Pioneering Neuroscience, 2013, **13**, 43-46.

Ruthenium Red Increases the Amplitude of EPSPs at the Crayfish Neuromuscular Junction

SARAH HOU, ANA KARIN KOZJEK, and YAOYANG CHEN
Department of Biology, Grinnell College, Grinnell, Iowa

ABSTRACT

Ruthenium red is an inhibitor of ryanodine receptors (RyRs). The inhibition of these receptors in the extensor cells of a crayfish was expected to result in the inhibition of Ca^{2+} release from internal stores and a decrease of excitatory postsynaptic potential (EPSP) amplitude. Having a better understanding of this process is important because calcium influences many cell processes and is involved in many pathological states and diseases. In the experiment, the nerve was stimulated at different frequencies and the EPSP amplitudes were measured intracellularly before and after the addition of Ruthenium red. The results collected were contrary to our hypothesis and showed an increase in EPSP amplitude under high frequency stimulation. This indicates that the consequences of Ruthenium red binding with other proteins may play a more significant role in altering the amplitude of EPSPs than the inhibition of RyRs. Ruthenium red is in a non-selective ligand, meaning it interacts with many proteins other than ryanodine receptors. At this point, further follow-up experiments are required to understand the specific effects of the inhibition of ryanodine receptors per se.

INTRODUCTION

Calcium is important in influencing many cell processes and is involved in many pathological states. The release of Ca^{2+} from internal stores is controlled by intracellular calcium receptors, which are proteins on the membrane of sarcoplasmic or endoplasmic reticulum (Wu et al., 2006). There exist two such families of Ca^{2+} release proteins: the inositol 1,4,5-trisphosphate receptors (IP3) and the ryanodine receptors (RyRs) (Zalk et. al., 2007).

RyRs are intracellular Ca^{2+} channels specialized for the rapid and massive release of Ca^{2+} (Zhao et al., 2001). They form a class of intracellular calcium channels, present in excitable animal tissue, and are known as a major cellular mediator of calcium-induced calcium release (Ryanodine receptors, n.d.). Intracellular calcium signaling mediated by IP_3 and RyRs (IP_3R/RyR) plays a central role in cell survival, but emerging evidence suggests that IP_3R/RyR are also important in apoptotic cell death (Hajnóczky et al., 2000). Since the invertebrate RyR is functionally similar to the vertebrate RyR (Quinn, 1998), it is important to further investigate the effect of the inhibition of RyRs on synaptic transmission in crayfish neuromuscular junctions.

Ruthenium red is known to inhibit the RyRs. Previous studies suggested that the blockage of intracellular calcium receptors could be an effective and well tolerated therapeutic strategy in conditions of excititoxicity or Ca^{2+} overload (Zhu et. al., 2011). Our hypothesis was that the inhibition of RyRs by Ruthenium red would result in a decrease of EPSP amplitude. We predicted that the inhibition of Ca^{2+} release from internal stores would lead to lowered levels of intracellular calcium, causing less neurotransmitter to be released into the synaptic cleft from the presynaptic vesicles. The final outcome can be observed in the amplitude change of EPSPs. High frequency of stimulation increases the role of Ca^{2+} ions, which means that the inhibition of RyRs should have more significant impact. Thus, the relative change before and after the inhibition should be greater when the nerve is stimulated at 20Hz in comparison to the stimulation frequency of 2Hz.

Our hypothesis was that the inhibition of ryanodine receptors by Ruthenium red would result in a decrease of EPSP amplitude. The outcomes of our research turned out to be the opposite of our main prediction: After the addition of Ruthenium red, an increase in the amplitude of EPSPs was observed. However, as expected, the relative change before and after the inhibition was greater at the stimulation frequency of 20 Hz.

MATERIALS AND METHODS

To test how the inhibition of RyRs with Ruthenium red influences the amplitude of EPSP, we stimulated the nerve at two different frequencies.

Crayfish Tail Dissection

A crayfish was immobilized and sedated by 15 minutes of immersion in an ice bath. The abdomen was removed with scissors to be dissected. Two vertical cuts were made

down the ventral side and the ventral tissue was removed, which allowed the dorsal exoskeleton and superficial extensor muscles to be exposed.

The crayfish tail was pinned down in a dish and bathed in 100mL of standard Ringer crayfish saline solution, which has a pH of 7.4 (Lindgren), and a composition consisting of 196 mM NaCl, 5.4 mM KCl, 3.5 mM CaCl2, and 2.6 mM MgCl2 (Mezochow, 2010).

Electrode-Making Process

Electrodes were made with glass capillaries by heat separation using a PUL-1 micropipette puller, and then filled with 3M KCl. They were used to record the amplitude of EPSPs in crayfish superficial extensor muscle cells given that the resistance was between 10-20MΩ. Suction electrodes were made by the same process with additional tip-dulling with sandpaper.

Inhibition of Ryanodine Receptors

In order to achieve the final concentration of 50 nM ruthenium red in 100 mL of saline solution, 25mL of the saline solution was removed and replaced with 25mL of 200nM Ruthenium red (purchasable from Sigma-Aldrich) mixture to block RyRs (Ben-Or).

Intracelullar Recording

With the microelectrode inside the cell, the nerve was stimulated at different frequencies through the suction electrode. The base frequency of the stimulation was 0.2 Hz. For the measurement of relative change of the amplitude of EPSPs before and after the inhibition, the nerve was stimulated at two different frequencies: 2 Hz and 20 Hz for duration of 10 seconds each. The latter created a condition known as *tetanic stimulation.* EPSP amplitudes of the intracellular cell responses to the stimulation were measured via microelectrodes.

Analysis

In order to observe the impact of the inhibition of RyRs on the neuromuscular transmission, we compared the relative change of the amplitudes of EPSPs before and after the inhibition. This allowed the comparison of the recordings taken from different muscle cells and different crayfish specimens. T-tests were used to determine whether the inhibition of RyRs resulted in a significant change in the amplitude of EPSPs between the control and experimental groups.

Control Groups:
a. without Ruthenium red under frequency of 2Hz.
b. without Ruthenium red under frequency of 20Hz.

Experimental Groups:
c. with Ruthenium red under frequency of 2Hz.
d. with Ruthenium red under frequency of 20Hz.

RESULTS

We wanted to know if inhibition of ryanodine receptors on the neuromuscular transmission at different stimulation frequencies had any effect on the EPSP amplitudes of the extensor muscles in crayfish tails. Our hypothesis was that the inhibition of RyRs by Ruthenium red would result in a decrease of EPSP amplitude. To examine our hypothesis, we recorded the amplitude of EPSPs in the crayfish muscle cell under different experimental conditions – two control groups and two experimental groups. Seen in the Chart 1 are the EPSPs amplitude changes normalized to the baseline frequency of 0.2 Hz in the crayfish muscle cell under different experimental conditions–two control groups and two experimental groups.

DATA				
	2 Hz		20 Hz	
Trial	**Base Line**	**Ruthenium Red**	**Base Line**	**Ruthenium Red**
20-Nov	9.53%	27.75%	45.57%	92.01%
22-Nov	46.43%	559.42%	194.99 %	1177.96%
27-Nov	10.19%	14.61%	12.17%	25.44%
Mean	22.05%	200.59%	84.24%	431.80%
Standard Deviation	21.12%	310.82%	97.35%	647.05%
Standard Error	12.19%	179.45%	56.21%	373.57%

Chart 1. Data analysis of three experimental samples, including the average change in EPSP amplitudes in control and experimental groups, mean value, standard deviation and standard error.

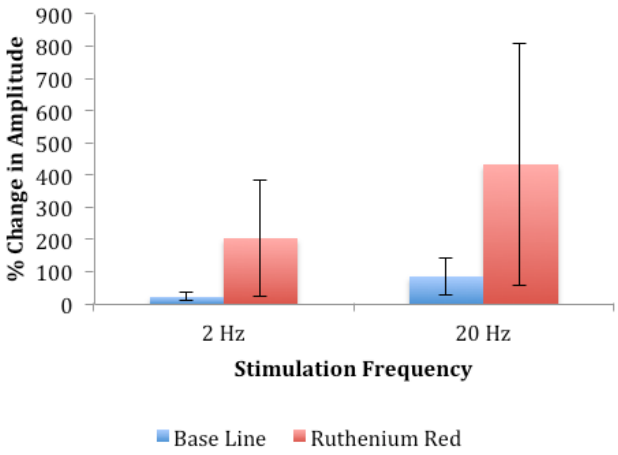

Figure 1. The average change in EPSP amplitude generated in crayfish muscle cells when stimulated in standard condition at a frequency of 2Hz, with Ruthenium red at a frequency of 2Hz, in standard condition at a frequency of 20Hz, and with Ruthenium red at a frequency of 20Hz (n=3 for each group). The bar graph reveals that EPSPs collected under conditions of 20Hz are greater in amplitude than those under 2 Hz. Regardless of the change in frequency, the average change in EPSP amplitude with Ruthenium Red is consistently greater than the one without under the same stimulation frequency.

The amplitude of EPSPs in the muscle cells increased when the stimulation frequency was raised from 2Hz to 20Hz. As seen in Figure 1, after the inhibition of RyRs by Ruthenium red, the amplitude of EPSPs in the muscle cells visibly increased under the stimulation frequency of both 2Hz and 20Hz.

In respect to stimulation of 0.2 Hz, the mean percent change in amplitude when being stimulated at 2 Hz and 20 Hz without Ruthenium red was 22.05% and 84.24% respectively. For the mean percent change with Ruthenium red, they were 200.59% and 431.80%. The results consistently show an increase in percent change in amplitude with both the addition of Ruthenium red and increase in frequency stimulation.

As seen in figure 1, there is a very visible jump in percent change of EPSP amplitudes. Even so, according to t-tests performed on the data, these results are statistically insignificant. The p-values of when comparing our data between our control and experimental groups were 0.38 and 0.40 for 2 Hz and 20 Hz respectively. This could very well be due to our small sample size and variation due to different crayfish specimen.

DISCUSSION

Our main prediction was that the inhibition of RyRs by Ruthenium red would result in a decrease of the amplitude of EPSPs. Contrary to our hypothesis, the amplitude of EPSPs increased with the addition of Ruthenium red. However, our measurements confirm that the impact of Ruthenium red is more significant under high frequency stimulation. The relative change of EPSPs' amplitude before and after the addition of Ruthenium red was larger at the frequency of 20Hz than the relative change as the nerve was stimulated at 2Hz.

The application of Ruthenium red is known to inhibit calcium-induced calcium release from intracellular calcium stores by blocking the RyRs on the membrane of endoplasmic reticulum (Phillipe et al., 1996). Thus, we expected that the amplitude of EPSPs would decrease due to the lower concentration of Ca^{2+} ions in the presynaptic cell caused by the inhibition of intracellular calcium release by RyRs.

If EPSPs had decreased with the application of Ruthenium red, this would imply that RyRs receptors were successfully inhibited, resulting in lower levels of intracellular Ca^{2+}. This would confirm the possibility that the blockage of these receptors could be an effective and well tolerated therapeutic strategy in conditions of excitotoxicity or Ca^{2+} overload (Zhu et. al., 2011).

However, our results indicate the opposite: the mean increase in the amplitude of EPSPs from the baseline (stimulation frequency of 0.2Hz) was larger after the inhibition in both experimental groups (at high and low frequency). The reason for this result could be that the Ruthenium red is in a non-selective ligand, which means that it interacts with a large number of proteins (Ruthenium red, n.d.). One possible explanation in our case is that Ruthenium red bound to the mitochondrial Ca^{2+} uniporter, which would inhibit the mitochondrial Ca^{2+} uptake performed by the uniporter (Hajnóczky et al., 2006). With the inhibition of the uptake, the Ca^{2+} ions would start to accumulate in the interior of the presynaptic cell, causing the release of more neurotransmitter vesicles into the synaptic cleft and so a higher amplitude of EPSPs.

On the other hand, the t-test we preformed revealed poor statistical significance between our control and experimental groups (p-values: p_1=0.38, p_2=0.40). The cause of this result might be our small sample number (3), a high variability between different crayfish specimen, or other difficulties regarding experimental conditions (cell death, muscle twitching and so on). Still, all the comparisons between the relative amplitudes of EPSPs before and after the inhibition in the same crayfish specimen indicated that the application of Ruthenium red caused an increase in the amplitude of EPSPs.

We can conclude that Ruthenium red affected the process of neuromuscular transmission, although further research has to be done. In the future, the sample size data collection should be greater in number and performed under highly controlled experimental conditions, especially, more research should be done about the side effects of the chemical used. Since Ruthenium red showed to increase the amplitude of EPSPs possibly due

to the inhibition of the calcium uptake performed by mitochondrial uniporters, leading in the accumulation of intracellular Ca^{2+}, it would be interesting to further investigate this phenomenon. The addition of cyanide would result in the disruption of the mitochondrial electron transport chain, which is the process crucial for all of the mitochondrial functions within the cell, including the calcium uptake (Cyanide, n.d.). The inhibition of this function prior to the addition of Ruthenium red, would give an answer to whether the blockage of the calcium uptake actually influenced neuromuscular transmission as our results suggest.

ACKNOWLEDGEMENTS

We would like to thank Professor Lindgren for teaching us the relevant knowledge about neuromuscular transmission in order to complete this lab experiment. Kaya Matson, our class mentor, and Jason Parks, our lab technician helped us locate and purchase appropriate lab materials and provided technical assistance and support during experiments. Without the help, guidance, and demonstrations of these people, this experiment would not have been possible.

REFERENCES

Bee JH, Park JW, & Kwon TK. 2003. Ruthenium red, inhibitor of mitochondrial Ca2+ uniporter, inhibits curcumin-induced apoptosis via the prevention of intracellular Ca2+ depletion and cytochrome c release. *Biochemical and Biophysical Research Communications*. 303(4), 1073-9.

Ben-Or, L. 2010. 5-HT-Activated Ryanodine Receptors Play an Active Role in Increasing Excitatory Post-Synaptic Potentials at the Crayfish Neuromuscular Junction. *Pioneering Neuroscience*, (11), 5-8.

Cyanide. n.d.. In Wikipedia. Retrieved December 4, 2013, from http://en.wikipedia.org/wiki/Cyanide

Excitotoxicity. (n.d.). In Wikipedia. Retrieved November 13, 2013, from http://en.wikipedia.org/wiki/Excitotoxicity

Hajnóczky G, Csordas G, Das S, Garcia-Perez C, Saotome M, Sinha Roy S, & Yi M. 2006. Mitochondrial calcium signalling and cell death: approaches for assessing the role of mitochondrial Ca2+ uptake in apoptosis. *Cell Calcium*. *40*(5-6), 553-60.

Hajnóczky, G. 2000. Control of apoptosis by ip3and ryanodine receptor driven calcium signals. *Cell Calcium*, 28(5-6), 349-363.

Phillipe, M., & Basa, A. 1996. The effects of ruthenium red, an inhibitor of calcium-induced calcium release, on phasic myometrial contractions. *Biochemical and Biophysical Research Communications*, *221*(3), 656–661. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8630017

Ruthenium red. n.d.. In Wikipedia. Retrieved December 4, 2013, from http://en.wikipedia.org/wiki/Ruthenium_red

Quinn, K. E., Castellani, L., Ondaris, K., & Ehrlich, B. E.. 1998. Characterization of the ryanodine receptor/channel of invertebrate muscle. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, 274(R494-R502), Retrieved from http://ajpregu.physiology.org/content/274/2/R494.article-info

Wu, H. 2006. Calpain and synaptic function. *Molecular Neurobiology*, 33(3), 215-236.

Zalk, R., Lehnart, S. E., & Marks, A. R. 2007. Modulation of the Ryanodine Receptor and Intracellular Calcium. *Annual Review Of Biochemistry*, 76(1), 367-385. doi:10.1146/annurev.biochem.76.053105.094237

Zhao, F., Pin, L., Chen, S. R. W., Luis, C. F., & Fruen, B. R. 2001. Dantrolene inhibition of ryanodine receptor ca21 release channels. *The Journal of Biological Chemistry,* 276(17), 13810. Retrieved from http://www.jbc.org/content/276/17/13810.long

Zhu, H., Bhattacharyya, B. J., Lin, H., & Gomez, C. M. (2011). Skeletal muscle ip3r1 receptors amplify physiological and pathological synaptic calcium signals. *The Journal of Neuroscience*, *31*(43), 15269-15283. doi: 10.1523/JNEUROSCI.3766-11.2011