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Effects of Argireline on EPSP amplitude at the Crayfish Neuromuscular Junction

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ABSTRACT

Argireline is an anti-wrinkle agent that is proposed to work in the presynaptic cell of the neuromuscular junction by mimicking the structure of a presynaptic protein and preventing the interaction between SNARE proteins, an interaction which is required for neurotransmitter release. We sought to discover how much of an effect Argireline has on inhibiting neurotransmitter release by measuring excitatory postsynaptic potentials (EPSP's) on crayfish tail muscles using intracellular recordings and subjecting them to this chemical. Hyaluronic Acid was used as our control because it is found in creams containing Argireline as the only active ingredient. Argireline had an opposite effect than what we had predicted raising the EPSP's of our crayfish muscles significantly.

INTRODUCTION

Argireline was recently introduced into cosmetics as an anti-wrinkle agent with effects similar to Botulin toxin type-A, (Botox). Botox works by decreasing the amount of muscle activity by blocking the release of acetylcholine. This is done by inhibiting the fusion of the vesicle storing the neurotransmitter to the cell membrane and thus rendering the muscle inactive for a period of three to four months (Kukreja and Singh 2009). Argireline (Ac-EEMQRR-NH2) is a synthetic hexapeptide that works in much the same way as Botox. Argireline mimics the effects of SNARE proteins, which cause the neurotransmitter containing vesicles to bind to the presynaptic membrane (Blanes-Mira et al., 2002).

According to C. Blanes-Mira et al (2002), the scientists who are credited with the discovery of Argireline, this substance is skin permeable and interferes with the assembly of the SNARE ternary complex inhibiting Ca^{2+}-dependent catecholamine release from chromaffin cells. Cosmetic companies claim that Argireline works by suppressing neurotransmission by imitating the SNARE-protein SNAP-25 and paralyzing muscle cells. SNARE-proteins act as a zipper to connect and fuse vesicles containing neurotransmitter to the cell membrane and thus, releasing the neurotransmitter (Mahal et al. 2002). By mimicking the structure of the SNARE-protein SNAP-25, Argireline binds to the SNARE-protein receptor cite and prevents the normal reaction (Jung, et al 2007).

By measureing EPSP's, it is possible to test the effect of Argireline on the neuromuscular junctions of crayfish. EPSP's are excitatory post-synaptic potentials and occur when neurotransmitter released by the presynaptic cell bind to receptors on the postsynaptic cell and cause an influx of positive ions and thus an increase in the voltage. EPSP's can be measured in the crayfish neuromuscular junction using microelectrodes and intracellular recording. This consists of a microelectrode in a postsynaptic muscle cell and a suction electrode, which fires an artificial stimulus of a presynaptic nerve.

Do to Argireline inhibiting the release of neurotransmitter into the synaptic cleft, we hypothesized that adding Argireline to our sample would cause a decrease in EPSP amplitude of the crayfish neuromuscular junction. Our results were quite contrary to our hypothesis, showing that Argireline actually significantly raises the amplitude of EPSP's.

MATERIALS AND METHODS

Crayfish

Carolina Biological Supply Company supplied us with a species of crayfish called *Procambarus clarkii*. These crayfish were stored at a temperature of 20°C and then placed into ice for 15 minutes before they were dissected. To begin the dissection, we detached the tail from the rest of the body. We then cut along both sides of the tail, staying as close to the ventral side of the tail as possible. We then got rid of the excess tissue so that only the exoskeleton of the dorsal surface and the four superficial extensor muscles remained. The remaining tail was then placed into our dissection dish, 10 ml in volume and then submerged in our testing solution.

Microelectrodes and Stimulation

Two types of electrodes were used for this experiment. Microelectrodes were used in the postsynaptic cell for recording EPSPs. These were made by pulling 1.2 mm diameter capillary glass tubes using a PUL-1 electrode puller. These electrodes were filled with 3M KCL solution. The resistance of these electrodes ranged from 4 MΩ to more than 10 MΩ. The microelectrodes were then connected to a computer so that the voltage difference between the microelectrode and the reference electrode could be measured and recorded using a computer program called SCOPE. Suction electrodes were used to fire an artificial stimulus onto the presynaptic cell. The suction electrode sucked up a nerve on the crayfish tail and then, using a GRASS stimulator, stimulated the presynaptic nerve with single repeating pulses at a frequency of 0.5 Hz. The lowest possible voltage in order to cause an EPSP was used.

Solutions

In the experiment we tested two different solutions. The first solution, the control, was Hyaluronic Acid. The other solution used was Argireline. We found that both of these solutions were 100% water soluble and therefore would dissolve into our saline solution. To make the solutions we added 10mL of normal saline, which was prepared from 5.4mM KCl, 19.6mM NaCl, 26mMMgCl_2H_2O, 10mM Sodium Hepes Buffer, and 13.5mM $CaCl_22H_2O$, was brought to a pH of 7.4 just before beginning our experiment and mixed with 2mL of both Hyaluronic Acid and Argireline to get respective readings. The Hyaluronic Acid and Argireline were both purchased from Cellbone.com.

RESULTS

We sought to determine the effect of Argireline on the crayfish neuromuscular junction. Argireline has been proposed to work by inhibiting SNARE-proteins from binding to one another and thus inhibiting the release of neurotransmitter into the synaptic cleft. Because of this, we predicted that the Argireline would decrease the amplitude of the crayfish EPSP. Using Hyaluronic Acid as our control, we hypothesized that the EPSP's recorded for the crayfish sample submerged in Argireline would be significantly lower than the EPSP's recorded for the crayfish sample submerged in our control. After measuring 41 EPSP's with our control and 50 EPSP's with Argireline, we found the opposite of our predictions to be true. Argireline significantly raised the amplitude of the EPSP's of the crayfish neuromuscular junction as shown in Figure 1.

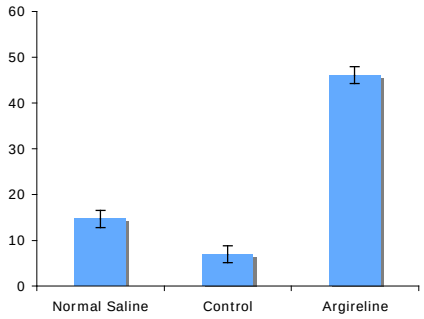

Figure 1: This graph shows the average value of EPSP amplitude for our control (Hyaluronic Acid) and for Argireline. The Average value for Argireline was significantly higher than that of the control. After performing a t-test on our data, we found our p-value for our control to be p=1.09E-26 and the p-value for Argireline to be p=2.17E-26. This statistical analysis shows our data to be very significant.

A t-test was then performed to compare the effects of the EPSP amplitudes measured in the presence of hyaluronic acid and argireline. This p-value was .04 which again shows our results to be significant. To find these results, we began a preliminary experiment by simply measuring EPSP's of crayfish tails submerged normal crayfish solution. We then measured EPSP's with crayfish tail submerged in our control, followed by our Argireline. We found the average EPSP submerged in normal saline to be around 15 mV. Our average EPSP amplitude of our control was unexpectedly smaller than that of the normal saline, being around 6.7 mV. Our average EPSP of our Argireline was significantly higher than our control having an average amplitude of 46.7 mV.

DISCUSSION

We hypothesized that because of the way Argireline is proposed to work, the application of it to the superficial extensor muscles of the crayfish tail would repress neurotransmitter release and thus decrease the amplitude of EPSP's. Because Hyaluronic acid is proposed to have no effect on anti-wrinkle activity, but added to a solution consisting of Argireline as an inactive ingredient, we predicted that it would have no effect on EPSP amplitude (Blanes-Mira 2002).

There is no doubt that use of Argireline affects the amplitude of EPSP's on the neuromuscular junction, although in a way quite contrary to what we had predicted. We found through our experimental procedure that Argireline actually significantly increases the amplitude of EPSPs in the crayfish neuromuscular junction. We also found that our control, Hyaluronic Acid, noticeably decreases the amplitude of the EPSP when compared to those of normal saline. This was an unexpected result but could be due the fact that hyaluronic has been found to decrease the ability for a protein to adhere to its receptor (Lord et al. 2009).

We do not know why Argireline had the effects it did, which were completely opposite to that which we expected, and therefore further research must be done to explain this result. Blanes-Mira et al. (2002) proposed that Argireline acts by inhibiting the effect of SNARE proteins and thus preventing vesicles containing neurotransmitter to fuse to the presynaptic membrane and release their contents. Through this proposed effect of Argireline, it would make sense that this chemical would cause the amplitude of EPSP to be reduced when applied to a sample muscle cell and nerve.

We found that Argireline has the opposite effect than what is proposed by Blanes-Mira et al. (2002). We do not know why this is, but it is known, however, that wrinkles are formed by excessive stimulation of muscle fibers, which in turn pull skin inwards, forming a wrinkle. Therefore, after analyzing our data, we concluded that Argireline increases amplitude of EPSP either by stimulating neurotransmitter release or through another method, such as affecting the activity of the innervating neuron.

Because our findings actually suggest that applying Argireline to skin would rapidly increase the amount of wrinkles instead of reduce them, further research must be done. If Argireline significantly increases the EPSP on the postsynaptic cell of the neuromuscular junction, then the muscle activity would be much more intense and would cause the muscle to fatigue and wrinkles to form at a much higher rate. This result completely contradicts the cosmetic claims made by cellbone.com and by the research of Blanes-Mira et al. (2002).

Further research should search to discover why Argireline and Hyaluronic Acid have the effect that they do on the crayfish tail neuromuscular juntion and would then relate these findings to the cosmetic significance of this chemical. It would also be important to know if Hyaluronic Acid has more of an effect in cosmetics than what is proposed by the findings of Blanes-Mira et al. (2002). It would be interesting to prove or disprove the claims made by the cosmetic industry about the effects of Argireline using scientific research at the neuromuscular level.

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