The Effects of 3,4-Diaminopyridine on Neurotransmitter Release in a Frog Model of
Lambert-Eaton Myasthenic Syndrome.

Honors Project

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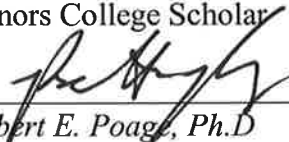

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May 7, 2005

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ACKNOWLEDGEMENTS

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ABSTRACT

THE EFFECTS OF 3,4-DIAMINOPYRIDINE ON NEUROTRANSMITTER RELEASE
IN A FROG MODEL OF LAMBERT-EATON MYASTHENIC SYNDROME.

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Lambert-Eaton Myasthenic Syndrome (LEMS) is a neuromuscular disorder characterized by muscle weakness in which the motor nerve has difficulty releasing enough neurotransmitter to cause muscle contraction. Through clinical trials, 3,4-Diaminopyridine (DAP) has been shown to successfully treat LEMS by increasing muscle contraction and strength. The precise effect of diaminopyridine on synaptic facilitation (an increase in transmitter release seen during high frequency stimulation) is not well understood. In order to study LEMS conditions, a well described neuromuscular synaptic preparation and a selective neurotoxin is used to model LEMS in an animal. Using the cutaneous pectoris muscle of the *Rana pipiens* (leopard frog), the electrical response of muscle cells to various frequencies of stimuli was measured using intracellular recording methods. The amplitude of the muscle cell's response to the nerve stimuli was used as an assay of neurotransmitter release in both LEMS-like and control (low calcium) conditions. The data indicate that diaminopyridine increases the amount of

neurotransmitter released in both control and LEMS conditions, but decreases the degree

of facilitation in both conditions. The preliminary results are not sufficient to determine

whether diaminopyridine causes a higher level of facilitation in LEMS conditions than in

the control (low calcium) conditions. The collected data are a foundation for the

continued studies on the mechanism of action of DAP, and the functional arrangement of

synaptic proteins in a model synapse. Additionally, the establishment of a research

laboratory in an undergraduate biology department where none had previously existed

was an important accomplishment of this project.

INTRODUCTION

The Neuromuscular Junction:

A review of the molecular events that occur at the neuromuscular junction is very important for understanding how it is studied. The neuromuscular junction (NMJ) refers to the site where a motor neuron innervates a muscle cell; it is also referred to as the endplate. There, the nerve terminus is enlarged and contains many synaptic vesicles filled with acetylcholine (ACh), a neurotransmitter. A synapse separates the presynaptic nerve membrane from the postsynaptic muscle membrane. When a nerve impulse, or action potential, reaches the nerve terminus, voltage-gated calcium channels are opened, and the influx of calcium triggers the exocytosis of ACh from the vesicles (Katz, 1967; Katz, 1969; Augustine et al., 1987). The ACh diffuses across the synapse to the muscle cell's membrane, the sarcolemma, which contains a high concentration of ACh receptors. These ACh receptors act as ligand-gated ion channels, so that when ACh binds, the receptors open to allow an influx of sodium across the local muscle membrane. This sodium influx causes a local depolarization that, if strong enough, will be propagated along the length of the sarcolemma, leading to muscle contraction (Purves et al., 2001).

In preparation for synaptic transmission, ACh is stored in many synaptic vesicles that "dock" to the inner layer of the nerve's presynaptic membrane (Neher, 1998). This docking involves the tight association of docking proteins, the vesicle, and nearby calcium channels (Simon and Llinas, 1985; Robitaille et al., 1990; Harlow et al., 2001). The physical connection of these elements primes the whole assembly for rapid

neurotransmitter release. When calcium enters the cell through calcium channels, it is thought to bind to the docking protein syntaxin, and somehow cause the membrane of the vesicle to fuse to the cell's and release the vesicle's contents into the synapse (Purves et al., 2001).

Importantly, neurotransmitter release at the neuromuscular junction exhibits the phenomenon of facilitation, as do all other synapses within the nervous system (Fisher et al., 1997). Facilitation is a mechanism of increased neurotransmitter release during repetitive nerve stimulation. As the nerve continues to be stimulated, more neurotransmitter is released per individual stimulus (Mallart and Martin, 1967). Higher frequency stimuli usually exhibit the highest levels of facilitation. There are 4 components to facilitation, identified through exponential stripping techniques and other mathematical evaluation of the decay rates of facilitation in nerve cells. The two components of facilitation relevant to this project includes F1, which rises and decays within tens of milliseconds after the first nerve impulse in a sequence, and F2, which takes longer, within hundreds of milliseconds, to rise and decay (Zengel et al., 1994; Fisher et al., 1997; Kuffler and Nicholls, 1997).

Lambert-Eaton Myasthenic Syndrome:

Lambert-Eaton Myasthenic Syndrome (LEMS) is a condition whose etiology is usually autoimmune or paraneoplastic. Cancer is often detected within a year of LEMS diagnosis in LEMS patients. The most common cancer found in these diagnoses is small cell lung carcinoma (Tim et al., 2000).

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LEMS results in decreased neurotransmitter release at the neuromuscular junction due to antibody-mediated destruction of the presynaptic calcium channels (Fukunaga et al., 1983; Kim et al., 1988). Patients with LEMS exhibit significant muscle weakness and have problems producing functional muscle contractions. Some patients have as much as a 25 fold reduction in normal levels of neurotransmitter release.

Clinical trials of 3,4-Diaminopyridine (DAP) show a significant increase in muscle strength and contraction in approximately 80% of LEMS patients (Sanders et al., 2000; Tim et al., 2000). DAP is the treatment of choice because patients usually exhibit only relatively minor side effects, perhaps due to its relative difficulty crossing the blood-brain barrier. It is also considered an "orphan drug", because LEMS is not considered to be common enough for the commercial development of its treatment to be a lucrative endeavor (Molgo and Guglielmi, 1996).

DAP acts as a blocker of voltage-gated potassium channels. By blocking potassium channels, the nerve cell isn't able to repolarize as quickly following a nerve impulse. This increases the duration of the action potential and indirectly, the amount of time the voltage-gated calcium channels are open (Molgo and Guglielmi, 1996; Sanders et al., 2000). More calcium flowing into the nerve terminus increases the amount of ACh release, so that the localized depolarization at the endplate is more likely to reach the threshold required to cause muscle contraction.

Rationale

It is important for the medical community to know how a treatment of DAP effects facilitation in LEMS patients, not only because facilitation is an important

physiological event of neurotransmission, but also because the effect of DAP on facilitation may shed light on the mechanisms of facilitation, an area of ongoing debate in the basic science of presynaptic neurophysiology. It is expected that DAP will increase neurotransmitter release in both control (low calcium) and LEMS-like (ω-Cgtx pretreated) conditions, while decreasing the relative levels of facilitation. Because LEMS patients have fewer functional calcium channels, and DAP keeps those channels open longer than normal, it is hypothesized that DAP will increase the level of facilitation in LEMS-like conditions when compared to control conditions.

A Frog Model of LEMS

In order to study the effects of DAP, a model for LEMS had to be developed. Previous studies have determined that the human NMJ is well modeled by the Leopard frog *Rana pipiens* (Kuffler, 1997; Purves, 2001). For this project, the thinness of the cutaneous pectoris muscle makes it ideal for the uniform treatment of the drugs necessary for the study. This model for LEMS was established by treating the *Rana pipiens* cutaneous pectoris with ω-conotoxin (ω-Cgtx), which mimics the effects of LEMS by irreversibly blocking the presynaptic calcium channels (Poage and Meriney, 2002; Wachman et al., 2004)

EXPERIMENTAL MATERIALS AND METHODS

The dissected muscle was pinned in a Sylgard recording chamber, either in low calcium normal frog Ringers (NFR) (0.6 or 0.8mM $CaCl_2$, 5mM $MgCl_2$, 2mM HEPES, 116mM NaCl, 2mM KCl, 5mM glucose, pH 7.3), or in normal calcium NFR (1.8mM $CaCl_2$, 5mM $MgCl_2$, 2mM HEPES, 116mM NaCl, 2mM KCl, 5mM glucose, pH 7.3) after ω-Cgtx treatment. Data was collected in these bathing solutions: control data was collected in low calcium NFR, before and after DAP treatment, and the LEMS-like conditions data was collected in normal calcium NFR after ω-Cgtx treatment, also before and after DAP treatment. DAP treatments varied from 0.5μM-2 μM, but it was determined that a 1μM treatment was optimal during this study. The concentrations of ω-Cgtx varied from 50nM to 500nM, however the best treatment for this study was revealed to be 400nM for 60-100 minutes.

A bipolar suction electrode was used to stimulate the motor nerve innervating the cutaneous pectoris. The stimulus rate and timing were controlled by a Pclamp6 Clampex software program (Axon Instruments). Signals from the Pclamp6 interface were used to trigger a stimulator (Harvard Instruments), which sent a super-threshold voltage pulse (2-5 V, 0.1 msec) through a signal isolation unit (SIU) (Grass Instruments) to the stimulating electrode.

The muscle's response to two-pulse stimuli trains at various frequencies was recorded as the change in voltage across the sarcolemma. The frequency of stimulation is

expressed as the interstimulus interval (ISI). The trains used in this project had ISI of 10, 20, 50, 100, 120, 150, 250, 500, and 1000ms.

Intracellular recording was used to observe the muscle's response to nerve stimuli. This method involves pulling a hollow glass capillary tube (World Precision Instruments, Inc., item TW120F-3) to a very fine point with a pipette puller (Narishige, model PP-830). The micropipette was then filled with 3M KCl. The microelectrode was manipulated to impale a single muscle cell near an endplate. The reference ground electrode was placed in the NFR bath that contained the prep.

The voltage change detected by the recording electrode was sent through a "high-impedence" amplifier (Dagan, model BBC 700), and to an oscilloscope (Kikusui, model 5020), and through an analog/digital conversion system (TL-1 interface, Axon Instruments). The digital signal was collected and analyzed using the Pclamp6 software suite (Axon Instruments) installed on a desktop computer (Gateway).

RESULTS

Figure 1 shows a typical recording of a muscle endplate potential (EPP). This example shows the activity across the muscle membrane in response to a two-pulse train with an ISI of 10ms recorded under various conditions. The sharp vertical spikes directly before the slower up-slope of the depolarizing response of the muscle is an artifact produced by the stimulus pulse. The response depolarization is observed in the peak that comes after the stimuli artifact, and measures the voltage change distributed across the muscle cell's membrane as a function of time. This figure shows that DAP treatment leads to an increase in muscle cell response to nerve stimuli by comparing the responses of the low calcium (control) pre-treatment trace (black) to the 3,4 DAP post-treatment trace (red) in Figure 1A and B. Keep in mind that the responses recorded after adding DAP were collected least 60 minutes after the treatment. Furthermore, in each of these recording conditions, facilitation can be observed, as indicated by the increase in the amplitude of the second response peak relative to the first response of the stimuli pair. This occurs even though the stimulus that produced each event is identical. However, the facilitation in the low calcium conditions appears to be greater than in the LEMS-like conditions, because of the relative differences in the peak amplitudes.
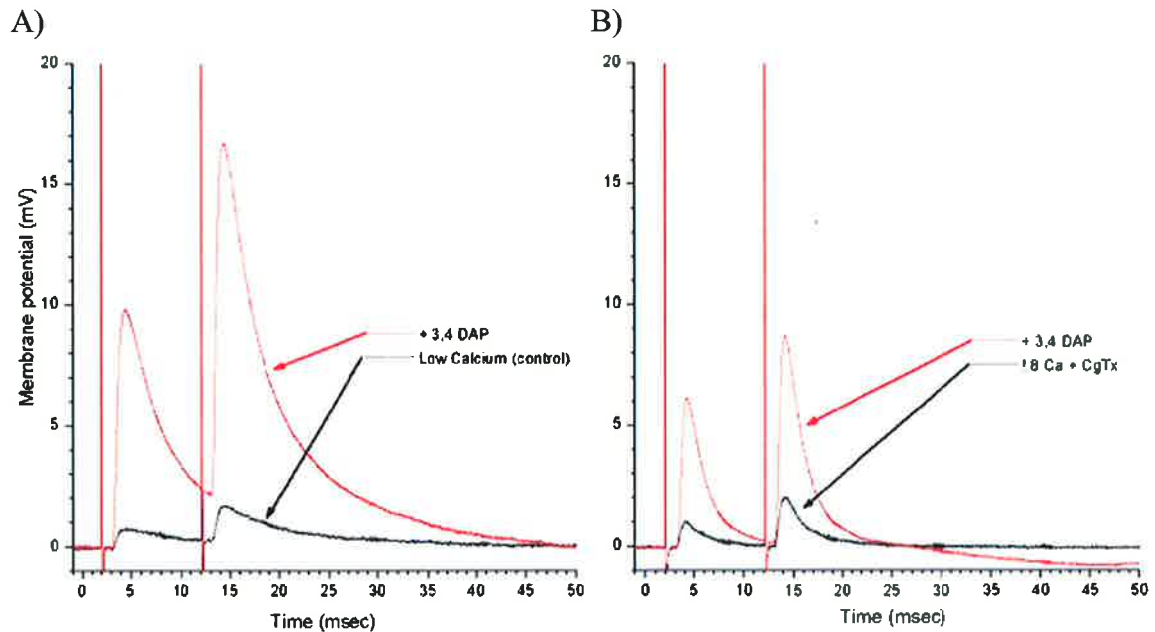
Figure 1: Intracellular recording of endplate potentials. A) Shows a recording obtained in low calcium (control) conditions before and after DAP treatment. B) Shows similar data obtained in the LEMS-like (ω-Cgtx) conditions before and after DAP treatment. Even though the stimulus that produced each event is identical, facilitation can be observed as the increase in the amplitude of the second response peak relative to the first response of the stimuli pair. The after-DAP responses were recorded at least 60 minutes after the treatment. DAP treatment appears to cause an increase in muscle cell response to nerve stimuli. Sometimes adding DAP in the LEMS-like conditions increased muscle response enough to produce muscle movement; in B) a movement artifact can be seen as a decrease in membrane potential after the second stimulus.

By graphing the magnitude of the control response (the first stimulus response) over time, it was established that DAP treatment causes a steady increase in EPP amplitude for approximately 60 minutes, at which time the effect appears to plateau (Figure 2). This is consistent with results from clinical trials of DAP, in which at least 1-3 hours were needed for it to be in full effect (Molgo and Guglielmi, 1996). Therefore, the muscle responses recorded at least 60 minutes after DAP treatment are best suited for analyzing DAP effects on neurotransmitter release.

In order to study the effect of DAP on facilitation of transmitter release, a measure of the percent increase in EPP amplitude is most often used (Mallart and Martin, 1967). The relative heights of the response peaks were expressed as a ratio, $V(t)$, where $V(t) = (V_{test} / V_{control})-1$. This ratio represents the percent change in voltage between the first (control) and second (test) responses, indicating the relative change in neurotransmitter release. A large body of work on facilitation has described the relationship between $V(t)$ and the interstimulus interval (ISI) (Fisher et al., 1997).

Figure 3 presents data collected from a single recording session in one cell, and shows the time course of the effect of DAP in decreasing facilitation, which is most notable during the highest frequency stimuli (shorter ISI). This can be seen as the plots of data from later times show decreased $V(t)$. This figure can only be studied for general trends because the sample size is only one cell, and the data show greater variability than averaged data should.
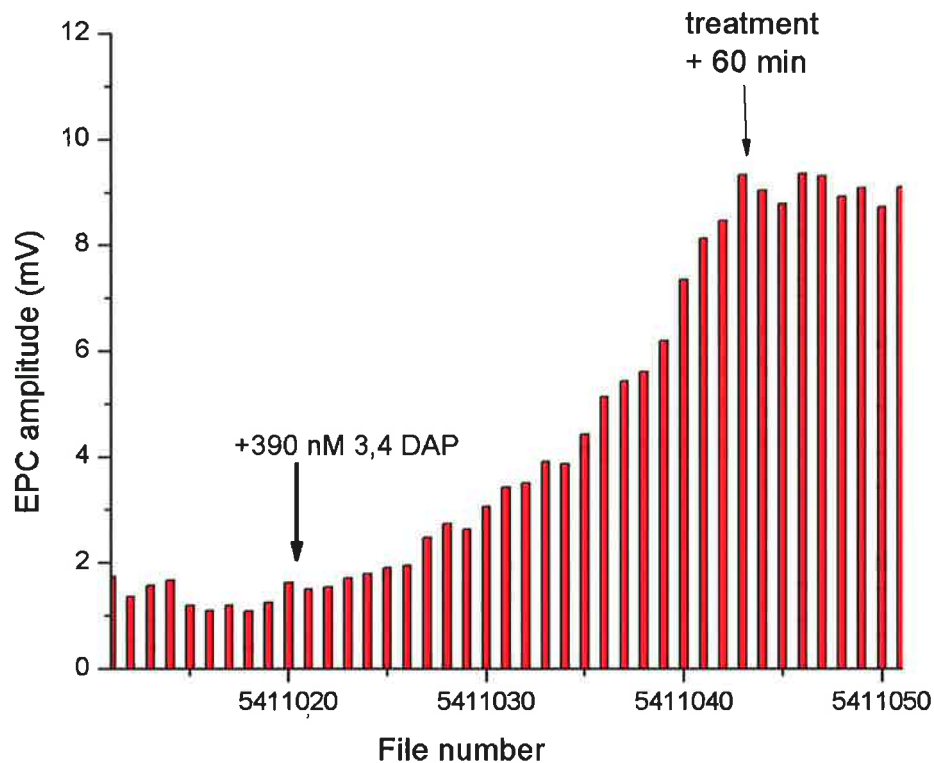
Figure 2: Increase in EPP amplitude after DAP treatment. The bars represent the magnitude of the first EPP of the two-pulse train responses. The first arrow marks the time of DAP treatment, and the second arrow marks 60 minutes afterward. DAP treatment causes a steady increase in EPP amplitude for 60 minutes, at which time the response appears to reach a plateau.
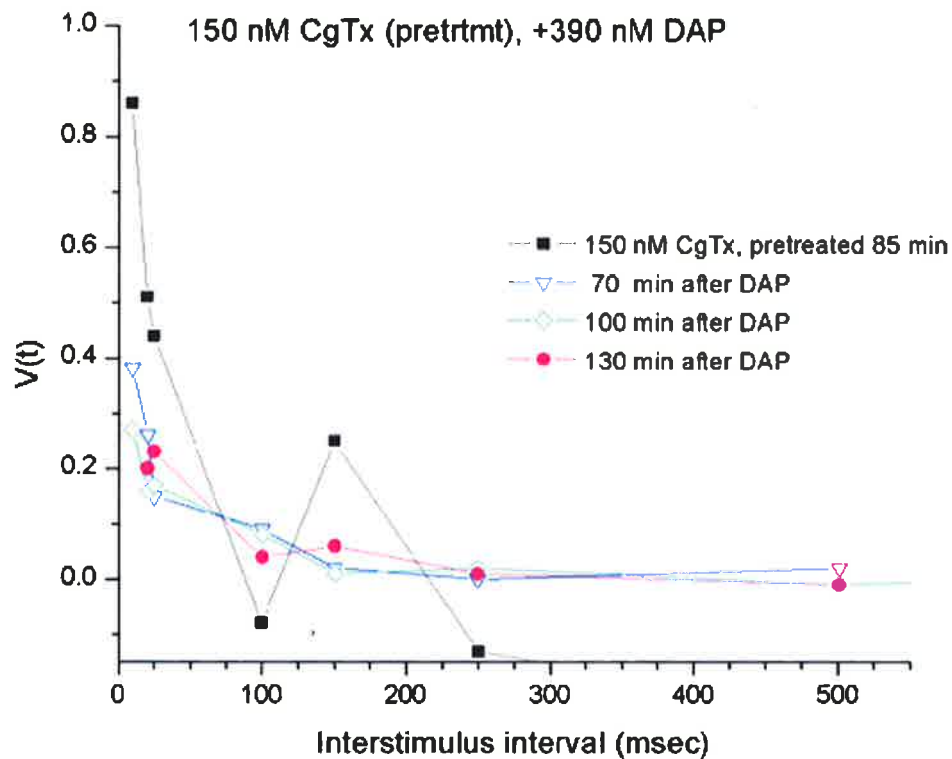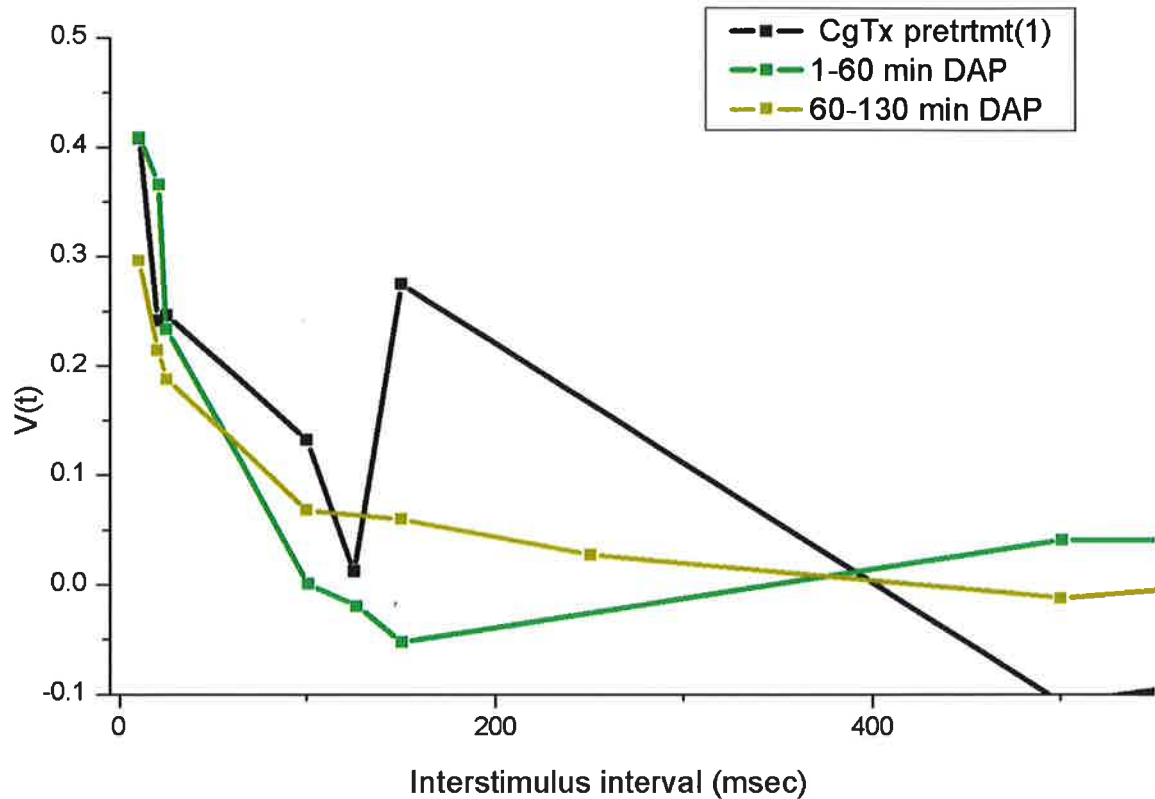
Figure 3: Effect of DAP treatment in LEMS-like conditions on facilitation in a single cell. Facilitation generally decreases as the interstimulus interval increases. DAP treatment generally decreases facilitation as well, especially in the shorter ISI time points. Note that the data is only from one sample, and exhibits larger variation than a group of averaged data.

Figure 4 shows averaged recordings from multiple cells under low calcium conditions. The data indicate trends of decreased facilitation after DAP treatment, including less facilitation at longer ISI. Figure 5 shows similar trends in the averaged data collected from cellular recordings in the LEMS-like conditions (low dose ω-Cgtx treatment) before and after addition of DAP.

Facilitation is known to be greater at higher frequency stimuli; therefore, as the time interval between the two-pulse stimuli train increases, facilitation should decrease. Figures 3, 4, and 5 exhibit this relative trend, which can be seen as decreasing facilitation at the longer ISI time points for all experimental conditions.
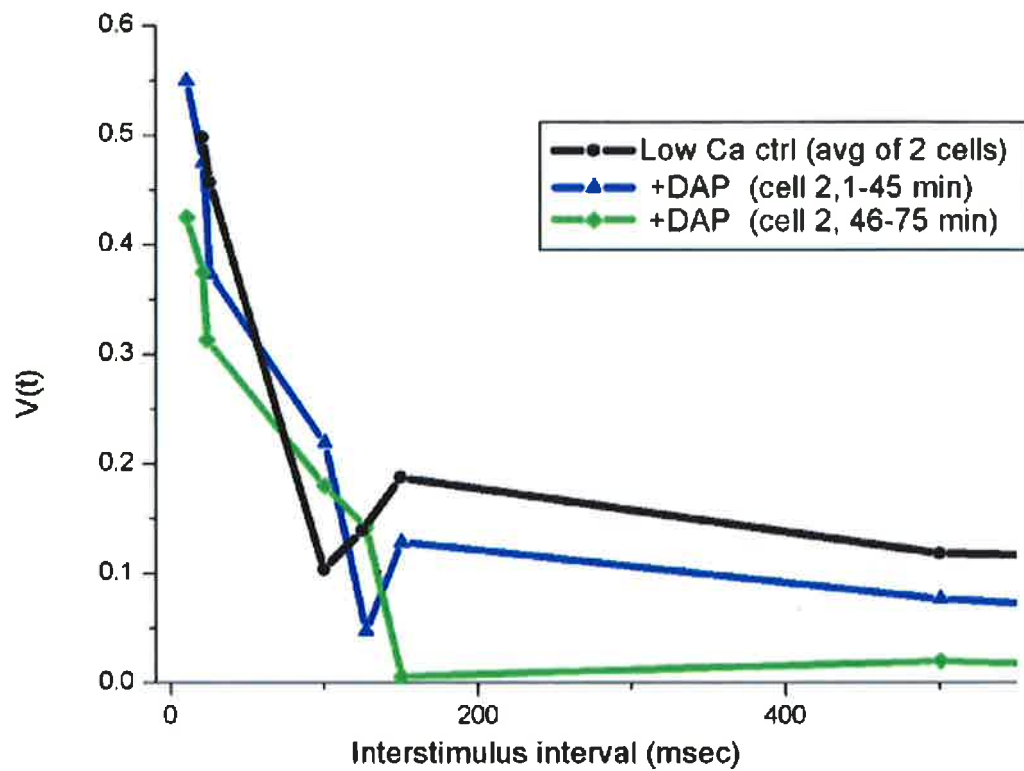
Figure 4: The effect of DAP treatment on facilitation in control (low calcium)

conditions. Facilitation is most pronounced at shorter interstimulus intervals. After DAP

treatment, facilitation generally decreases.

Figure 5: The effect of DAP treatment on facilitation in LEMS (ω-Cgtx pretreated) conditions. Facilitation is most pronounced at shorter interstimulus intervals. After DAP treatment, facilitation generally decreases. (An anomalous point at 150ms ISI of the pre-treatment data [black] is believed to be due to the low sample size in these experiments.)

DISCUSSION

These data are consistent with clinical findings that 3,4-DAP ameliorates muscle weakness by increasing the release of ACh. Here, it is verified that similar effects occur at the neuromuscular junction of the frog in both control and LEMS-like conditions. Furthermore, the data trends indicate that DAP reduces facilitation in both control and LEMS-like conditions, especially at higher frequency stimuli. However, it has yet to be determined whether DAP causes a difference in facilitation in the two conditions studied. Finally, this project establishes the feasibility of a *Rana pipiens* model for the study of human neuromuscular disease.

In order to quantify the effects of DAP on facilitation in the low calcium control and LEMS conditions, the quantal content of the collected recordings will need to be determined (Duffer and Nichols, 1977). Quantal content is related to the precise number of synaptic vesicles thought to have fused to the presynaptic nerve membrane and released acetylcholine (Del Castillo and Katz, 1954; Heuser et al., 1979). This is measured by collecting the change in voltage that occurs when a single vesicle randomly fuses with the presynaptic membrane. Because these events are in the millivolt range and the recording setup was not optimized for recording small amplitude events, these small voltage changes were not collected with sufficient rigor during the course of this project. Therefore, in order to test the second part of the hypotheses, additional experiments must be performed under conditions, and with equipment, that will produce recordings with better signal-to-noise ratio.

The data that went into the construction of the averaged figures was collected from a relatively low sample size (<10 replications in all conditions). While these graphs can be used to generalize on trends, they are far from concrete evidence. The low sample size results from the unforeseeable problem of equipment compatibility that resulted in a relatively short amount of time to collect raw data, while fortuitously providing an excellent opportunity to troubleshoot the equipment and produce results under a deadline.

Adding to the mix, the control data was more erratic than expected, perhaps because the data collected prior to DAP treatment was recorded immediately after successful cell impalement. Many times, piercing a cell with the recording electrode caused a temporary instability in the membrane potential. During brief recordings, that could have added an unexpected source of variability to the small amount of control data collected.

It is important to note that this project took shape before a research laboratory had been established, and that some components of the recording apparatus are still not functioning properly. This crude beginning was hindered by the lack of reasonable funds to support an electrophysiology research lab, and even by the lack of simple utilities such as electrical wiring and sinks in the lab space. Even though these resource problems were largely overcome with ingenuity and borrowed equipment, the compatibility problems arising from the use of outdated electronic equipment were more difficult to resolve.

The data collected here are the foundation for the continued studies, not only on the mechanism of action of DAP, but also on the functional arrangement of synaptic

proteins in a model synapse. Additionally, the establishment of a functioning research laboratory in an undergraduate biology department where previously there had been none was an important accomplishment of this project.

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