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Nitric Oxide Mediates the Effect of DF_2 on EPSP Amplitude.

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ABSTRACT

This experiment looked at the role of nitric oxide in the mechanism by which the peptide DF_2 increases neurotransmitter release at the crayfish neuromuscular junction. We hypothesized that if DF_2 increases neurotransmitter release through a pathway involving nitric oxide as a retrograde signal, then when L-NAME, an inhibitor of nitric oxide synthesis, was applied, DF_2 will not increase neurotransmitter release. To test this we exposed crayfish superficial dorsal extensor muscles to DF_2, which was amplified with IBMX. We then submerged the preparation into saline solution that contained DF_2, IBMX, and L-NAME. Neurotransmitter release was measured by the amplitude of EPSP traces recorded from the postsynaptic muscle cells via intracellular recording. Our data shows an increase in neurotransmitter release after exposure to DF_2 and IBMX, as well as a decrease in neurotransmitter release after the addition of L-NAME, a trend that supports our hypothesis. Further testing is needed to draw conclusions about our hypothesis.

INTRODUCTION

DRNFLRFamide, AspArgAsnPheLeuArgPhe-NH_2, (DF_2), is a peptide that enhances synaptic transmission at neuromuscular junctions. However, the mechanism by which DF_2 accomplishes this is still unclear (Friedrich *et al.* 1998; Badhwar et al 2006). Badhwar et al (2006) suggest that protein kinase A (PKA) and protein kinase G (PKG) are involved in the pathway by which DF_2 increases EPSP amplitude. Friedrich *et al.* (1998) claim that protein kinase C (PKC) is needed in this process. Calcium-calmodulin dependent protein kinases, such as CaMKII, also have apparent roles in mediating the effects of DF_2 in the presynaptic terminal (Noronha and Mercier 1995). According to Badhwar *et al.* (2006), it is plausible that nitric oxide is required in the DF_2 signaling pathway as a retrograde signal because of its small molecular size, high membrane permeability, and the presence of membrane-bound guanylyl cyclase in crustaceans. Badhwar *et al.* (2006) hypothesized that nitric oxide increases cGMP levels, which is involved in the DF_2 pathway, via soluble guanylyl cyclase. Our study explores the role of nitric oxide as a possible retrograde messenger in the mechanism by which DF_2 increases neurotransmitter release.

The antagonist L-NAME was utilized in this study because of its ability to block nitric oxide production. L-NAME blocks nitric oxide production because the structure is similar to that of the amino acid L-Arginine. This similarity in structure allows L-NAME to act like L-Arginine and bind with nitric oxide synthase, the nitric oxide production enzyme, to stop nitric oxide production. We hypothesized that if DF_2 increases neurotransmitter release through a pathway involving nitric oxide as a retrograde signal, then when L-NAME is applied to inhibit nitric oxide synthesis, there will be a decrease in neurotransmitter release.

To test this hypothesis we exposed crayfish superficial dorsal extensor muscles to L-NAME to block nitric oxide production. We expected DF_2 to increase the neurotransmitter release, and that L-NAME would cause a decrease in neurotransmitter release. Our results show that DF_2, when enhanced with IBMX, causes an increase in neurotransmitter release. Our results also suggest that exposure to L-NAME prevents this increase in neurotransmitter release.

MATERIALS AND METHODS

Crayfish Specimen

We used crayfish (*Procambarus clarkii*), which were stored at 20 °C, and put on ice before the experiment. We cut the tail off a crayfish and then cut along the sides of the tail, cutting as close to the ventral part of the tail as possible. The cephalothorax and surrounding tissues and muscle cells were removed so that only the exoskeleton of the dorsal surface and the superficial extensor muscles along the dorsal surface remained. The tail was placed into the dissection dish, pinned, and covered with 25mL of crayfish saline solution.

Saline Solutions

Ringer solutions were prepared with three different chemicals by dilution with a low calcium crayfish ringer solution (Table 1). This control solution had a pH of 7.4 and consisted of 5.4mM KCl, 200.7mM NaCl, 12.3mM $MgCl_2 \cdot 6H_2O$, 5mM Sodium Hepes Buffer and 5mM

$CaCl_2 \cdot 2H_2O$. We used a lower calcium solution to inhibit cells from triggering action potentials.

Table 1. Composition of Saline Solutions

Saline	DF_2 (mM)	IBMX (mM)	L-NAME (mM)
Control	0	0	0
A	2.0	-	-
B	2.0	.01	-
C	2.0	.01	0.3

The first preparation was exposed to the control saline followed by saline A. The second preparation was exposed to the control, then saline B, followed by saline C. Each preparation was submerged into 25mL of saline solution, which was replaced with new solution every 15-30 minutes. Tests involving DF_2 were completed without changing the saline solution until a new chemical was added because we had such a small amount of DF_2 to work with. These tests were completed as quickly as possible to protect against fatigue and cell death.

Electrodes

We used two kinds of electrodes, suction electrodes for nerve stimulation and microelectrodes for recording. Both electrodes were fitted to manipulators and their respective reference electrodes were submerged in the saline solution. The suction electrode was put into an electrode holder that allowed saline to be drawn through the holder by a syringe. Recording electrodes were pulled from glass capillary tubes with 1.2mm diameter, filled with 3M KCl, and inserted into an electrode holder, which was also filled with 3M KCl. Recording electrodes had resistances ranging from 4MΩ to more than 10MΩ.

Nerve Stimulation and Recording

The suction electrode was attached to a stimulator, which stimulated the pre-synaptic nerve that was sucked into the electrode. The nerve was stimulated with single pulses at a frequency of 0.5Hz, and at the lowest voltage possible to measure an EPSP.

Using a microscope and micromanipulator, we inserted the microelectrode into a muscle cell in the same segment and on the same side as the nerve that was being stimulated. The recording microelectrode recorded the signals in the post-synaptic muscle cells. The signals passed through an amplifier and the membrane potentials and EPSP traces were viewed using the Scope program.

RESULTS

We tested the involvement of nitric oxide in the DF_2 signaling pathway, through which DF_2 increases EPSP amplitude. First, we exposed one preparation to DF_2 to see if DF_2 affects EPSP amplitude. We then applied DF_2 and IBMX, followed by L-NAME to another preparation to see if blocking nitric oxide production alters the effect of DF_2 on EPSP amplitude.

We compared the EPSP amplitudes recorded before and after we applied DF_2. Our data shows that DF_2 does not increase EPSP amplitude when applied alone. DF_2 decreased the EPSP amplitude by 28.4% (Figure 1).

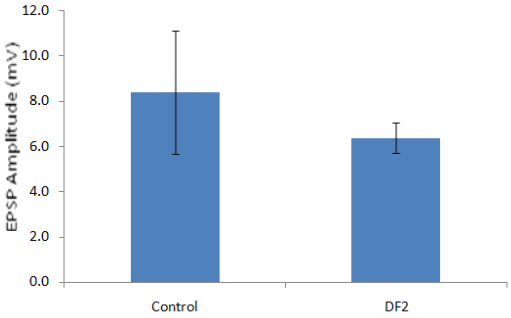

Figure 1. Average EPSP Amplitude Before and After Exposure to DF_2 The average control amplitude was 8.4 mV (n=4) and the average DF_2 amplitude was 6.4mV (n=3). After exposure to DF_2, the amplitude dropped 24% ($p>.05$, student t-Test). Error bars indiciate standard error of 2.72 for the contrl and 0.66 for DF_2.

We compared the change in EPSP amplitude before and after we applied both DF_2 and IBMX on another crayfish preparation. The average EPSP amplitude for the control was 5.61 mV, and the average EPSP amplitude for DF_2 and IBMX was 6.58 mV. The average EPSP amplitude increased 17.3%, which demonstrates the effect of DF_2 and IBMX on EPSP amplitude. We then compared the EPSP amplitudes before and after L-NAME was added. Our results showed that the average EPSP amplitude after the sample was exposed to L-NAME was 3.22 mV, 51% lower than the average amplitude for DF_2 and IBMX (Figure 2). The average amplitude for L-NAME was also 42.5% lower than the average for the control (Figure 2).

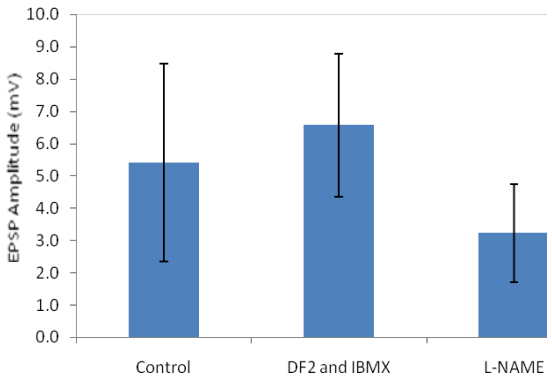

Figure 2. Average EPSP Amplitudes.
The average EPSP amplitude for DF_2 and IBMX trials (n=5) is higher than the control average (n=4, p>.05), and the L-NAME trials (n=5) have a lower average than both DF_2 and control trials (p>.05 for both comparisons). Error bars show standard errors, control: 3.06, DF_2 and IBMX: 2.21, and L-NAME: 1.51.

DISCUSSION

In our experiment, we observed that DF_2 did not increase neurotransmitter release when applied alone. Although we do not have much data to support this result, these results are contrary to the results presented by Badhwar *et al*. (2006). This data could suggest a flaw in our assumptions that DF_2 should increase EPSP amplitude per Badhwar et al (2006), but more data is needed for such a conclusion to be drawn from these results.

As suggested by Badhwar et al (2006), when a significant increase in EPSP amplitude was not observed after adding DF_2, we added IBMX, which should have enhanced the effects of DF_2. When DF_2 and IBMX were both added to the preparation the EPSP amplitude increased, which indicates an increase in neurotransmitter release. Although we do not have a significant amount of data to draw conclusions, this result is consistent with the findings of Badhwar et al (2006).

Our data shows that the EPSP decreases when L-NAME is added to a preparation that contains DF_2. This preliminary data suggests that nitric oxide is involved in the pathway by which DF_2 increases neurotransmitter release. Further study is needed to support or refute this trend. This data would support our hypothesis that nitric oxide is involved in the mechanism by which DF_2 increases EPSP, as the necessary nitric oxide should be unavailable because L-NAME blocks the synthesis (Newman et al, 2007).

The average EPSP amplitude for L-NAME was also lower than the average control amplitude. This could be due to a few reasons. One possibility is that inhibiting nitric oxide production with L-NAME may affected mechanisms other than the DF_2 mechanism. Our design did not allow for targeted application of chemicals, so it is possible that the application of L-NAME affected more than just the effect of DF_2 and IBMX. The other very plausible option is that the amplitudes were generally lower because the cells were fatigued and beginning to die. Although saline solutions were replaced at relatively regular intervals, it is possible after about one and a half hours, the crayfish muscle cells were beginning to die.

Future research on this topic would include more trials using L-NAME and DF_2 to see if more data supports our hypothesis the way our preliminary data suggests. If our hypothesis is supported, the next step would be to test the role of nitric oxide as a retrograde signal using carboxy-PTIO. This would also support the suggestion that nitric oxide is involved in a retrograde signaling pathway that Badwar *et al.* (2006) present in their paper. More tests should be done to measure nitric oxide levels, and to test if the DF_2 receptors are located on the post-synaptic membrane using fluorescent markers.

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