 3 in all cases.

DISCUSSION

Ruthenium red appeared to cause a decrease in EPSP amplitude relative to the control condition, although the difference was not statistically significant. Ruthenium red also displayed a potential trend of increasing PPF, but the difference between the control and ruthenium red conditions was not significant. In both cases, there are a number of possible explanations for the lack of statistical significance. First, the small sample size (n = 3) likely played a role. Second, our experimental concentration of ruthenium red (50µM) may have been at fault, as it matched departmental recommendations but did not match Hou et al.'s reported ruthenium red concentration of 50nm. Third, ruthenium red is a non-selective ligand, so it may have biological activity that we are unaware of that affected our results.

While the results were statistically insignificant, they seemed to contradict the conclusion of Hou et al. (2013) that application of ruthenium increases the amplitude of EPSPs in crayfish neuromuscular junctions. They also seem to contradict Silveira et al. (2005), who demonstrated that inhibiting the ryanodine receptors in the frog neuromuscular junction decreases PPF and tetanic depression. The apparent facilitation of PPF parallels the conclusion of Wang and Kelly (1997) that inhibition of RyRs "prevented…. PPF attenuation." However, because our ruthenium red condition was not significantly different from the control condition, the scope of possible conclusions drawn from our results is limited.

Future research could focus on more fully determining the biological activity of ruthenium red, as well as testing various concentrations of ruthenium red to determine which are the most effective. Research on chemicals that inhibit PPF or increase EPSP amplitudes instead of decreasing them would also be welcome.

Furthermore, given the trends that our data show, future research could focus on expanding the scientific community's ability to inhibit intracellular calcium release in response to repeated stimulation. This could translate into new therapies for certain neurodegenerative disorders, such as Parkinson's and ALS, in which overstimulation of neurons causes cell damage or death via excessively high calcium concentrations (Pivovarova and Andrews 2010). For example, a drug could ameliorate symptoms by targeting overactive neurons and weakening their calcium responses.

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