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Exogenous Nitric Oxide Enhances Synaptic Transmission at the Crayfish Neuromuscular Junction

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ABSTRACT

Nitric oxide (NO) plays a critical role in the CNS in higher animals and at the lizard neuromuscular junction (NMJ), modulating synaptic transmission. However, whether this role is conserved at the crayfish NMJ remains unclear. In this study, we attempted to determine the exogenous and endogenous role of NO in synaptic transmission by recording excitatory post synaptic potentials (EPSPs) at the crayfish NMJ. Application of DEA-NO, a NO donor, significantly increased EPSP amplitude. However, the application of L-NAME, a Nitric Oxide Synthase (NOS) inhibitor, alone did not significantly change EPSPs. We therefore conclude that exogenous nitric oxide enhances synaptic transmission at the crayfish NMJ; however, endogenous NO is not produced tonically during low-frequency stimulations.

INTRODUCTION

Recently, diffusible gases have begun to receive attention as potential neurotransmitters/ modulators in both vertebrates and invertebrates. Although there is less than a consensus for these gases' function, it is generally accepted that they serve a modulatory role in the nervous system (Baranano and Snyder, 2001). Nitric oxide (NO), for example, participates in long-term potentiation (LTP) and other forms of synaptic plasticity, by modulating the outputs of many neural networks, including those involved in swimming in *Xenopus*, feeding in the locust, or in producing stomach movements of lobsters (Araki *et al.*, 2003). NO has been demonstrated to play a key modulatory role in the olfactory, visual, and mechanosensory systems, as well as to play a key role in memory formation in many animals (Schupe *et al.*, 2001).

The role of NO in the presynaptic terminal as well as the postsynaptic terminal has been widely studied in Central Nervous System (CNS) and endothelial cells of higher eukaryotes. Under normal physiological conditions, NO is an unorthodox, non-polar neurotransmitter, that diffuses from its site of synthesis in three dimensions (Philippides *et al.*, 2000). Since NO can permeate through cell membranes, its influence is based on its diffusional constant and life span (Ahern *et al.*, 2002). NO is produced during the conversion of L-arginine to L-citrulline by the calcium/calmodulin-activated enzyme Nitric Oxide Synthase (NOS), in a reaction requiring oxygen and NADPH (Moncada *et al.*, 2001). Previous studies at the lizard neuromuscular junction (NMJ) have identified NOS in nerve terminal, Perisynaptic Schwann Cells (PSCs), and muscle cells via NADPH-diaphorase staining (Graves *et al.*, 2004). NO has been shown to act directly on neuronal and muscle targets by S-nitrosylation of proteins and/or indirectly by means of activation of sGC, which works to augment levels of the second messenger cyclic guanosine 3',5' monophosphate (cGMP) (Ahern *et al.*, 2002). There are also indications that NO from nNOS could play a role as a retrograde messenger, diffusing rapidly to the presynaptic terminal and affecting neurotransmitter release (Arancio *et al.*, 1996). However, whether this mechanism is conserved at the crayfish NMJ remains unclear.

Because of the multiplicity of effects of NO action on mechanosensory processing, determining the specific role it may have on the outputs of local circuits remains a difficult and ongoing task. In the crayfish, NO is known to be involved in modulating the activity of local circuits that control and generate movements of the appendages of the tail fan (Alraki *et al.*, 2003). Previous imaging studies with a NO-specific indicator have shown that NO is synthesized endogenously in the terminal abdominal ganglion and that NOS inhibitors can lessen the level of NO synthesis (Schuppe *et al.*, 2002). Furthermore, through histochemistry and intracellular double labeling, neurons that contain the enzyme NOS and synthesize NO, have been identified as intersegmental ascending interneurons with somata in the terminal abdominal ganglion (Araki *et al.*, 2003). It has been shown that up-regulating endogenous or exogenous NO levels by bath application L-arginine or SNAP leads to an enhancement of the reflex responses of the blades of the crayfish's tailfan, known as the uropods (Araki *et al.*, 2003). Bath application of the sGC inhibitor, ODQ, and an analogue of cGMP to the tailfain indicate that one of

the targets of NO in the terminal ganglion is the enzyme sGC, and that the actions of NO are mediated through cGMP (Araki *et al.*, 2003). When L-arginine and ODQ were applied simultaneously to the terminal ganglion, the L-arginine-induced increase in reflex response was blocked by ODQ, implying that NO exerts its modulatory effects through sGC and cGMP (Araki *et al.*, 2003). Mean responses from six preparations showed that bath application of NOS inhibitor L-NAME caused a significant reduction in spike frequency of motor neuron response in approximately 50% of trials (Araki *et al*., 2003).

Previous studies have only looked at the frequency of local circuit response to endogenous NO and not the strength of synaptic transmission at the NMJ (Araki *et al*., 2003). Therefore, in our study, we attempted to directly determine the role of both endogenous and exogenous NO on synaptic transmission at the crayfish NMJ by measuring the amplitude of Excitatory Post-Synaptic Potentials (EPSPs) as the indicator of the strength of synaptic transmission and neurotransmitter release. Our results suggest that exogenous NO enhances synaptic transmission. Additionally, our data suggest that there is no endogenous production under basal experimental conditions (low-frequency stimulation) at the crayfish NMJ.

MATERIALS AND METHODS

Crayfish preparation

We used adult crayfish obtained from Carolina Biological Supply Co. and maintained in freshwater tanks until use. Before experiments, the crayfish was placed in ice for at least 10 mins to facilitate quick and accurate removal of the distal part of the abdomen. Unnecessary exoskeleton and muscle fibers were removed to clearly expose the extensor muscle on the ventral side of the crayfish tail. Then, the preparation was pinned in a Sylgard®-coated dish containing fresh crayfish saline solution (5.4 mM KCl, 196 mM NaCl, 13.5 mM $CaCl_2$, 2.6 mM $MgCl_2$, and 10 mM HEPES). The bathing solution was changed every 15 mins to increase the longevity of the muscle. All procedures were in accordance with the guidelines by the Institutional Animal Use and Care Committee at Grinnell College.

Drug application

All pharmacological agents were obtained from Sigma Chemical Co. including L-N^G-Nitroarginine methyl ester (L-NAME) and 2-(N,N-Diethylamino)-diazenolate-2-oxide diethylammonium salt (DEA/NO). All chemicals were stored in a -20℃freezer. Immediately prior to this application, L-NAME and DEA/NO were diluted in crayfish saline to the desired concentration at 5 mM and 100 µM, respectively.

Electrophysiology and data analysis

Excitatory Post-Synaptic Potentials (EPSPs) were evoked by stimulating the motor nerve axon with square pulses at 1-10 V for 0.2 ms at 0.2 Hz. EPSPs were recorded using a glass micropipette filled with 3 M KCl (resistance 10-20 MΩ), and were collected with a Maclab data acquisition system (ADInstruments, Colorado Springs, CO, USA). Mean amplitude of EPSPs were recorded from four different randomly chosen muscle cells after averaging 16 individual EPSPs in each cell. n represents the number of experiments. We used one way ANOVA to analyze the data, taking $p<0.05$ as significant.

RESULTS

No endogenous production of nitric oxide

To determine whether there is endogenous production of Nitric Oxide (NO) and whether the endogenous NO alters synaptic transmission, L-NAME, a Nitric Oxide Synthase (NOS) inhibitor, was applied at 5 mM and Excitatory Post-Synaptic Potentials (EPSPs) were recorded at the crayfish Neuromuscular Junction (NMJ). The preparation was pre-incubated in L-NAME-containing saline for 10 mins before the recording of L-NAME group. As shown in Figure 1, L-NAME did not cause significant changes in EPSP amplitude when compared with the control group (144.99% ± 50.35% of control, n=3, p=0.42). We did not obtain enough data for the wash group and thus data from the wash group was excluded from our data analysis.

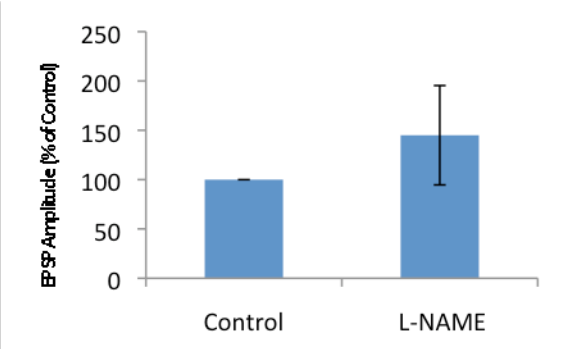

Figure 1. EPSP amplitude in the presence of 5 mM of L-NAME. EPSPs were recorded at the crayfish NMJ. The preparation was pre-incubated in L-NAME-containing saline for 10 mins before the recording of L-NAME group. L-NAME did not cause significant change in EPSP amplitude(144.99% ± 50.35% of control, n=3, p=0.42). Error bars represent S.E.M.

Exogenous NO enhances synaptic transmission

To determine the effect of exogenous NO on synaptic transmission, DEA/NO, a NO donor, was applied at 100 µM and EPSPs were recorded at the crayfish NMJ. As shown in Figure 2, DEA/NO enhanced EPSP amplitude when compared with the control group (137.63% ± 5.10% of control, n=1, p=0.01) and washout of DEA/NO reversed the enhancement (83.70% ± 3.30% of control, n=1, p=0.14).

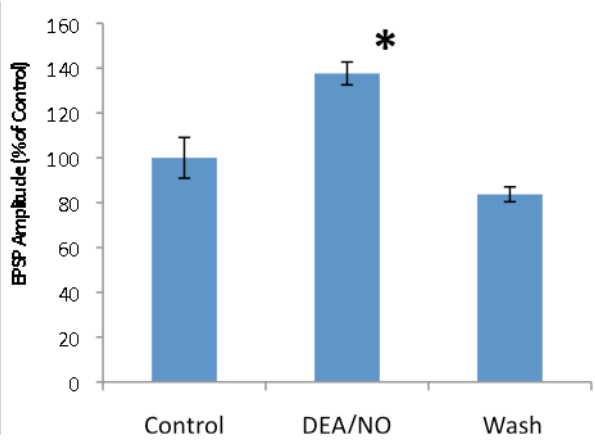

Figure 2. EPSP amplitude in the presence of 100 µM of DEA/NO. EPSPs were recorded at the crayfish NMJ. DEA/NO enhanced EPSP amplitude significantly (137.63% ± 5.10% of control, n=1, p=0.01). Washout of DEA/NO reversed the enhancement (83.70% ± 3.30% of control, n=1, p=0.14). Error bars represent S.E.M.

DISCUSSION

In this experiment, we attempted to determine the role of both endogenous and exogenous Nitric Oxide (NO) on synaptic transmission. We recorded excitatory post-synaptic potentials (EPSPs) at the crayfish neuromuscular junction (NMJ) with the addition of L-NAME and DEA-NO. With the application of L-NAME, a decrease in EPSP amplitude compared to control levels was expected while an increase in EPSP amplitude compared to control levels was expected with the application of DEA-NO.

In comparison to control levels, there was no significant change in EPSP amplitude upon application of the Nitric Oxide Synthase (NOS) inhibitor L-NAME. This was inconsistent with previous studies that showed that bath application of 5 mM L-NAME significantly decreased the spike frequency of the evoked motor neuron response from 230 $\pm$ 46 to 125 $\pm$ 33 Hz (Araki et al., 2003) at tailfan of the crayfish. Moreover, we did not have enough data to determine if a further 15 min wash with normal saline would return the response to control levels, as it did in previous studies (Araki et al., 2003). More data is needed to adequately assess our hypothesis that L-NAME serves as an inhibitor of NO synthesis at the crayfish NMJ.

In contrast, application of DEA-NO enhanced EPSP amplitude compared to control levels. Washout reversed EPSP amplitude to near control levels. These results were consistent with our hypothesis that DEA-NO would increase EPSP amplitude and suggests that the addition of exogenous NO may enhance synaptic transmission at the crayfish NMJ. Similarly, previous studies have indicated that application of DEA-NO in the abdominal ganglion of the *aplysia* increases the amplitude of the EPSP by 155% ($p<0.01$) compared to control levels (Antonov *et al*, 2007). In addition, application of DEA-NO rescued EPSP amplitude in the presence of L-NAME at the lizard NMJ (Graves *el al*, 2004). Although these studies indicate that exogenous NO enhances synaptic transmission, further experiments are required to confirm whether NO enhancement on EPSP occurred via pre or post-synaptic mechanisms. Previous studies have suggested that NO can act directly on neuronal and muscle targets by S-nitrosylation of proteins and/or indirectly by means of activation of a soluble guanylate cyclase (sGC) (Ahern *et al.*, 2002). For future studies, we can inhibit or activate endogenously both upstream and downstream components of the NO synthesis pathway with L-arginine, NMDA, and ODQ. L-arginine is an NOS agonist and has been indicated to increase spike frequency of evoked response in motor neurons by 106 to 172 Hz at the tailfan of the crayfish (Araki *et al*, 2003). NMDA is specific agonist of the NMDA receptor upstream of NO synthesis. ODQ is an inhibitor of sGC, and thus inhibits the downstream effects of NO.

NO has been indicated to modulate long-term potentiation (LTP) as well other forms of synaptic plasticity in the hippocampus (Hopper and Garthwaite, 2006). Additionally, previous studies have found evidence of LTP with tetanic stimulation at the crayfish NMJ (Dixon and Atwood, 1989). Since presynaptic restructuring has been indicated during LTP (Wojtowitz *et al, 1989)*, continued studies utilizing the crayfish NMJ may contribute valuable information to our understanding of the role of NO modulation on learning and memory.

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