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The nitric oxide inhibitor L-NAME prolongs synaptic facilitation

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ABSTRACT

The retrograde messenger, nitric oxide, has been shown to modulate the release of acetylcholine in the presence of Ca at the frog neuromuscular junction (nmj). NO and Ca are also thought to regulate synaptic plasticity. The nitric oxide synthase inhibitor, L-NAME, has been shown to downregulate the production of endogenous NO. In this study we tested for facilitation at the frog nmj in the presence and absence of L-NAME. We found that L-NAME prolonged higher levels of synaptic facilitation in paired pulse stimulations at low frequency (0.2 Hz).

INTRODUCTION

In recent years nitric oxide (NO) has emerged as a novel signaling molecule in many tissues (Schuman and Madison, 1994). Particularly, NO has been implicated as a modulator of neurotransmitter release in both the central (CNS) and peripheral (PNS) nervous systems (Schuman and Madison, 1994, Boehning and Snyder, 2003). NO is synthesized primarily at the motor end plate by the enzyme, nitric oxide synthase (nNOS) (EC 1.14.23), upon conversion of L-arginine to L-citrulline (Kusner and Kaminski, 1996). Increases in free cyotsolic Ca^{2+} has been shown to be the most influential regulator of nNOS activity (Zefirov et al., 2002; Esplugues, 2002). Action potentials in the presynaptic terminal stimulate the fusion of acetylcholine (ACh) containing vesicles to the presynaptic membrane (Esplugues, 2002). ACh activates nicotinic acetylcholine receptors (AChR) in the muscle, leading to the influx of small, positively charged ions and the depolarization of the muscle cell (2002). These effects, in turn, induce the influx of Ca^{2+} through voltage-dependent calcium channels (VDCC) (2002). Ca^{2+} is also thought to be released endogenously from the sarcoplasmic reticulum (SR). Intracellular Ca^{2+} then binds to calmodulin tetrahydrobiopterin, which subsequently interacts with and activates nNOS (Ko and Kelly, 1999).

NO has also been identified as a retrograde messenger that modulates synaptic plasticity (Bredt and Snyder, 1989). Etherington and Everett (2004) found that postsynaptic production of nitric oxide induces long-term depression of neurotransmitter release at the mature amphibian neuromuscular junction. NO produced in the muscle diffuses to the presynaptic terminal and binds to the heme active site of soluble guanylyl cyclase (sGC) (Stamler and Meissner, 2001; Bredt et al., 1992). It is believed that sGC converts GTP to cyclic GMP (cGMP), which, by an unknown mechanism, modulates synaptic depression (Aonuma et al., 2000, Ahern et al., 2002). NO has also been implicated in long-term potentiation in hippocampus cells (Ko and Kelly, 1999).

The effects of NO are antagonized by the exogenous administration of the nNOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) (*NOS inhibitors*). L-NAME is chaperoned across the plasma membrane via the L-leucine transport system, where it is hydrolyzed by esterases to form N^G-nitro-L-arginine (L-NA), a structural analog of L-arginine. L-NA inhibits nNOS through competition with L-arginine (Frandsen et al., 2001).

Although there is compelling evidence supporting the possibility that NO is an important modulator of long-term synaptic depression and potentiation, there is little known about the role of NO and Ca^{2+} in synaptic facilitation. Thus, in this experiment we aimed to determine if synaptic facilitation is regulated by NO and/or Ca^{2+} at the frog neuromuscular junction.

We tested for the involvement of NO signaling in a form of synaptic facilitation induced at the frog neuromuscular junction by paired stimulation pulses at low frequency (0.2 Hz) in the presence and absence of L-NAME. We assessed the role of Ca^{2+} by utilizing buffer solutions, which contained high and low concentrations of Ca^{2+}. We hypothesized that introducing L-NAME would increase facilitation, because neurotransmitter release would not be hampered by NO mediated inhibition. We also hypothesized that muscle cells in a low Ca^{2+} environment would also increase facilitation, since Ca^{2+} is required for nNOS activation.

MATERIALS AND METHODS

Northern Leopard Frogs (*Rana pipiens*) were killed by double pithing according to procedures approved by the

Institutional Review Board of Grinnell College. The sartorius muscle was dissected with nerves attached. Muscle preparations were pinned down to a gel-coated recording chamber where they were maintained in amphibian Ringer solution (NaCl,112mM; KCl, 3.2mM; $CaCl_{2,}$ 2.7mM; $Na_2HPO_{4,}$ 0.5mM; Tris Buffer, 2.0mM; glucose, 2.0grams; adjusted to pH of 7.2 with either NaOH or HCl). Experiments were performed at room temperature.

Electrophysiology

The sartorius muscles were pinned down onto the recording chamber in a bath of Ringer solution. Muscles were stimulated via the nerve using a suction electrode controlled by a Grass SD9 stimulator. Electrophysiological recordings were made using an intracellular borosilicate microelectrode filled with 3M KCl. All recording were made from muscle fibers with membrane potentials ranging from -40mV to -100mV. The threshold for the muscle cells was about (0.1-0.8mV) and muscle contractions were removed by incubating the muscles cells with 7μM curare for 20 minutes for EPP recordings. EPPs were evoked at frequencies ranging from 0.2Hz to 0.6Hz by delivering stimuli to the nerve about four times the threshold of the cell via the suction electrode, to ensure activation of proper ion channels. EPP amplitude values were recorded as 8 sweep averages to remove ambient electromagnetic noise from our results. Synaptic potentials were recorded via Mac Lab recording hardware and Scope 3.6.3 recording software. Analysis of synaptic potentials was performed using peak parameters in the scope software. Control end plate potentials (EPP) were made in either high or low calcium ringer's solutions depending on the condition.

Drugs

After several sets of control EPP amplitude values were recorded, 100mL of L-NAME was added to 30 ml of curarized Ringer's solution to make a final concentration on 0.3mM L-NAME. EPPs were recorded at 5 and 10 minutes post introduction. EPP was also recorded in low calcium Ringer solution (NaCl,112mM; KCl, 3.2mM; $CaCl_{2,}$ .3mM; 4.8mM $MgCl_2$; Na_2HPO_4, 0.5mM; Tris Buffer, 2.0mM; glucose, 2.0grams; adjusted to pH of 7.2 with either NaoH or HCl) with and without the same concentration of L-NAME in regular Ringer's solution.

Induction of Synaptic Facilitation

After recording single pulse control EPPs, we introduced paired pulse stimuli at the same voltage and modulated the delays (60, 80, and 120ms) to test for facilitation. Facilitation was recorded in all experiments. The presence or absence of facilitation was determined by percent change from control to normalize EPP amplitudes between experiments.

RESULTS

Following the addition of L-NAME, the EPP amplitudes did not change significantly from the control (p-value = 0.144). However, the facilitated EPP amplitudes were maintained at a higher level for a longer period of time. Figure 1 shows that the reduction in facilitation over time was more gradual in L-NAME than in the control.

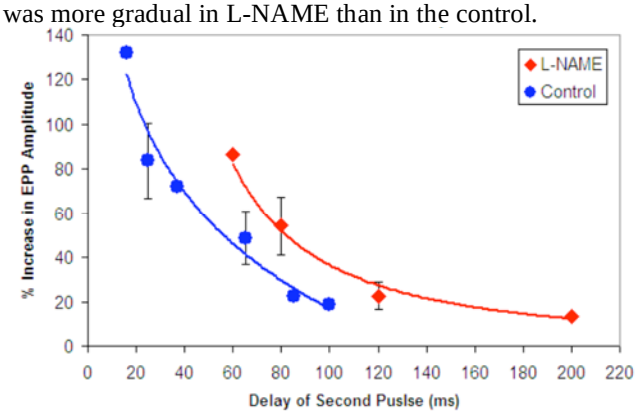

Figure 1. Twin pulse facilitation of frog neuromuscular junctions in the presence and absence of L-NAME. The facilitated increase in EPP amplitude drops as a function of time. L-NAME prolongs this drop in facilitation. We report the EPP amplitudes as percent increases from the initial stimulus pulse so that all the data could be normalized and directly compared. Error bars represent +/-1 standard deviation from the mean. All other data points represent single values and, therefore, do not have error bars.

DISCUSSION

We report that the relative increase in EPP amplitude that regularly occurs during synaptic facilitation is amplified by the addition of L-NAME. These results support our hypothesis that facilitation increases in the presence of L-NAME, due to its inhibitory effects on nNOS. nNOS synthesizes the retrograde messenger, NO, which diffuses from the postsynaptic terminal to the presynaptic terminal (Bredt and Snyder, 1989). NO then depresses neurotransmitter release, resulting in postsynaptic depression (Etherington and Everett, 2004; Thomas and Tobitaille, 2001). Therefore, following treatment with L-NAME, we expected to see facilitation instead of depression.

Ca^{2+} enters the nerve terminal when it is depolarized (Nicholls et al., 2001). Synaptic facilitation occurs when residual Ca^{2+} in the presynaptic terminal leads to an increase in the efficacy of quantal

neurotransmitter release upon the repetitive invasion of action potentials (Thomas and Robitaille, 2001). It has been shown that the effects of NO are Ca^{2+} dependent (Zefirov et al., 2002). Ca^{2+} is, thus, a necessary component and regulator of facilitation at the adult frog neuromuscular junction.

We investigated the role that Ca^{2+} plays in facilitation by bathing the muscle in a buffer solution with either high or low concentrations of Ca^{2+}. Unfortunately, we were unable to obtain data for our low calcium study. We are unsure as to why this happened; however, we suspect that our 0.3mM Ca^{2+} concentration was too low for neurotransmitter to be released at a noticeable level. This suspicion seems consistent with previous studies. Zefirov et al. (2002) demonstrated that externally derived Ca^{2+} at low concentrations (0.2 – 0.4mM) elicits a greater depression of neurotransmitter release than Ca^{2+} at high concentrations. Another problem with our low Ca^{2+} experiment was that we attempted to measure EPPs more than 50min after the muscle was excised. In low Ca^{2+} preparations the muscle deteriorates substantially after 30min.

In the future we would like to attempt this experiment again, varying Ca^{2+} concentration by degrees. The data obtained from this study would further elucidate the complex mechanism of NO mediated synaptic plasticity.

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