INTERACTION BETWEEN PRESYNAPTIC INHIBITION AND FACILITATION AT THE CRAYFISH NEUROMUSCULAR JUNCTION

by

Colin Don Malcolm DeMill
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APPROVAL

Name: Colin Don Malcolm DeMill

Degree: Master of Science

Title of Thesis:

Interaction between presynaptic inhibition and facilitation at the crayfish neuromuscular junction.

Examining Committee:

Chair: Dr. B.D. Roitberg

Dr. K.R. Delaney, Professor
Department of Biological Sciences, S.F.U.

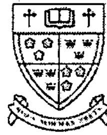
Dr. A.P. Farrell, Professor
Department of Biological Sciences, S.F.U.

Dr. L.M. Quarmby, Associate Professor
Department of Biological Sciences, S.F.U.

Dr. C. Krieger, Professor
School of Kinesiology, S.F.U.
Public Examiner

April 2, 2004
Date Approved

Simon Fraser University



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Abstract

The crayfish claw opener (abductor) muscle was used to investigate the dynamics of calcium entry into excitor nerve terminal boutons during presynaptic inhibition and its effects on the kinetics of muscle contraction. I measured the force of contraction of the whole muscle in conjunction with intracellular recording from individual muscle fibers while stimulating the excitor axon either alone or in conjunction with the inhibitor axon. The amplitudes of postsynaptic excitatory junction potentials were greatly reduced during inhibition to a point below the threshold to initiate contraction. Upon cessation of inhibition their amplitude increased, initiating contraction more quickly. This can be explained by a buildup and maintenance of presynaptic facilitation in excitor motor terminals during inhibition.

Co-activation of the inhibitor axon caused reductions in calcium entry into presynaptic excitor terminal boutons (range 0 to 50 %, mean ± SEM = 20 ± 1 %, n = 122 terminals), and reductions in the amplitude of postsynaptic excitatory junction potentials (range 30 to 70 %, mean ± SEM = 51 ± 2 %, n = 27 cells). Neurotransmitter release was related to calcium entry by the third power. Presynaptic inhibition likely reduces the number of calcium channels that are activated by an action potential rather than the flux of calcium per channel. Thus, my data suggests significant co-operativity between calcium channel micro-domains to initiate vesicle fusion and release.

Analysis of the anatomical relationship between excitor and inhibitor terminal boutons revealed 6 % greater inhibition to excitor boutons on branches off the main varicose structure. Excitor and inhibitor axons travel approximately in parallel with each other. However, the amount of inhibition at a bouton was not correlated with the distance of an excitor bouton from either an inhibitor bouton or the main branch. Bath application of GABA increased the level of inhibition from proximal to distal terminal boutons on a branch.

This was not observed for inhibition delivered with electrical stimulation of the inhibitor axon. I propose a model where presynaptic inhibition causes local shunting of an actively propagated action potential, which then recovers outside the shunted region.

Dedication

To Lee.

Quotation

"Common sense is science exactly in so far as it fulfills the ideal of common sense; that is, sees facts as they are, or, at any rate, without the distortion of prejudice, and reasons from them in accordance with the dictates of sound judgment. And science is simply common sense at its best; that is, rigidly accurate in observations and merciless to fallacy in logic."

T. H. Huxley, 1880.

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I would like to thank Dr. Kerry Delaney for introducing me to the fascinating world of neurobiology. Kerry's endless optimism to try the impossible and constantly improve one's experimental design has been valuable to my scientific growth.

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My parents are responsible for inspiring my scientific curiosity and providing me with the tools to explore it. Finally, I would like to thank Lee for her personal and intellectual support throughout this endeavor.

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Chapter 1: Introduction

The crayfish claw opener (abductor) muscle (Figure 1) is innervated by a glutamate-releasing, excitatory axon and a GABA releasing, inhibitory axon (van Harreveld, 1939). There is also an inhibitory neuron common to all muscles in the limb which innervates the first 3 to 10 proximal fibers of the opener muscle (Wiens, 1985), and was not investigated in this study. The claw opener muscle fibers are non-spiking and the tension that develops in the muscle is proportional to the average depolarization that results from summation of excitatory postsynaptic junction potentials (EJPs) (Orkand, 1962; Bittner, 1968). Unlike vertebrate neuromuscular junctions, individual EJPs produced in response to single presynaptic action potentials are small (50 μV to 2 mV), so temporal summation by itself cannot readily generate sufficient depolarization to cause contraction. Therefore, in addition to temporal summation, repeated stimulation produces a large degree of activity-dependent, short-term facilitation of release of neurotransmitter that is essential for generating muscle contraction (for reference see Figure 14). While the amount of facilitation produced is impressive, up to 10 to 100 fold with repetitive high frequency stimulation, there is an inherent time delay required to deliver enough action potentials required to induce contraction (Atwood, 1976). The inhibitor axon releases GABA onto the muscle and the presynaptic excitor terminals where it activates a shunting chloride conductance through $GABA_A$ (γ-aminobutyric acid) receptors (Dudel and Kuffler, 1961). Most of the reduction of the EJP is mediated through presynaptic inhibition with postsynaptic inhibition estimated to contribute only 5 to 10 % (Fatt and Katz, 1953; Atwood and Bittner, 1971; Baxter and Bittner, 1980).

While the majority of axo-axonal inhibitory synapses are found on terminal varicosities (Atwood et al., 1984), some occur at constricted regions of the excitor axon termed bottlenecks (Atwood and Morin, 1970; Jahromi and

Atwood, 1974). It has been proposed that presynaptic inhibition at these vulnerable regions could effectively block the propagation of an action potential to entire branches of terminal boutons of the excitor axon (Dudel and Kuffler, 1961; Florey and Cahill, 1982; Atwood and Tse, 1988), and this phenomenon has been modeled (Atwood et al., 1984, Segev, 1990). However, in practice this phenomenon is rarely seen physiologically. As most experiments have been conducted at low frequencies, to avoid movement, it has been suggested that branch point failure may occur at high frequencies with prolonged periods of stimulation (Govind et al., 1995). I conducted my experiments with high, physiologically significant frequencies of stimulation and evaluated the extent to which specific geometry of axonal branching influenced the efficacy of presynaptic inhibition at different excitor axon terminal boutons.

Crayfish neuromuscular junction as a model

The crayfish opener neuromuscular junction (NMJ) is an attractive model for physiological research because of its apparent simplicity, ability to produce ample activity dependent synaptic enhancement and similarities to the vertebrate central nervous system (CNS). As the opener muscle is only stimulated by one excitor and one inhibitor axon (Figure 2), the activity of the entire muscle can be controlled by stimulating the axons independently. This muscle is an ideal system for studying short-term synaptic plasticity since it produces synaptic facilitation, augmentation and potentiation without significant depression over short stimulus periods. These processes increase the probability of transmitter release with activity. Fatigue of transmitter release is not evident even over long periods of stimulation at moderate, physiologically relevant frequencies due to efficient recycling of neurotransmitter vesicles (Bittner, 1968; Bittner and Kennedy, 1970).

Crayfish muscle, like other arthropod muscles, is poly-innervated with on the order of 50 synaptic release sites per muscle fiber distributed along their length (Atwood and Bittner, 1971). The crayfish NMJ shows significant regional variation with respect to the amount of synaptic enhancement produced by

different synapses. As many of these synapses run along the surface of the muscle fibers, they are available for study using techniques such as loose (macro) patch recording (Dudel and Kuffler, 1961) and calcium imaging (Figure 2). Anatomical and physiological differences between the synapses have shed light on the fundamental mechanisms for synaptic plasticity.

General properties of muscle contraction

In general, crustacean muscle fibers have diverse structural, electrical and contractile properties. Fast acting fibers such as the lobster antennal remotor muscle are usually characterized by short sarcomeres (2 to 4 μm), a low ratio of thin to thick myofilaments in the contractile apparatus and a well developed T-tubule system. Electrical characteristics of these fibers are typical of a phasic type of activity and include low resistance, high threshold for excitation-contraction coupling (20 to 30 mV) and sometimes generate all-or-nothing spikes. In contrast, slow acting tonic fibers such as crayfish abdominal flexor and extensor muscles, are generally characterized by long sarcomeres (10 to 15 μm), a high ratio of thin to thick myofilaments and a lesser developed T-tubule system. Tonic fibers usually have a low threshold for excitation-contraction coupling of a few millivolts and develop tension slowly, which is ideal for postural and repetitive locomotory activity (reviewed by Atwood, 1976).

The crayfish opener muscle of the first walking leg is characteristic of a tonic crustacean muscle (Figure 1b). An action potential stimulated in the excitor motor axon causes depolarization of the motor terminals leading to activation of voltage gated calcium channels. Calcium entry triggers release of glutamate into the NMJ, which then binds to receptors on the postsynaptic membrane. This triggers a depolarization of the muscle membrane, termed an EJP. This depolarization is the electrical sum of all of the synaptic activity, estimated to result from 40 to 50 release sites, on the muscle fiber (Bittner and Harrison, 1970). Tension develops in a graded fashion with increasing EJP amplitude. Muscle contraction is a monotonic function of membrane

depolarization and is produced by direct or indirect stimulation (Orkand, 1962; Bittner, 1968).

Depolarization of the muscle induces activation of an L-type voltage gated calcium current (Araque et al., 1994). As calcium flows into the muscle it triggers contraction through a calcium induced calcium release mechanism (Gyorke and Palade, 1992) and activates a calcium induced potassium current (Araque et al., 1998). The rate of membrane depolarization, due to activation of calcium current, is regulated by a negative feedback mechanism controlled by the potassium channel. Therefore, the potassium current prevents calcium spiking and controls the graded depolarization, thus regulating the force of contraction of the muscle fiber (Araque et al., 1998).

Regional variation in the tension produced by muscle fibers to a given stimulus was shown to be a property of the presynaptic motor synapses and not a property of the muscle fibers themselves (Bittner, 1968). It was found that muscle fibers from different regions had similar electrical characteristics and when stimulated independently and equal amounts of depolarization produced equal amounts of tension. Thus, presynaptic heterogeneity is functionally significant for the crayfish, because different regions of the muscle are activated at different frequencies allowing tension to be produced in a graded fashion over a wide range of stimulus frequencies. As crustaceans have relatively few neurons, nerve terminal differentiation provides peripheral control for whole muscle tension (Atwood et al., 1965).

The range over which this NMJ can operate was shown by Atwood (1962) who measured contraction of the opener muscle via a force transducer attached to the dactyl tip of the propodite (Figure 3) with stimulation ranging from 5 to 100 Hz. During prolonged stimulation of the excitor at high frequency (100 Hz for 3 s), a muscle fiber was observed to depolarize to a plateau of 15 mV above the resting membrane potential. In contrast, EJPs recorded from single stimuli range from 50 μV to 2 mV. It appears that potentiation of transmitter release with repeated stimulation of the motor axon is essential for operation of the opener muscle.

Muscle contraction in the intact animal occurs with doublets (pairs) of action potentials from the excitor motor axon at frequencies of 10 to 50 Hz (Wilson and Larimer, 1968). These patterns of activation may be functionally important to contraction of the muscle (Wilson and Davis, 1965; Smith, 1974).

Anatomy of the system in detail

The inhibitor neuron is larger than the excitor with a main trunk diameter of about 14 μm versus 10 μm (Dudel and Kuffler, 1961). The excitor motor neuron conducts an action potential of approximately 100 mV in amplitude, with a duration of 1 ms (half peak amplitude) with a 30 to 50 ms duration depolarizing after potential of about 10 mV (Baxter and Bittner, 1981). The axons run along the surface of the muscle fiber and are highly varicose in structure with terminal boutons joined by bottlenecks (Figure 2). The numerous varicosities range in diameter from 1.5 to 10 μm and the connecting regions (bottlenecks) are approximately 0.5 μm in diameter (Florey and Cahill, 1982). Secondary branches of smaller diameter, frequently less than 0.25 μm, extend from the main varicose structure (Jahromi and Atwood, 1974).

In general, the physiological properties of motor synapses along a single muscle fiber are uniform, but vary between different fibers (Bittner, 1968). Synapse bearing nerve terminals are accompanied by glial cells and are enfolded by specialized regions of the muscle fiber (Jahromi and Atwood, 1967; Sherman and Atwood, 1972). The terminals have restricted areas for synapses. Electrophysiological evidence has predicted 50 terminals per muscle fiber (Bittner and Harrison, 1970), but detailed anatomical analysis by Florey and Cahill (1982) estimates this number to be much higher. In the active zones are clusters of calcium channels which have been shown to be P-type voltage gated calcium channels (Araque et al., 1994).

The inhibitor axon forms synapses on the muscle fiber and on the excitor axon near its release sites. While the majority of axo-axonal synapses are formed on excitor terminal boutons, some have been found at branch points. It

has been determined that the excitor does not form reciprocal axo-axonal synapses on the inhibitor (Pearce and Govind, 1993).

Although presynaptic GABA receptors differ pharmacologically from their postsynaptic counterparts on the same muscle cell (Dudel and Hatt, 1976), they both open chloride channels (Takeuchi, 1976).

Calcium and transmitter release

Transmission at the crayfish opener NMJ is rapid, with transmitter release occurring within a fraction of a millisecond after the arrival of an action potential at the terminal (Dudel and Kuffler, 1961). Transmitter release is triggered by brief, localized, high calcium concentrations (Katz and Miledi, 1967) of about 100 μM in active zones (Zucker, 1999). It has been demonstrated that there is a highly non-linear dependence of release on extracellular calcium concentration (Dodge and Rahamimoff, 1967; Dudel, 1981; Augustine et al., 1985). Zucker proposes a simple model for release (1999), in which five calcium ions bind to a trigger molecule. The trigger molecule would have a low affinity for calcium because transmission is not saturated as residual calcium levels rise in the terminals. Binding of calcium to the trigger molecule is weakly affected by EGTA, a buffer with a slow forward rate of calcium binding, but strongly affected by BAPTA, a much faster buffer (Adler et al., 1991; Swandulla et al., 1991). Thus, binding of calcium to the trigger molecule is fast, and transmitter release occurs shortly after calcium flows into presynaptic terminals (Llinas et al., 1981). Vesicle fusion to the presynaptic membrane and transmitter release then occurs. Fusion is segregated into active zones (Robitaille et al., 1990; Cohen et al., 1991) and occurs about 50 nm from calcium channels (Heuser et al., 1979).

Activity dependent short-term synaptic enhancement

Repetitive stimulation produces enhancement of release of neurotransmitter with three phases: facilitation, with a time constant of less

than one second; augmentation, with a time constant of several seconds; and potentiation, with a time constant of several minutes (Zucker, 1999). The probability of release at synapses increases with stimulation as a result of higher background intra-terminal calcium (Katz and Miledi, 1968; Zucker and Lara-Estrella, 1983; Delaney et al., 1989; Zucker et al., 1991). Post-tetanic potentiation is defined as enhanced release remaining after the aforementioned phases and any concurrent depression have worn off. All of these phenomena require calcium entry to be induced (Zucker, 1999).

The probability of transmitter release from a stimulus is enhanced for one to several seconds from a synapse following one or a brief train of stimuli. This phenomenon results from facilitation and augmentation and is important in grading muscle contractions (Atwood, 1967; Atwood and Wojtowicz, 1986). Synapses at the crayfish NMJ show pronounced facilitation (for example see Figure 14) with variation observed between synapses in different regions. Facilitation is measured as the ratio of the amplitude of the postsynaptic EJP from each impulse in a train of stimuli in relation to the first (see for example Figure 14). Zucker (1999) predicts a model for facilitation with calcium binding to a second site with higher affinity and slower kinetics than the secretory trigger molecule.

Presynaptic inhibition

Presynaptic inhibition is an heterosynaptic form of synaptic plasticity where an inhibitory neuron depresses release from another neuron though an axo-axonal synapse. In the crayfish opener muscle, the inhibitor neuron depresses release from the excitor motor neuron through presynaptic axo-axonal synapses. The phenomenon of presynaptic inhibition, which is also found in the CNS (Eccles, 1964; Nicoll and Alger, 1979), was first demonstrated definitively at the crayfish NMJ by Dudel and Kuffler (1961). With quantal analysis it was found that the decrease in EJP amplitude resulted from a decrease in quantal content with no change in quantal size, thus it was a presynaptic phenomenon (Dudel and Kuffler, 1961). Inhibitory axo-axonal

synapses on excitatory motor synapses were then anatomically demonstrated with electron microscopy (Atwood and Jones, 1967; Atwood and Morin, 1970).

Early experiments on the crayfish opener muscle reported complete elimination of contraction with co-activation of the excitor and inhibitor axons (Marmont and Wiersma, 1938; Kuffler and Katz, 1946). Most crustacean muscles receive inhibitory input from a GABA-releasing neuron acting directly on the muscle that inhibits depolarization by reducing the membrane time constant to inhibit summation of EJPs (Atwood and Wojtowicz, 1986). However, Fatt and Katz (1953) noted that the conductance increasing mechanism they reported could not fully account for the depression of the EJP following inhibitor stimulation. The idea that this inhibition could be mediated presynaptically was proposed by Dudel and Kuffler (1960).

Inhibition was found to be exerted presynaptically via electrical shunting of the excitor action potential. GABA opens presynaptic chloride channels (Takeuchi and Takeuchi, 1966) and shunts some of the action potential depolarization (Dudel, 1963; Baxter and Bittner, 1991), reducing the number of calcium channels opened by an excitor action potential (Zucker, 1999). Presynaptic inhibition reduced the amplitude of the excitor action potential measured in the excitor axon near the Y branch by 4 to 8 mV. This was due to a shunting mechanism rather than a direct reduction in voltage. This is because inhibition only lasts for 2 to 6 ms while the decay of the inhibitory potential lasts for 20 to 50 ms. Also, depolarizing or hyperpolarizing inhibitory potentials both reduce the amplitude of the excitor action potential by the same extent. Thus, presynaptic inhibition reduces the amplitude of the excitor action potential by a mechanism independent of the sign, amplitude and duration of the inhibitory potential, which occurs from a conductance change associated with the inhibitory potential (Baxter and Bittner, 1981). At the actual terminal bouton one might expect a reduction in the amplitude of the excitor action potential of 6 to 20 mV, which would be predicted to greatly reduce the amount of transmitter released (Charlton and Bittner, 1978; Katz and Miledi, 1967).

Inhibitor and excitor interactions and anatomy

Axo-axonal synapses are sometimes found to occur at branch points and constrictions of the motor axon (Jahromi and Atwood, 1974). These regions of the axon may induce biophysical limitations for conduction because of higher axial resistance. Thus, the reliability of conducting an action potential through these points, i.e. the safety factor for the propagation of an impulse, would be predicted to be low (Parnas, 1972; Grossman et al., 1973; Spira et al., 1969). Therefore, there may be some functional significance to the location of axo-axonal synapses (Atwood, 1976).

It was proposed by Dudel (1963) that presynaptic inhibition worked by blocking the action potential before reaching the nerve terminal. However, this result is not typically observed (Atwood and Bittner, 1971). It has been proposed that a chloride conductance at or near bottlenecks and branch points may provide the conditions to block active propagation of the action potential (Dudel 1963; Spira et al., 1969, 1976; Dudel, 1983; Atwood et al., 1984). Although some axo-axonal synapses are found at these vulnerable regions (Jahromi and Atwood, 1974), the majority have been found to synapse more directly onto excitor terminals (Smith, 1978; Atwood et al., 1984). Furthermore, if impulse blockade occurred, then one would expect to see an elimination of the facilitation of transmitter release. In the crab leg, a system which shows intense presynaptic inhibition, a decrease in facilitation has been observed (Wiens and Atwood, 1975). However, facilitation typically persists during presynaptic inhibition at the crayfish NMJ (Atwood and Bittner, 1971; Baxter and Bittner, 1981), demonstrating that most of the terminals are invaded by an action potential.

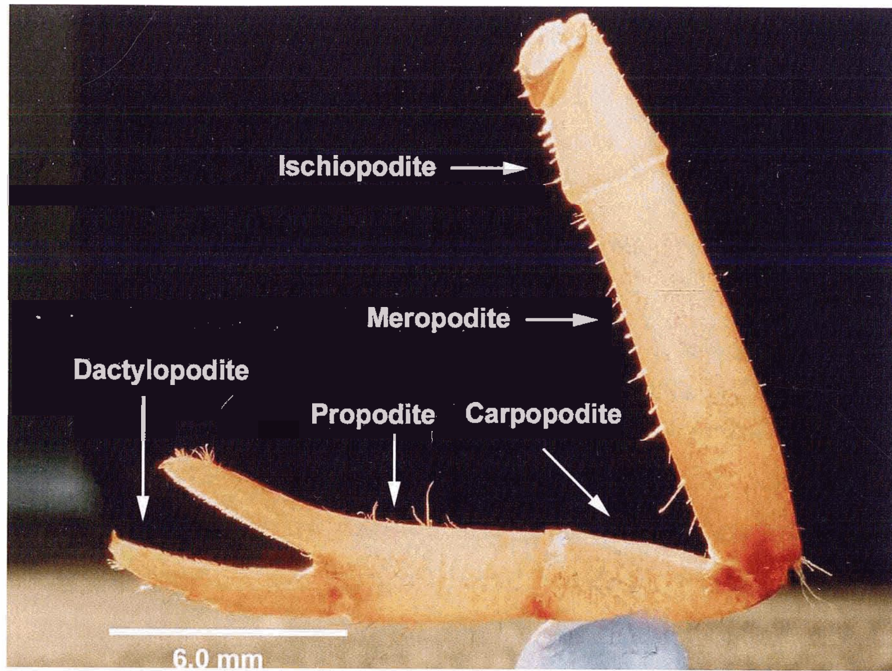
In this study I have specifically investigated the interaction between activity dependent facilitation in the excitor axon and the reduction of neurotransmitter release by presynaptic inhibition. It has been shown that facilitation develops during co-activation of the excitor and inhibitor axons is masked by presynaptic inhibition and unmasked within a few milliseconds of the offset of inhibitor activity provided the excitor axon continues to fire (Dudel

and Kuffler, 1961; Atwood and Bittner, 1971; Baxter and Bittner, 1980). I have found that while presynaptic inhibition can effectively block muscle contraction during co-activation of the excitor and inhibitor axons, it does so without eliminating facilitation. I have demonstrated that the functional consequences of this interaction are to increase the temporal response of muscle contraction. This agrees with predictions of Atwood and Wojtowicz (1986) that one purpose of presynaptic inhibition may be to increase the kinetics of an ensuing contraction. I have explored the mechanism for this differential effect on release and facilitation by measuring the action potential evoked calcium influx into excitor axon terminal boutons in the presence and absence of inhibitor axon activity.

Figure 1 The first walking leg of the crayfish.

(A), shows the segments of the leg of the crayfish. The opener muscle is located in the ventral portion of the propodite. Contraction of this muscle causes the dactylopodite to open (abduct). Axons were stimulated from the meropodite segment. (B), shows the dorsal surface of the opener muscle divided into regions: proximal, central and distal. The excitor and inhibitor axons run parallel with each other, branching along the surface of the muscle fibers.

A



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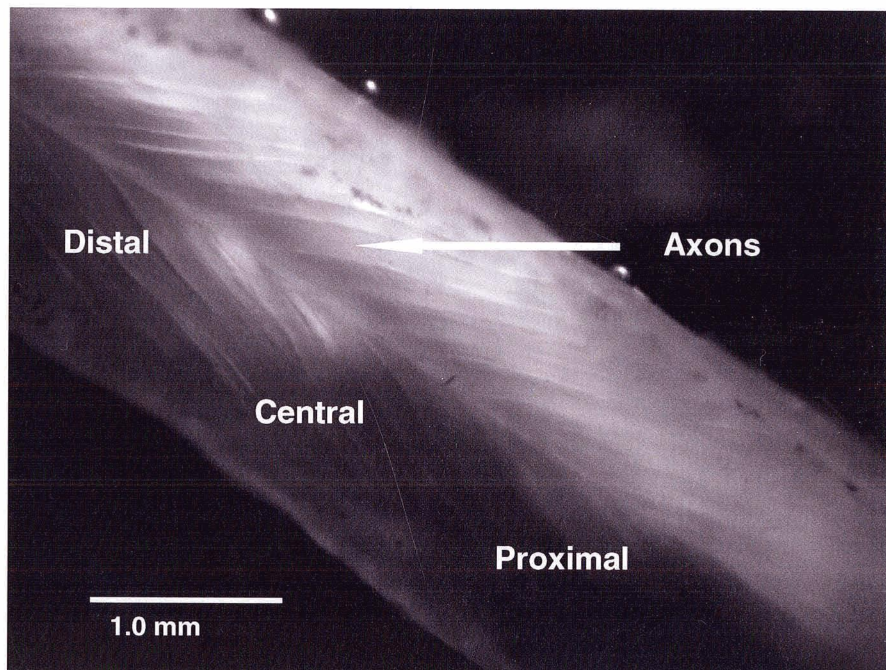
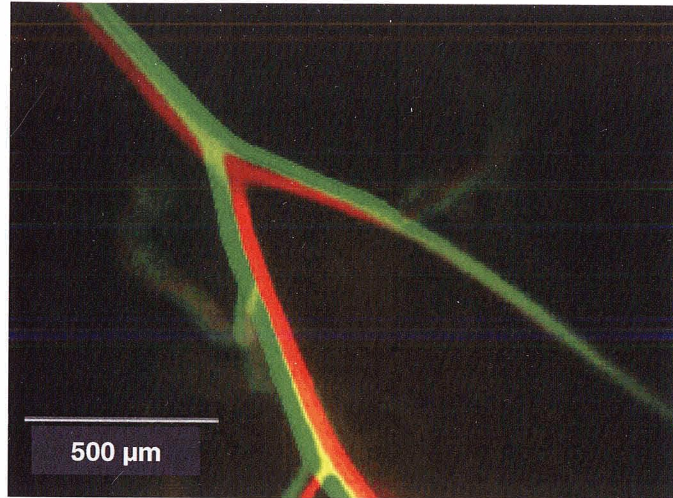


Figure 2 Anatomical relationship between the excitor (Calcium Green-1) and inhibitor (Alexa 568, red) axons on the surface of the crayfish opener muscle.

(A), depicts the main 'Y' branch of the excitor and inhibitor axons and that they run parallel on the surface of the muscle. (B), shows the axons branching to form varicose terminal boutons. (C), shows the complex anatomical relationship between excitor and inhibitor terminals at the level of magnification used to image the activity of excitor terminals. Images of the excitor and inhibitor axon were taken separately, using a dual dichroic mirror, and overlaid.

A



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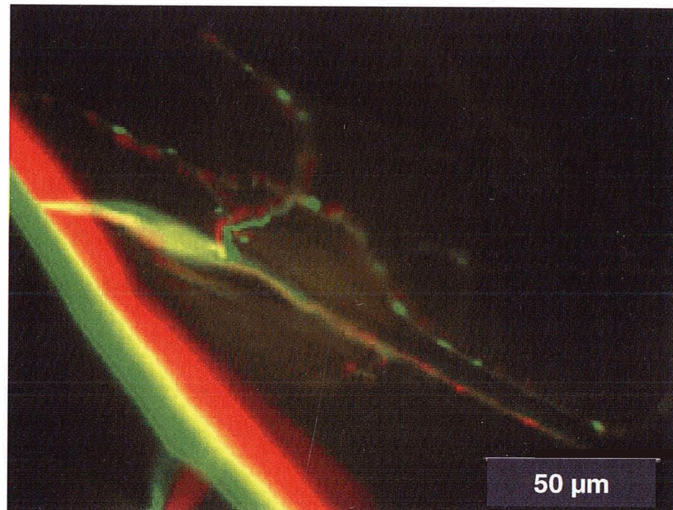

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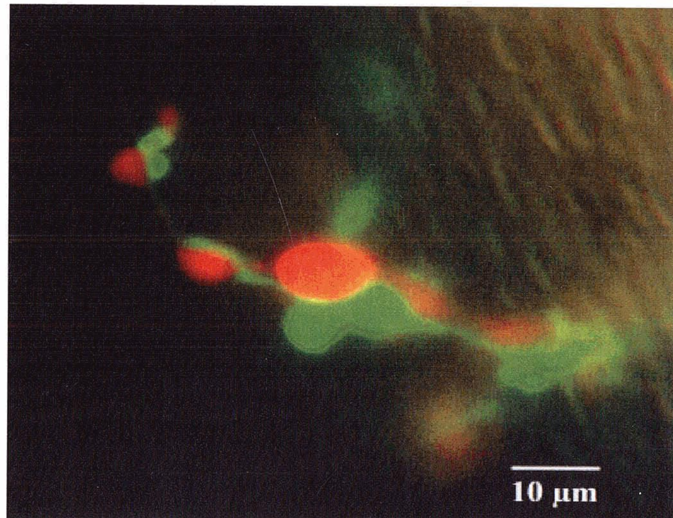


Chapter 2: Materials and Methods

Animals and preparation

Crayfish, *Procambarus clarkii*, ranging from 6 to 8 cm in length were obtained from Atchafalaya Biological Supply (Raceland, LA) and housed at 18°C in tanks with flowing, filtered water. They were maintained on a 12h:12h light:dark cycle, provided glass jars and black plastic ABS tubing for housing and were fed carrots. Experiments were performed on the first walking leg of intermolt stage animals removed at the joint between the basipodite and ischiopodite. The leg was placed in a 35 mm sylgard lined Petri dish dorsal side up. The dactylopodite was secured with dental periphery wax and then a small drop of cyanoacrylate glue was applied to the base of the propodite and carpopodite to secure the claw firmly. The meropodite and top portion of the propodite were removed to expose the excitor and inhibitor axons and opener muscle respectively (Figure 1, 3). Great care had to be taken when removing the closer muscle from the preparation to avoid damage to axons running along the surface of the opener muscle (Kuffler and Katz, 1946). The excitor and inhibitor axons were stimulated using glass suction electrodes made from capillary patch glass that was broken to achieve the desired diameter and fire polished.

Preparations were bathed with modified Van Harreveld's solution (Van Harreveld, 1936), which consisted in mM: 205 NaCl, 5.4 KCl, 13.5 $CaCl_2$, 2.6 $MgCl_2$ and 1.0 N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), titrated to pH 7.4 with NaOH. The temperature of the bath was monitored with a thermister (Fluke, model 51K/J) and maintained at 20 ± 1°C using an external water bath that surrounded the experimental dish.

Force transduction and electrophysiology

For force transduction experiments, the leg was attached in a similar manner to a plexiglas recording chamber with the dactylopodite left free to move vertically over a ledge (Figure 3). Force measurements were obtained using a Grass force-displacement transducer (Model FT03). The dactylopodite was attached to the force transducer with #9-0 surgical silk (Deknatel). The force transducer was mounted to a mechanical manipulator that was used to raise the transducer above the claw until the thread was taut and the dactylopodite was held level with the propodite in a neutral position, being neither open nor closed. The signal from the force transducer was amplified (Grass Instruments Low Level D.C. Amplifier, 7P122; Grass Regulated Power Supply, RPS 107) filtered between 35 Hz and 2 KHz, and digitized at a rate of 2 KHz.

The excitatory and inhibitory postsynaptic junction potentials (EJPs and IJPs) were recorded with sharp electrodes filled with 3 M KCl with a resistance ranging from 10 to 15 MΩ. Muscle fibers selected for recording were intermediate fibers primarily located proximal to the major Y branch of the motor axons, distal and lateral to the tight midline proximal bundle (Delaney et al., 1991). Postsynaptic potentials were recorded with a commercial high impedance head stage amplifier (Neurodata, IR283), amplified a total of 1000-fold, filtered between 0.05 Hz and 1 KHz and digitally sampled at 4 KHz.

Axons were stimulated via glass suction electrodes with 500 µs constant current pulses delivered from a Master-8 pulse generator through stimulus isolators (Neuro Data SIU90). The kinetics of muscle contraction produced from stimulating the excitor axon alone, 25 stimuli at 20 Hz (which was the minimum frequency which produced a consistent contraction), were compared to that produced following a period of co-activation of both the excitor and inhibitor axons (Figure 4a). For co-activation, the inhibitor axon was stimulated with 4 action potentials at frequency of 100 Hz, with the final inhibitor stimulus in the train preceding each excitor stimulus by 2 to 3 ms. The inhibitor to excitor stimulus delay was adjusted to produce maximal

inhibition and was determined at the beginning of each experiment. Several trials (5 to 10) were averaged for each condition with each animal.

The time to onset of contraction was measured for each condition and compared with a paired t-test between animals. The amplitude of the last inhibited EJP was compared to the first non-inhibited EJP with a paired t-test. The amplitude of the last inhibited EJP was also compared to the corresponding control EJP with a paired t-test between animals. Enhancement (facilitation and augmentation) was calculated by dividing the amplitude of the second EJP by each successive EJP for the control and inhibited condition. The result was compared with a two sample t-test.

Calcium imaging

The effect of presynaptic inhibition on facilitation was studied further by measuring calcium entry into the presynaptic terminal boutons of the excitor axon via the transients produced by a calcium sensitive fluorescent dye. A different stimulation paradigm was employed in order to avoid movement of the muscle: 3 excitor stimuli were delivered at 50 Hz and compared to excitor stimuli with inhibition (Figure 4b). Studies of presynaptic inhibition are typically not conducted at high rates to avoid movement of the muscle (Govind et al., 1995), but I wanted to test this phenomenon at a physiologically relevant frequency for the muscle and thus chose to use a short train of 3 stimuli. Terminal boutons measured were located in the vicinity of the main Y branch of the axon.

The excitor axon was penetrated distal to the main Y branch (Figure 2a) with a sharp electrode, containing 15 mM membrane impermeable Calcium Green-1 (Molecular Probes, Eugene OR), 300 mM KCl and 1.0 mM HEPES, with a final resistance of approximately 40 MΩ. To measure changes in calcium concentration in the terminal boutons, the axon was iontophoretically filled with Calcium Green-1 using 5 to 10 nA of negative, continuous current for a total of approximately 250 nA•min. To visualize the position of inhibitory terminal boutons at the end of the experiment, the inhibitor axon was

iontophoretically filled with 1 mM Alexa 568 using approximately 300 nA•min of 10 nA negative, continuous current. Terminal boutons on the surface of the muscle in the intermediate fiber region were observed using an custom-made epifluorescent microscope with a 60 X NA 0.9 water immersion lens (Leica). The preparation was illuminated using a 75 W xenon arc lamp. A switching monocrometer (Polychrome II, T.I.L.L. Germany) was used to select excitation wavelengths. A CCD camera (Imago TILL-Photonics) and image acquisition system was used to record images of fluorescent transients controlled and synchronized to the electrophysiological recordings using TILL-Photonics software (TILLVision™) on a PC. The image of the terminal bouton was vertically and horizontally binned 4X during recording to reduce noise levels.

In order to stimulate the excitor axon at rates that would be observed in the animal, but avoid movement of the muscle, short trains were used to observe calcium entry into the terminal boutons. The change in fluorescence in the terminals was measured while stimulating the excitor axon either alone, 3 times at 50 Hz, or with the excitor stimulation preceded by inhibitor stimuli at 100 Hz (for a total of 8 inhibitor stimuli) (Figure 4b). Fifty-five images were taken, each with a 20 ms exposure, for each train. Because many trials had to be averaged, the excitor alone condition and the excitor plus inhibitor condition were interleaved to avoid any confounding effects with a change in the properties of the dye. Effects could include a change in concentration as the dye diffuses through the axon and photo-bleaching from extended periods of imaging. A 10 s delay was incorporated between each trial to prevent a build-up of short term synaptic plasticity. Twenty to forty trials of the excitor alone condition and the excitor plus inhibitor condition were averaged and compared. Several terminals were imaged at a time, typically within a 60 X 60 µm field of view. During the experiments the inhibitor axon was monitored by penetrating it with a sharp electrode and recording the resulting action potentials to ensure faithful stimulation of inhibitor axon.

A final control measure was taken to ensure that the calcium transients were linearly related to the change in calcium entry. As I was only stimulating with 3 action potentials, I was well below a level of calcium that would saturate

the dye. In order to accurately state that a 20 % reduction in the fluorescent transient corresponded to a 20 % reduction in calcium entry I had to ensure that I was working in the linear range of the dye. This was tested by comparing transients from 3 and 6 stimuli of the excitor axon. If the second transient was approximately twice the amplitude of the first then the response was considered linear. This was routinely done for each experiment to ensure accuracy of the results obtained.

At the end of the experiment the anatomical relationship between the excitor and inhibitor axons was determined by filling the inhibitor axon with Alexa 568. Images were obtained from the live claw in the physiology apparatus using a dual FITC – Texas Red dichroic (Chroma Optical, 51006) with 488 and 564 nm excitation (Polychrome II) and 510 to 550 nm band pass and 600 nm long pass emission filter (Chroma Optical Corp. VT).

Fluorescent transients were analyzed, using TILLVision™ software, by drawing a region of interest around a varicosity where an average level of fluorescence was calculated for each frame. The same ROI was used for the excitor alone condition (control) and the excitor plus inhibitor (inhibited) condition. Changes in fluorescence ($\Delta F / F$) were calculated as a difference between background corrected fluorescence at time t to the average resting fluorescence (Fr) of the terminal recorded 10 frames prior to the stimulus. The equation used was $((F - Fr)/ (Fr - B))$ where B is the background fluorescence, measured from a region of muscle adjacent to the terminals. The control fluorescence transient was then compared with the inhibited transient to determine the reduction in calcium entry into terminals with presynaptic inhibition. Fractional fluorescence changes were < 5 %, which is well within the range where changes in the concentration of calcium are linearly related to the fluorescence change, so calibration of the absolute change in the concentration of calcium was unnecessary; percentage changes in $\Delta F / F$ are equal to percentage changes in calcium influx.

As the majority of imaging and electrophysiological experiments were conducted separately, it was necessary to conduct several experiments where

terminals were imaged and the resulting EJPs in corresponding muscle fibers were recorded concomitantly. This allowed presynaptic and postsynaptic data to be pooled from different experiments for comparison.

To test if inhibition was occurring, the amplitude of the fluorescent transient of the inhibited condition was compared to the control, non-inhibited, condition for each terminal bouton with a paired t-test. Postsynaptically, the amplitude of the EJPs were also compared with a paired t-test. Experiments in which both electrophysiology and imaging were performed were compared with those with either imaging or electrophysiology with t-tests.

In order to test if branch point failure could be induced with increased inhibition, the imaging and electrophysiological experiments described above were repeated with greater stimulation of the inhibitor axon. Twenty inhibitor stimuli at 100 Hz were delivered before the first excitor stimulus for a total of 24 inhibitor stimuli. This was to ensure that the inhibitor was fully facilitated before excitation.

Further testing of the effect of inhibition on the presynaptic terminals of the excitor was achieved with bath application of GABA. The preparation was exposed to moderate concentrations of GABA, between 10 and 30 μM, for at least 10 minutes before recording began. The excitor action potential was recorded with an intracellular electrode to ensure faithful following of the axon to stimuli. A high concentration of GABA, 50 μM, was used to knock out the action potential entirely. The preparation was rinsed with ringer and a final control recording taken. Most experiments utilized 100 stimuli at 5 Hz which produced a large calcium transient without contraction of the muscle and eliminated the need to average trials. One experiment was conducted with the standard protocol used to test presynaptic inhibition, 3 excitor stimuli at 50 Hz, to allow comparison.

Anatomical analysis of presynaptic inhibition

Anatomical analysis was achieved by overlaying images taken of the excitor axon (Calcium Green-1) with images of the inhibitor axon (Alexa 568). The following measurements were obtained: distance of excitor terminal boutons from the nearest branch, distance of excitor bouton from the nearest inhibitor bouton and whether the bouton was on the main portion of the branch or on a secondary branch. The amount of inhibition at each terminal bouton was then compared to these anatomical measures to observe possible trends. Each terminal bouton may have a number of synapses and it cannot be determined from this analysis whether an inhibitory terminal bouton has axo-axonal synapses on the excitor axon or synapses on the muscle.

A correlation between the amount of inhibition at one terminal bouton and the distance from the nearest branch or the distance to the nearest inhibitor bouton was tested by plotting the data and attempting to fit a regression line. The amount of inhibition at terminal boutons on the main varicose structure to those on secondary branches was compared with an two sample t-test.

Figure 3 Apparatus for the force transduction experiments.

Excitor and inhibitor axons were isolated and stimulated independently with suction electrodes. Contraction of the opener muscle (in the propodite) was measured with a force transducer via a suture thread attached to the claw (dactylopodite). Simultaneous electro-physiological recordings were taken, with an intracellular electrode, of the activity of individual muscle fibers.

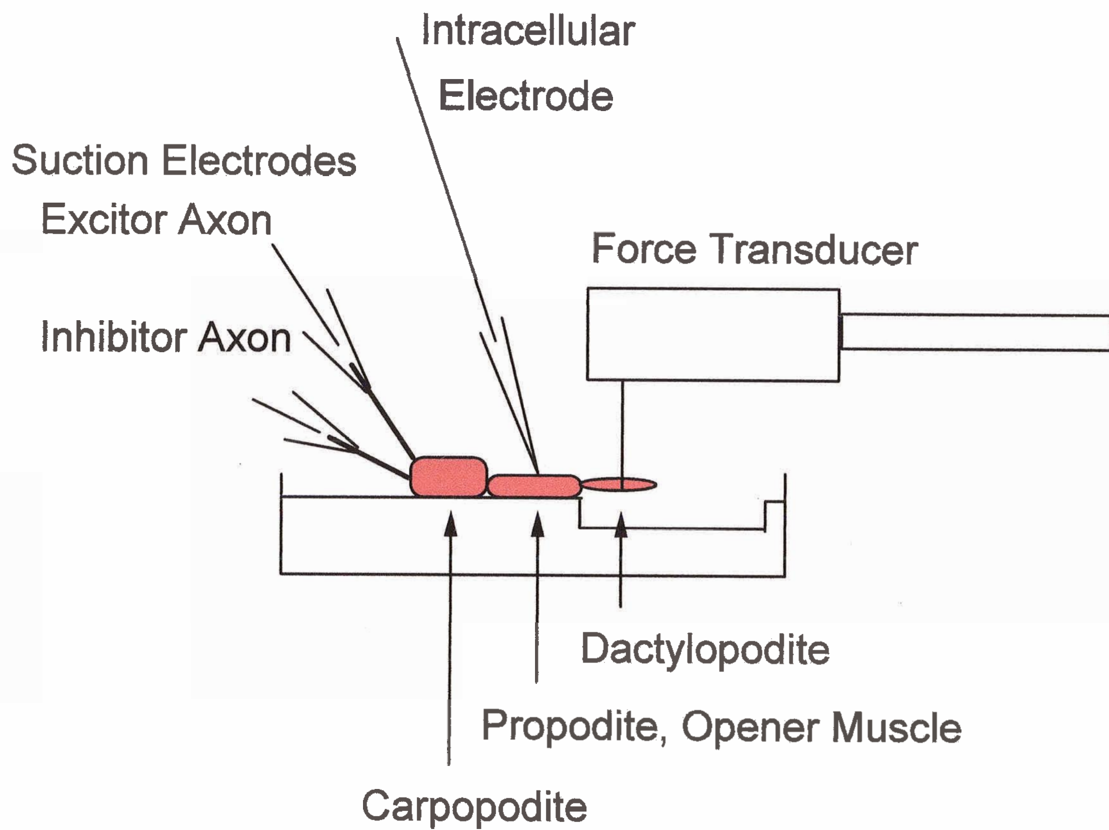
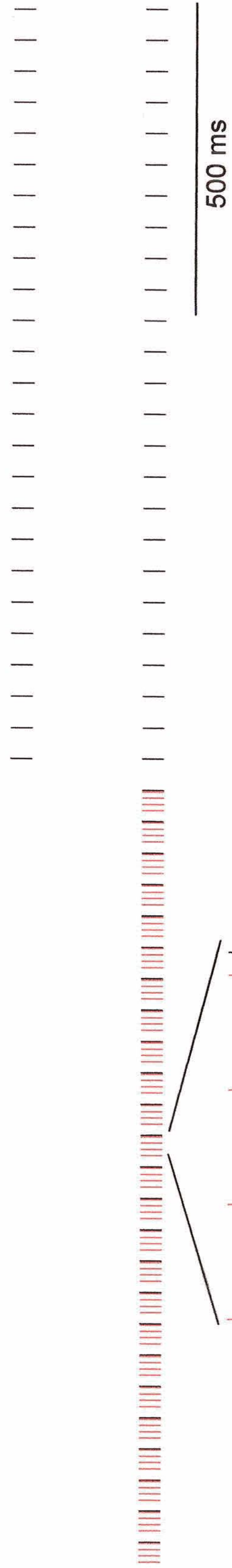
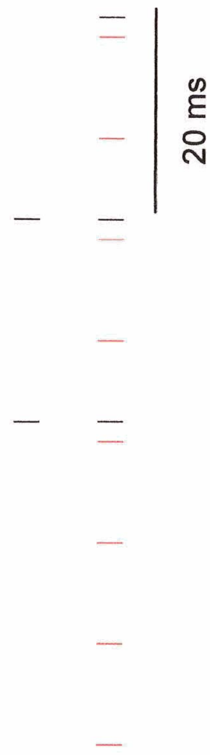

Intracellular
Electrode
Suction Electrodes
Excitor Axon
Force Transducer
Inhibitor Axon
Dactylopodite
Propodite, Opener Muscle
Carpopodite

Figure 4 Stimulus paradigm used for force transduction and imaging experiments.

For the force transduction experiments (A), the exciter (black) was stimulated at 20 Hz and the inhibitor (red) was stimulated four times before each excitor stimulus at 100 Hz. The inhibitor stimuli were timed such that the final inhibitor stimulus arrived 2 to 3 ms before each excitor stimulus. Contractions resulting from 25 excitor stimuli (top trace) were compared to 25 excitor stimuli following 25 excitor stimuli with co-activation of the inhibitor (bottom trace). Imaging experiments (B), used 3 excitor stimuli at 50 Hz (top trace), which was enough to observe a fluorescent transient but not enough to initiate contraction. Inhibitor stimuli were delivered at 100 Hz, timed so that 4 inhibitor stimuli occurred before the first excitor stimulus and an additional 2 inhibitory stimuli occurred for each of the next 2 excitor stimuli (bottom trace).



A
500 ms
10 ms



B
20 ms

Chapter 3: Kinetics of Contraction

Introduction

Due to the fact that both the opener and stretcher muscle in the legs of all decapods share the same excitor axon (Atwood, 1976), it has been thought that the purpose of the specific inhibitor axon (tested in this study) is to allow independent control of these muscles. This does not explain why the main mode of action of the specific inhibitor axon is presynaptic, because postsynaptic inhibition could segregate the stretcher from the opener effectively (Atwood and Wojtowicz, 1986). One benefit of a presynaptic mechanism for inhibition is the conservation of neurotransmitter in excitor terminals that would be released with a postsynaptic mechanism (Bryan and Kranse, 1977). A functional purpose for presynaptic inhibition may be due to the complex relationship between inhibition and facilitation, where presynaptic inhibition may allow facilitation to build, but not be expressed until desired. This is the hypothesis tested in this study.

It has been observed that short-term facilitation builds up in excitor terminals during presynaptic inhibition (Dudel and Kuffler, 1961; Atwood and Bittner, 1971; Baxter and Bittner, 1981). The accepted mechanism for presynaptic inhibition involves the reduction in amplitude of an invading action potential from a chloride mediated shunt. The moderately reduced action potential activates fewer voltage-gated calcium channels, reducing calcium entry and thus reducing transmitter release. A moderate reduction in calcium entry will have a significant impact on transmitter release but not on facilitation, because calcium-triggered release of transmitter is a highly non-linear process. It has been observed at this and other synapses (Magleby and Zengel, 1982), that reducing calcium influx by reducing the single channel

calcium current by reducing extracellular calcium, has little to no negative effect on facilitation. In the squid giant synapse, facilitation of transmitter release occurs even with rather small depolarizations of presynaptic terminals (Charlton and Bittner, 1978).

Atwood and Wojtowicz (1986) suggested that with both conservation of transmitter and short-term facilitation, the rate of muscular contraction would increase just after a period of inhibition. This would result from facilitated release from a large pool of available vesicles. To test this phenomenon, I chose to measure the force produced by the whole muscle in conjunction with intracellular recording of postsynaptic EJPs, while stimulating at a frequency that would produce a contraction. The time to onset of the contraction produced from stimulating the excitor alone was compared with the time to onset of contraction after a period of co-activation of the excitor and inhibitor axons. Thus, the functional implication of the interaction between presynaptic inhibition and facilitation to the crayfish could be explored.

Results

Kinetics of muscle contraction

I found that stimulating the excitor axon 25 times at 20 Hz produced a contraction of the opener muscle, commencing 158 ± 22 ms after the first stimulus. If the inhibitor axon was stimulated at the same time, 100 times at 100 Hz with each excitor stimulus preceded by an inhibitor stimulus and a 2 to 3 ms delay, no contraction was observed. If the excitor axon was stimulated (25 times at 20 Hz) immediately after a period of excitor and inhibitor activation, the contraction occurred with a delay of 38 ± 15 ms. Thus, a period of co-activation of the excitor and inhibitor axons effectively primed the muscle allowing it to contract significantly faster (paired t-test, $P < 0.001$, $n = 6$ preparations) than if it had been activated from rest (Figure 5).

Postsynaptic response during muscle contraction

During contraction, intracellular recordings of the electrical activity of individual muscle fibers in the proximal region were taken and an example of data obtained from one cell is depicted in Figure 6a. While only 25 excitor action potentials were delivered to observe the control measurement for muscle contraction, here 50 stimuli were delivered (black) to allow comparison of 25 EJPs after cessation of stimulation of the inhibitor axon (red). In the control trace (black) an initial rapid build up of the amplitude of the EJP occurred for the first 10 to 12 stimuli followed by a slower increase in EJP amplitude. The rapid build up in EJP amplitude is characteristic of facilitation, while the slower increase is characteristic of augmentation. With inhibition, the first 25 stimuli of the red trace, EJP amplitude is greatly reduced but does increase in amplitude with each successive stimulus. When the inhibitor is turned off, the last 25 stimuli of the red trace, the amplitude of the EJP immediately increases.

Data from several experiments was normalized, averaged and plotted (Figure 6b) to allow comparison. The amplitude of EJPs during co-activation of the excitor and inhibitor axons was greatly reduced but not eliminated, and facilitation of the reduced EJPs was observed. The amplitude of the final EJP before cessation of inhibition was reduced by 64 ± 12 %. Furthermore, the amplitude of the first EJP following cessation of inhibition was significantly larger (paired t-test, $P < 0.001$, $n = 6$ preparations) than the EJP preceding it. However, the first EJP following the cessation of inhibition does not immediately reach the same amplitude that it would have reached in the absence of preceding inhibitor activity. Within 25 stimuli after cessation of inhibition, the amplitude of EJPs reached the amplitude of EJPs from the control, non-inhibited, condition.

By plotting the force transduction measurement with the postsynaptic EJPs recorded from an individual muscle fiber (Figure 7), it is possible to directly observe the effect of maintaining facilitation during inhibition. Stimulating the excitor axon alone at 20 Hz resulted in contraction of the muscle after approximately 4 EJPs. Conversely, stimulating the excitor axon

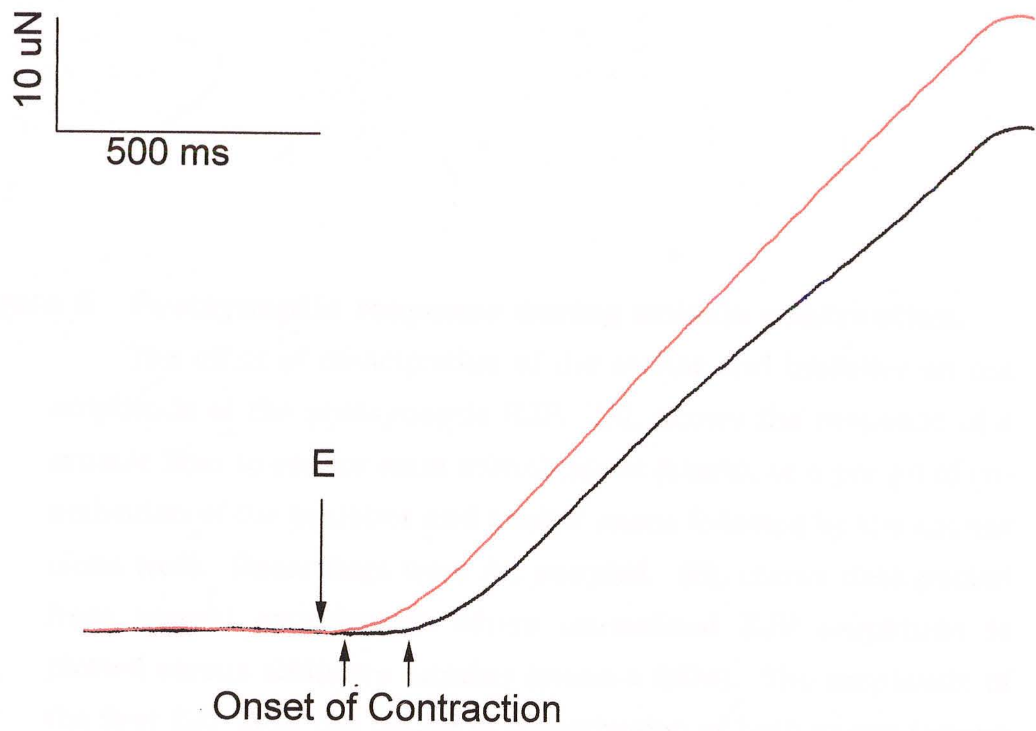
after a period of co-activation of the excitor and inhibitor axons resulted in contraction commencing after only 1 or 2 EJPs.

Enhancement of the EJP (facilitation and augmentation) was measured from the amplitude of EJPs from the control and inhibited condition (Figure 6), during inhibition potentiation of transmitter release for excitor synapses was slightly reduced. Facilitation and augmentation were observed to build during inhibition, but at a reduced level (two sample t-test, $P = 0.01$, $n = 6$ preparations). Enhancement was measured relative to the amplitude of the second EJP because the amplitude of the first EJP could not be accurately determined (Figure 8).

Figure 5 Effect of previous co-activation of excitor and inhibitor axons on the kinetics of muscle contraction.

(A), shows that the time to onset of contraction was reduced when a period of co-activation of the excitor and inhibitor axons precedes excitor stimulation alone (red), versus excitor axon stimulation from rest (black). The trace represents force produced by the opener muscle measured from the dactylopodite from one experiment (see Figure 4 for stimulus paradigm). 'E' represents either the start of excitation for the control, or the first stimulus after inhibition for the test condition. (B), shows the time to onset of contraction averaged for the two conditions. Control contractions commenced after 158 ± 22 ms, and contractions that were preceded by a period of co-activation of the excitor and inhibitor axons commenced after 38 ± 15 ms (paired t-test, $P < 0.001$, $n = 6$ preparations).

A



B

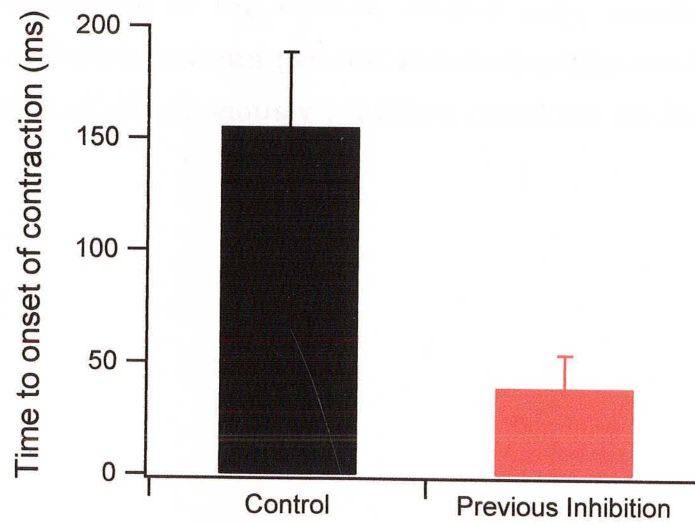
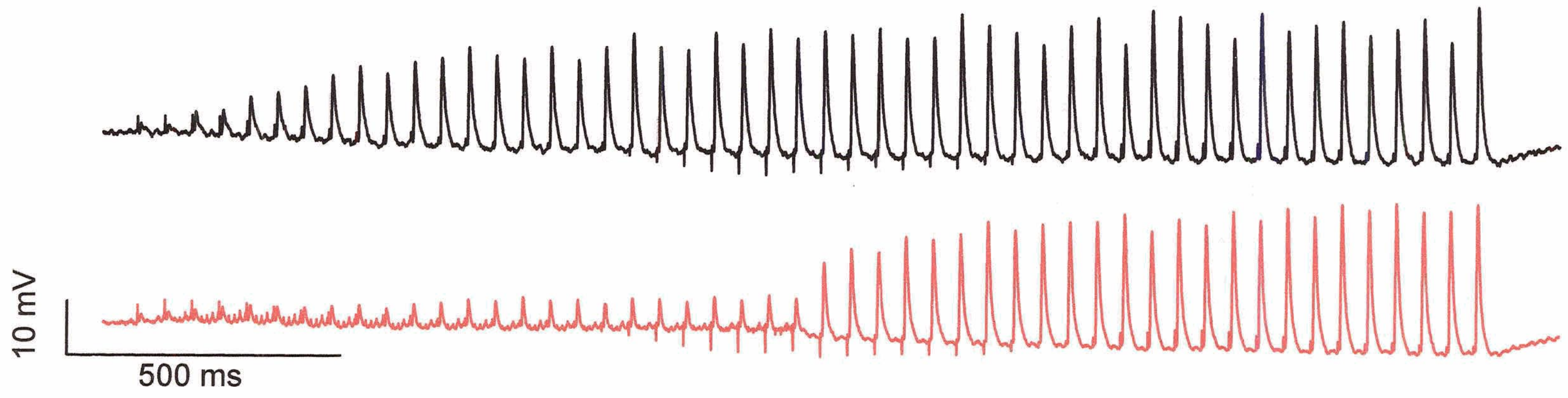


Figure 6 Postsynaptic response during muscle contraction.

The effect of co-activation of the excitor and inhibitor on the amplitude of the postsynaptic EJP. (A), shows the response of a muscle fiber to excitor axon stimuli alone (black), or a period of co-activation of the inhibitor and excitor axons followed by the excitor alone (red). Recordings were AC coupled. (B), shows data pooled from several experiments where normalized EJP amplitude is plotted versus stimulus number (mean ± SEM). The amplitude of the first EJP after the period of co-activation of both axons (arrow) is significantly larger than the previous, inhibited, EJP (paired t-test, $P < 0.001$, $n = 6$ preparations). However, it is not equivalent to the amplitude of the control, excitor only, condition. With several additional stimuli (several hundred milliseconds), the EJP amplitudes of the previously inhibited condition do reach control levels.

A



B

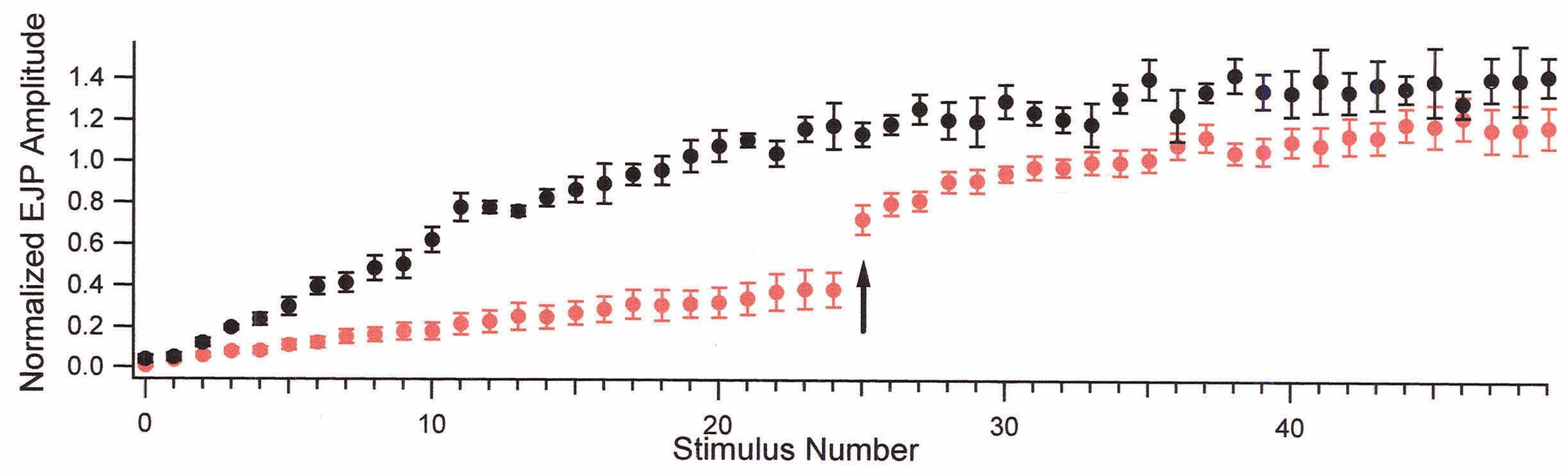
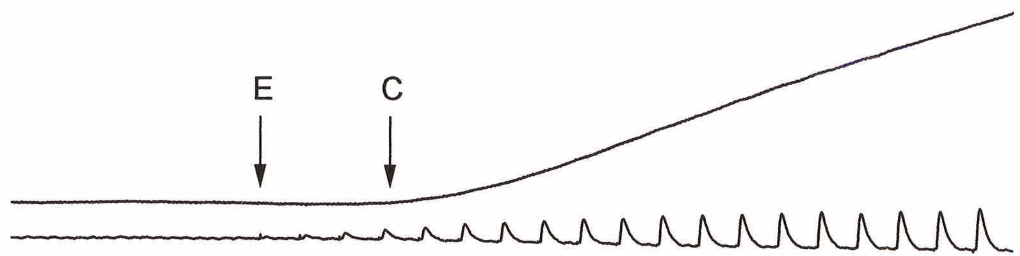


Figure 7 Stimuli needed to initiate contraction after a period of presynaptic inhibition.

A comparison between the postsynaptic EJP from a muscle fiber (bottom trace of each set) and the force produced by the muscle (top trace) shows that less EJPs are necessary to elicit contraction of the muscle if a period of co-activation of the excitor and inhibitor precedes excitation. (A), shows the contraction commencing after approximately 4 EJPs when stimulated from rest. (B), shows the contraction commencing after 1 or 2 EJPs with previous co-activation of the excitor and inhibitor axons. 'E' indicates the first stimulus for the control (A), or the first stimulus after a period of co-activation of the excitor and inhibitor axons (B). 'C' represents the onset of contraction (See Figure 4a for the stimulus paradigm).

A



B

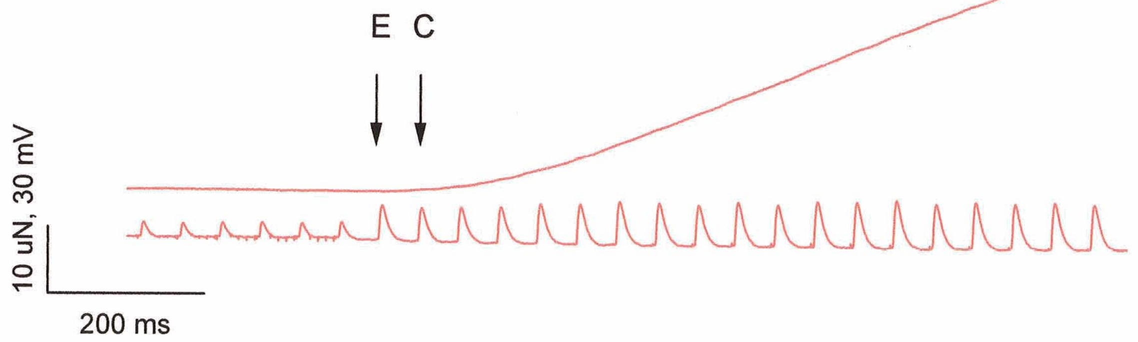
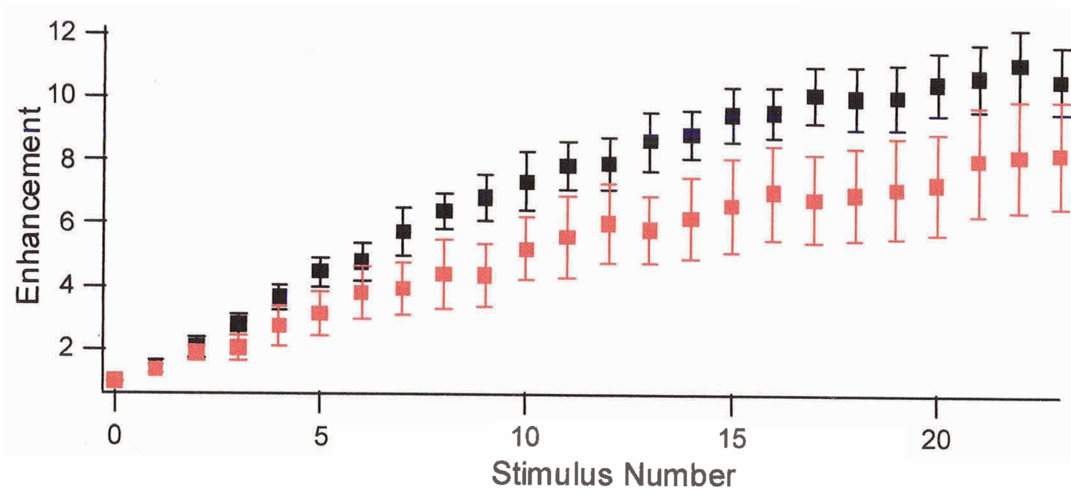


Figure 8 Enhancement of the EJP during force transduction experiments.

The relationship between enhancement (facilitation and augmentation) and stimulus number when stimulating the excitor axon at 20 Hz with and without the inhibitor. Facilitation and augmentation build during the first 10 to 12 stimuli and then taper off for both the excitor alone (black), and excitor and inhibitor together (red) conditions. Overall, there was slightly less enhancement of transmitter release with concomitant activation of the excitor and inhibitor (two sample t-test, $P < 0.01$, $n = 6$ preparations). Enhancement was estimated by dividing the amplitude of each successive EJP by the amplitude of the second EJP.



Enhancement
12
10
8
6
4
2
0
5
10
15
20
Stimulus Number

Discussion

The results of my study support Atwood and Wojtowicz's (1986) hypothesis that the rate of muscular contraction should increase after a period of inhibition. Indeed, the time to onset of contraction was consistently reduced when excitor stimulation followed a period of presynaptic inhibition compared to excitor stimulation alone. This indicates that processes such as facilitation and augmentation may build in excitor terminals during presynaptic inhibition, but the full extent of this buildup is only revealed when inhibition ceases and excitation continues. This provides the synapse with a powerful mechanism to build facilitation while not eliciting contraction of the muscle until desired. At this point transmitter release is facilitated enough to have an immediate effect, whereas normally in this tonic motor system, several stimuli have to occur in order to build up enough facilitation to elicit contraction of the muscle.

Direct evidence for the maintenance of short-term synaptic plasticity during inhibition was shown with simultaneous recording of postsynaptic EJPs from individual muscle fibers during force transduction experiments. Facilitation and augmentation build during the control stimulation of the excitor alone, wheras during co-activation of the excitor and inhibitor axons, the EJPs were reduced to a level below the threshold for contraction while the amplitude of the EJPs were still observed to build. Furthermore, the amplitude of the first EJP after inhibition was significantly larger than the previous inhibited EJP, demonstrating the maintenance of facilitation during presynaptic inhibition. Finally, the synapse was effectively 'primed' so that when inhibition ceased, transmitter release was potentiated with the next action potential, resulting in a nearly full amplitude EJP.

This phenomenon is probably functionally significant to the crayfish because it increases the kinetics of contraction. I have demonstrated that maintenance of facilitation could be an important mechanism in the normal operation of this neuromuscular system, and a fundamental mechanism to synapses that utilize presynaptic inhibition in general.

I did not see a full return to control, non-inhibited, EJP amplitudes with the first EJP stimulated after cessation of inhibition as Baxter and Bittner (1981) observed, which could indicate that there was some loss of short-term synaptic plasticity during presynaptic inhibition. A graph of facilitation does show less facilitation during presynaptic inhibition than the control condition. However, the EJP did return to the amplitude that it would have reached had there been no inhibition after several additional stimuli. The stimulation paradigm used in my experiments differed from Baxter and Bittner's study in that they stimulated with a short train (11 excitor stimuli at 100 Hz), while I used a longer, lower frequency train (25 excitor stimuli at 20 Hz). One explanation for the difference between the results of my study and previous work may be that during inhibition I had some loss of augmentation, which would have built up with the longer stimulus train used to here. Some loss of augmentation during presynaptic inhibition is expected because it is directly dependent on the free calcium concentration in these terminals (Delaney et al., 1994) which is presumably reduced, a hypothesis tested in chapter 4. Another explanation could be the activation of a $GABA_B$ receptor. My estimation of facilitation shows only a small reduction in facilitation during presynaptic inhibition, yet the EJPs take several hundred milliseconds to reach the control amplitudes. This time course would be consistent with a $GABA_B$ mediated contribution to inhibition. Activation of metabotropic $GABA_B$ receptors would inhibit transmitter release for several hundred milliseconds. $GABA_B$ receptors have been demonstrated pharmacologically to play a role in transmitter release from excitor motor terminals (Fischer and Parnas, 1996a, b; Parnas et al., 1999). Further experiments would have to be conducted to verify this possibility.

It is unlikely that there is a long lasting postsynaptic effect of inhibition. Postsynaptic inhibition at this synapse works by the same principle as presynaptic inhibition: a $GABA_A$ mediated chloride conductance is activated that reduces the effectiveness of an ensuing EJP. As postsynaptic inhibition functions with the same short time constant as presynaptic inhibition, it is suspected that the postsynaptic conductance will have decayed by the time the next EJP arrives. Muscle contraction kinetics could have been enhanced by a

small build up of calcium in the muscle fibers in response to the small amplitude of inhibited EJPs. It is possible that, during inhibition, a low concentration of calcium may build in the fiber that is below the threshold for contraction. When inhibition stops and excitation continues, calcium entry from the large EJP would add to the calcium in the muscle fiber causing a larger contraction. This would have to be measured in order to verify that the increase in the kinetics of contraction observed resulted entirely from a presynaptic mechanism.

To determine how the crayfish might use the maintenance of facilitation during presynaptic inhibition, it would be useful to conduct experiments using complex stimulus paradigms observed *in vivo.* Wilson and Davis (1965) examined neural activity in freely behaving animals and found that activity in motor axons was irregular and activity in the exciter was usually accompanied by activity in the inhibitor. Other researchers have observed doublet and triplet patterns of motor axon activity (Wilson and Larimer, 1968). More detailed analysis of the effect of presynaptic inhibition and facilitation on excitor motor terminals would be conducted utilizing macropatch recording of the activity from individual terminals directly. Shorter trains of excitor stimulation would have to be used in order to minimize movement of the preparation.

Chapter 4:
Imaging Individual Terminal Boutons

Introduction

In order to test the hypothesis proposed in the previous section, that the kinetics of muscle contraction were increased due to the maintenance of facilitation in excitor motor terminals, I measured the change in calcium entry into motor terminal boutons. A moderate reduction in calcium entry could drastically reduce transmitter release as several calcium ions act cooperatively to trigger release (Dodge and Rahamimoff, 1967), without affecting facilitation (Magleby and Zengel, 1982).

In order to determine the activity at individual terminals, I chose to use calcium imaging rather than extracellular macropatch recording. Macropatch recording involves placing an electrode directly over a terminal varicosity and directly measuring voltage changes. However, it does not easily allow detailed anatomical analysis of multiple terminal boutons. With calcium imaging, I was able to simultaneously estimate the activity from many terminal boutons and could interpret the effects of the anatomical relationship between terminals on physiology. Although I had to infer changes in voltage from changes in calcium, calcium imaging was a better choice for my experiments because I was interested in the relationship between calcium entry and transmitter release.

While most studies of presynaptic inhibition have been conducted at low frequencies to avoid movement (Govind et al., 1995), I stimulated at a high (50 Hz), physiologically relevant frequency observed *in vivo* (Wilson and Larimer, 1968), but with only 3 action potentials. This was also the minimum number of action potentials I could use to obtain a reasonable fluorescent transient for analysis of inhibition. I could thus observe the effect of presynaptic inhibition on calcium entry in relation to the geometry of the excitor axon.

Results

Imaging and electrophysiology

I investigated the effect of presynaptic inhibition on calcium entry into excitor terminal boutons with short trains of 3 stimuli at 50 Hz (Figure 4b). The inhibited condition showed a moderate reduction in calcium entry, although variation was observed between terminals ranging from 0 to 50 %. Overall, the fluorescent transient decreased significantly with presynaptic inhibition with an average reduction of 20 ± 1 % (paired t-test, $P < 0.001$, 122 terminals from 12 preparations) (Figure 9). One experiment was analyzed in detail, to determine the accuracy of an estimate of the percent inhibition for a particular terminal. Where the percent inhibition was greater that 20 %, a significant difference between the control and the inhibited fluorescent transients was observed with only 5 trials averaged (paired t-test, $P < 0.05$). With an increased number of trials (20 to 30), I was able to observe a significant difference between the control and the inhibited fluorescent transients of up to 6 % (paired t-test, $P < 0.05$).

Postsynaptically, a more dramatic reduction in the amplitude of the EJPs was observed. Data from a proximal muscle cell (Figure 10a) illustrates a reduction in the amplitude of the EJPs. Normalized and pooled data for 27 proximal cells from 18 experiments (Figure 10b), revealed the percent inhibition for the first, second and third EJPs to be 42 ± 4 %, 51 ± 4 % and 59 ± 2 % (mean ± SEM), respectively. The amplitude of the first, second and third inhibited EJPs were significantly smaller than the respective control conditions (paired t-test, $P < 0.001$, $n = 27$ cells). The increase in percent inhibition with each subsequent stimulus was significant (paired t-test, $P < 0.05$) and raises the question of whether or not the inhibitor was fully facilitated for the first excitor stimulus. The third EJP was inhibited 59 ± 2 %, similar to the percent inhibition (64 ± 12 %), observed in the previous set of experiments investigating the kinetics of contraction, indicating a similar amount of inhibition.

The 20 % decrease in calcium entry presynaptically resulted in, on average, a 51 % decrease in the amplitude of the EJP postsynaptically (Figures 9, 10). From these data, I calculated the exponent for the non-linear relationship between calcium entry and transmitter release. It has been demonstrated that 5 to 10 % of the inhibition, measured as a reduction in the amplitude of the EJP, is mediated directly on the muscle fiber (Fatt and Katz, 1953; Atwood and Bittner, 1971; Baxter and Bittner, 1980). Thus, I subtracted this from the total inhibition to estimate the effect of presynaptic inhibition (41 to 46 %). Using these estimates with the equation $(Ca^{2+})^n$ = release, I calculated an approximate value for n of 2.6 ± 0.2. Due to the non-linear nature of this relationship and the fact that variation was observed for each terminal, one cannot simply use the average of the percent inhibition observed presynaptically to estimate the exponent for release. One must calculate the resulting postsynaptic effect for a specific value of n for each terminal, $([Ca^{2+}]_{1..122})^n$ = release. The resulting values are plotted (Figure 11) where 41 to 46 % inhibition of the EJP (presynaptically mediated) corresponds to an n value of approximately 2.8 ± 0.3.

The facilitation of the first, second and third EJP was calculated relative to the amplitude of the first EJP (Figure 12). There was a significant decrease in the facilitation of the third EJP with co-activation of the excitor and inhibitor axons versus the excitor axon stimulated alone (paired t-test, $P < 0.01$, $n = 27$ cells from 18 preparations). This result was unexpected because previous researchers found facilitation to be maintained during presynaptic inhibition (Baxter and Bittner, 1981).

The majority of imaging and electrophysiological experiments were conducted separately. To allow comparison of pre- and postsynaptic data between experiments, several experiments were conducted where terminal boutons were imaged and the resulting EJPs in muscle fibers were recorded at the same time. The calcium transient was reduced by 18 ± 2 % (mean ± SEM, n = 60 terminals from 4 preparations) for the combined experiments, versus 21 ± 2 % (mean ± SEM, n = 62 terminals from 8 preparations) for experiments without electrophysiology. In experiments where imaging and electrophysiology

were combined, the EJP was inhibited 51 ± 8 %, 55 ± 6 % and 59 ± 6 % for the first, second and third stimuli, respectively (mean ± SEM, n = 5 cells from 4 preparations), versus 40 ± 4 %, 50 ± 5 % and 60 ± 3 % (mean ± SEM, n = 22 cells from 14 preparations) for experiments without imaging (Figure 13). The results of these experiments were not significantly different from experiments conducted alone, thus allowing comparison of all data (Table 1).

Table 1 Comparison of data from imaging and electrophysiological experiments to combined experiments (mean ± SEM).

	Presynaptic % Inhibition	Postsynaptic EJP1	EJP2	EJP3
Either imaging or electrophysiology	18 ± 2 % n = 62 term.	40 ± 4 % n = 22 cells	50 ± 5 %	60 ± 3 %
Combined experiments	21 ± 2 % n = 60 term.	51 ± 8 % n= 5 cells	55 ± 6 %	59 ± 6 %

In order to determine the relative contributions of temporal summation and facilitation on increasing the amplitude of the EJP, the phenomena were modelled separately (Figure 14). One way to increase the amplitude of the EJP and reach the threshold for contraction would if each EJP occurred before the previous one decayed. Their amplitudes would add together increasing the total depolarization of the muscle cell (temporal summation); this is not required for facilitation. Typical EJPs decayed with a time constant of 5 to 7 ms, thus a 7 ms time constant for decay was used for the model. From the model it was found that 50 Hz stimulation does not result in appreciable temporal summation of the EJP. Higher stimulus frequencies would result in temporal summation. When facilitation was added to the model, it produced a result which resembles my experimental data.

Figure 9 Reduction in calcium entry into excitor terminal boutons during presynaptic inhibition.

The relationship between calcium entry (observed as a change in fluorescence), during stimulation of the excitor alone (black) or the excitor and inhibitor (red). There was on average a 20 ± 1 % (ranging from 0 to 50 %) reduction in the fluorescent transient in excitor motor terminal boutons during presynaptic inhibition. Presynaptic inhibition caused a significant reduction in the fluorescent transient in excitor terminals (paired t-test, $P < 0.001$, 122 terminals from 12 animals).

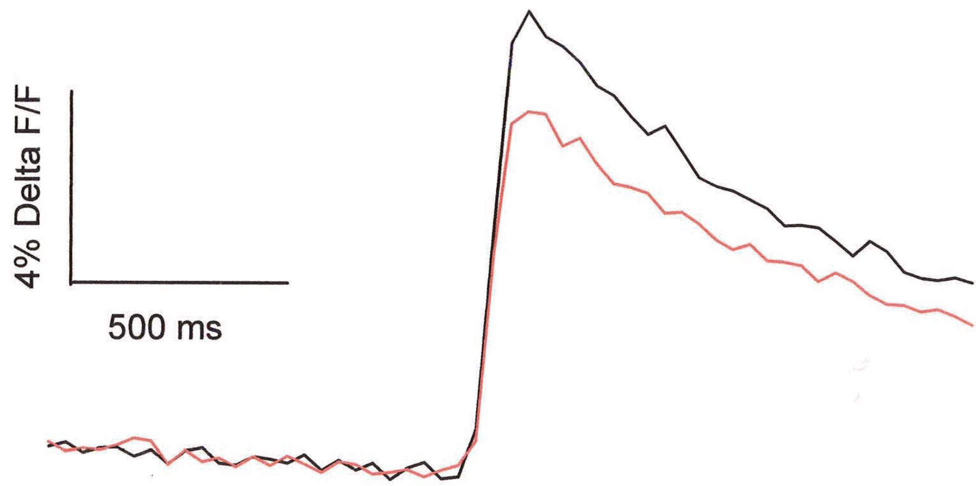
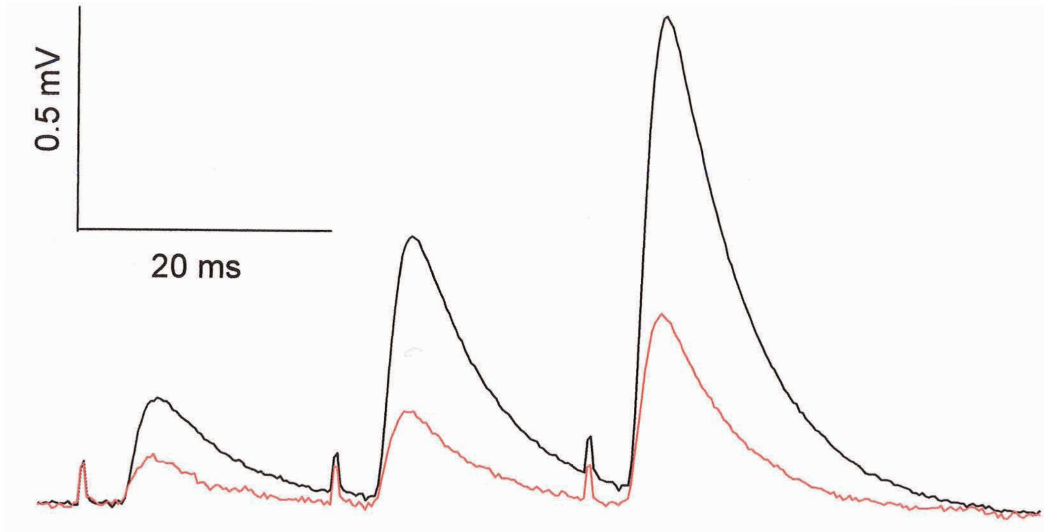

4% Delta F/F
500 ms

Figure 10 Effect of presynaptic inhibition on the EJP.

(A), shows an example of EJPs recorded from a muscle fiber when stimulating only the excitor (black) or the excitor and inhibitor together (red). (B), pooled data, shows that on average the first, second and third EJPs were inhibited by 42 ± 4 %, 51 ± 4 % and 59 ± 2 % respectively (n = 27 cells from 18 animals). The amount of inhibition increased with each successive stimulus.

A



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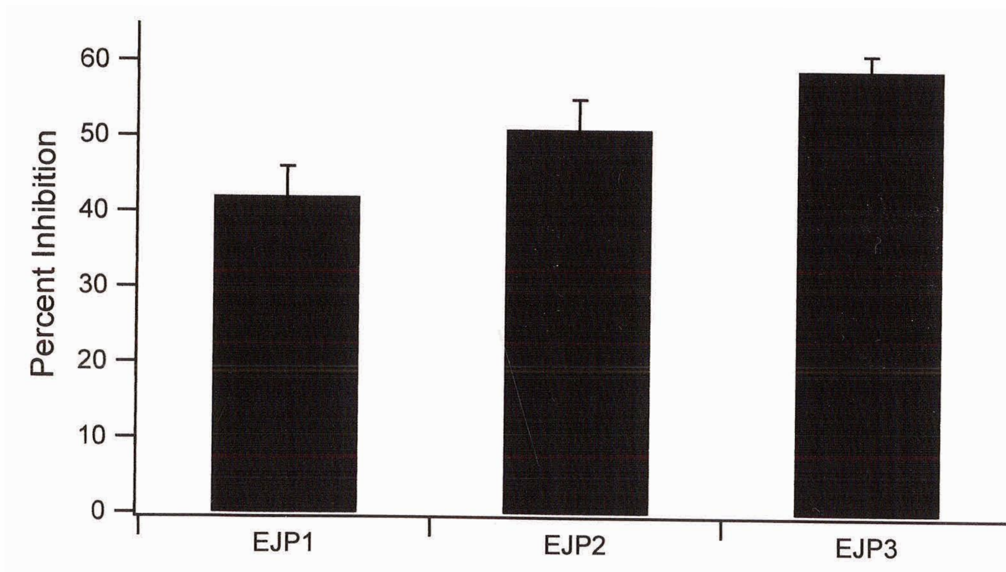


Figure 11 Relationship between percent inhibition observed postsynaptically and the exponent for release.

It is estimated that a 20 % decrease in presynaptic calcium entry led to a 41 to 46 % decrease in transmitter release. Using the equation: $(([Ca^{2+}]_{1..122})^{n}$ = release), the reduction in transmitter release, for a given value of n for each terminal bouton imaged, was calculated. The relationship between percent inhibition versus the exponent for release was plotted. From this relationship it is observed that a 41 to 46 % reduction in transmitter release corresponds to an n value of 2.8 ± 0.3.

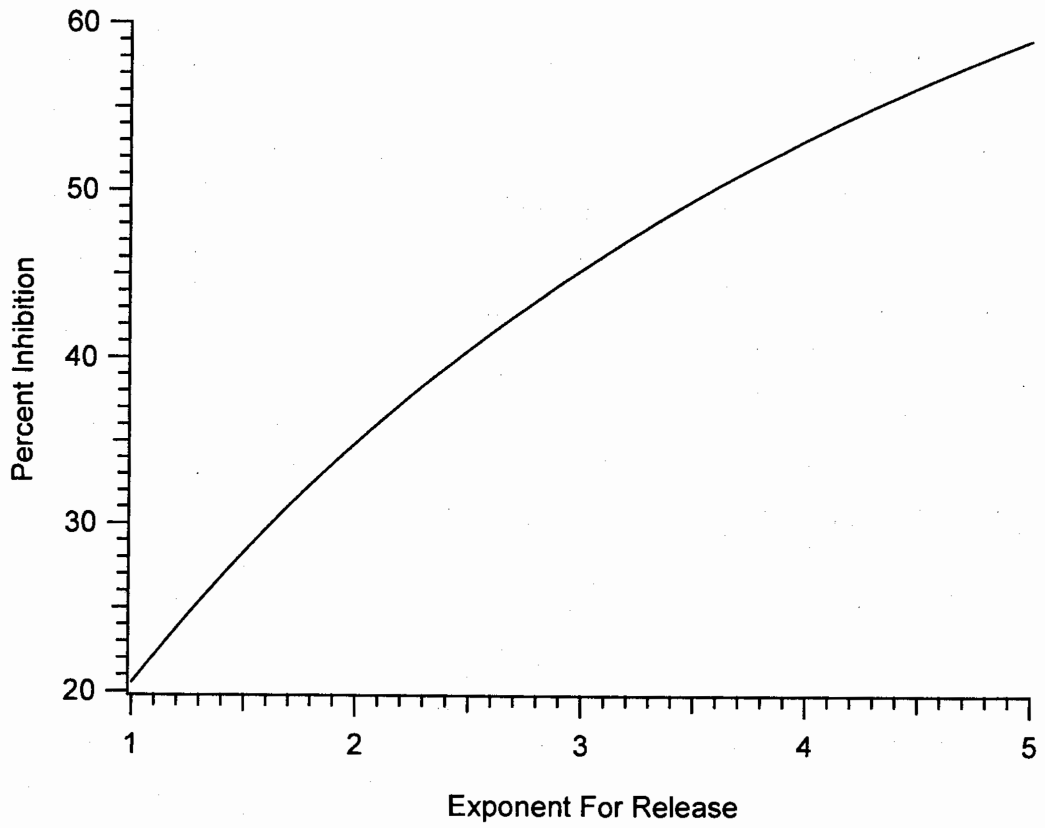

60
50
40
30
20
Percent Inhibition
1
2
3
4
5
Exponent For Release

Figure 12 Effect of presynaptic inhibition on facilitation.

Facilitation of transmitter release from excitor terminal boutons, as measured from EJPs resulting from stimulation of the excitor alone (black) or the excitor with the inhibitor (red). There was significantly less facilitation of the third EJP with inhibition (paired t-test, $P < 0.01$, $n = 27$ cells from 18 preparations). Facilitation was calculated by dividing the amplitude of the first, second and third EJP by the amplitude of the first.

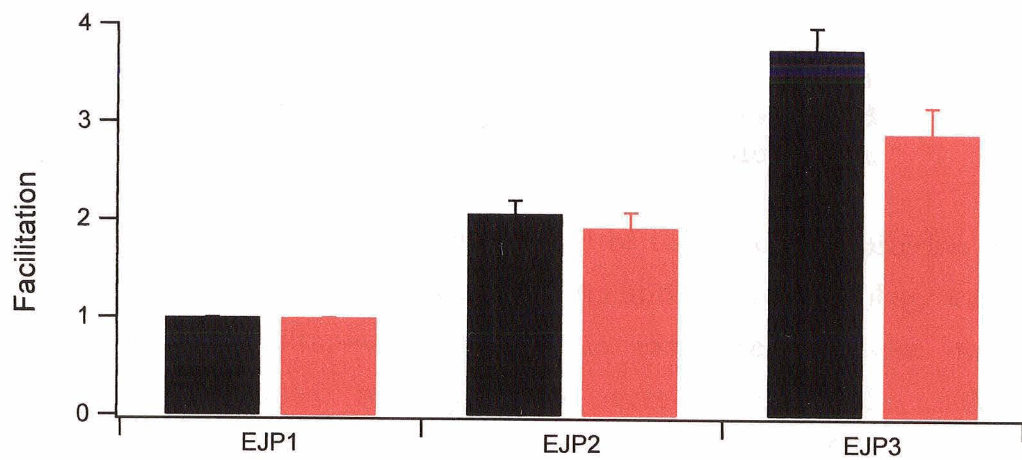

4
3
2
1
0
Facilitation
EJP1
EJP2
EJP3

Figure 13 Comparison between percent inhibition induced in experiments with only electrophysiology and experiments with both electrophysiology and imaging.

This figure shows a comparison of the percent inhibition of the EJP for experiments where imaging and electrophysiology were conducted simultaneously (black) to experiments where only electrophysiological measurements were taken (red). For combined experiments, the percent inhibition of the first, second and third EJPs were 51 ± 8 %, 55 ± 6 % and 59 ± 6 % respectively (n = 5 cells from 4 preparations). For those experiments with only electrophysiology, EJPs were inhibited by 40 ± 4 %, 50 ± 5 % and 60 ± 3 % for the first, second and third EJPs respectively (n = 22 cells from 14 preparations). The percent inhibition between the two types of experiments was not significantly different. See Table 1 for a comparison with imaging data.

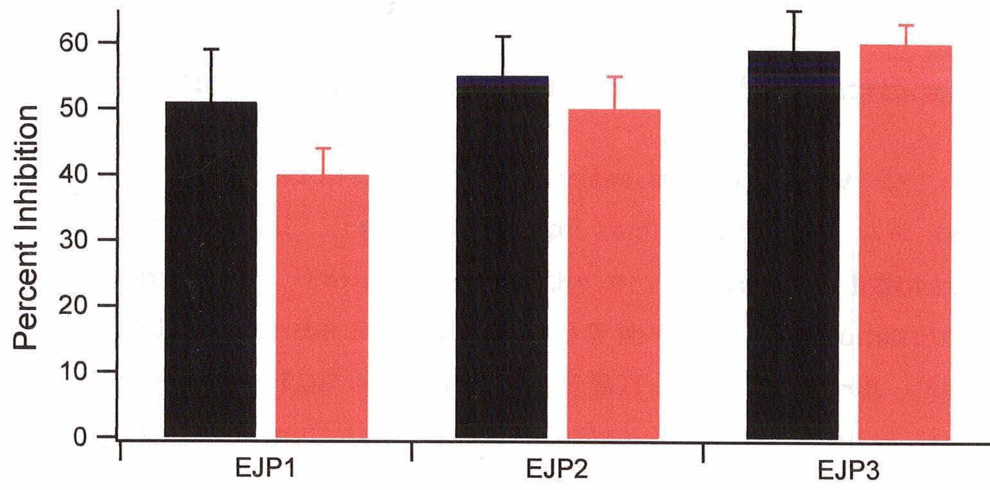
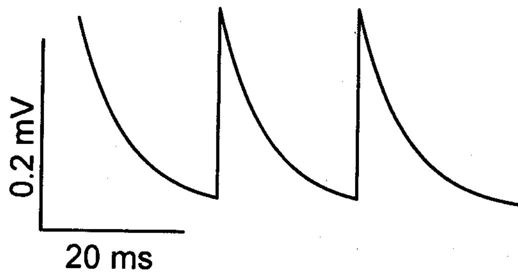

Percent Inhibition
60
50
40
30
20
10
0
EJP1
EJP2
EJP3

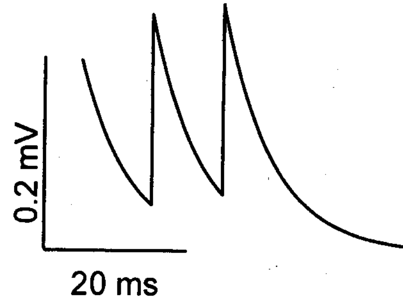
Figure 14 Model of temporal summation and facilitation of the EJP.

Model shows that the increase in EJP amplitude, observed experimentally, is a result of facilitation and not temporal summation. Parameters for the model were as follows: initial amplitude of the first EJP was 0.2 mV, the time constant for the decay of the EJP was 7 ms and 3 EJPs were delivered. (A), shows that at a stimulation rate of 50 Hz with no facilitation there was almost no increase in EJP amplitude. Increasing the stimulation frequency to 100 Hz (B), does produce some temporal summation. Stimulating at 50 Hz with a facilitation value of 2 (C), shows an increase in successive EJP amplitude similar to experimental data (D).

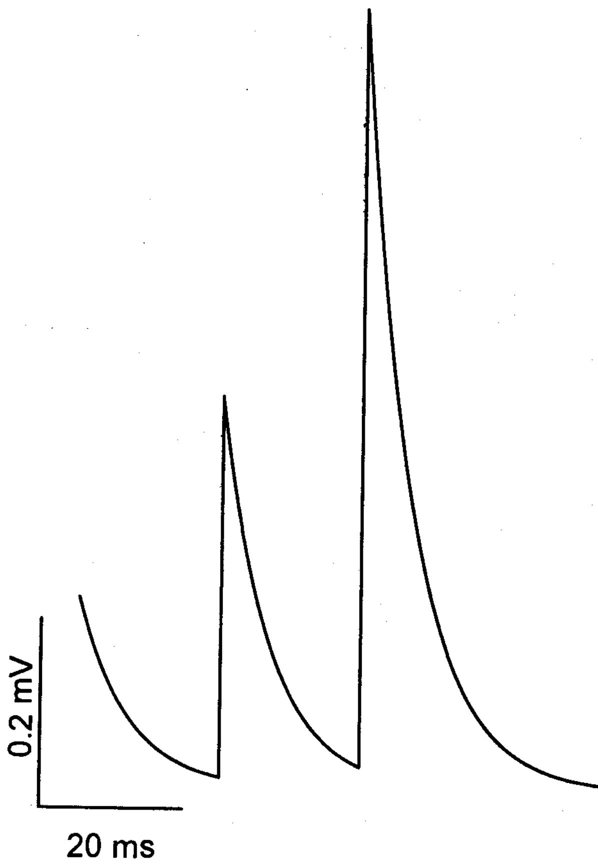
A



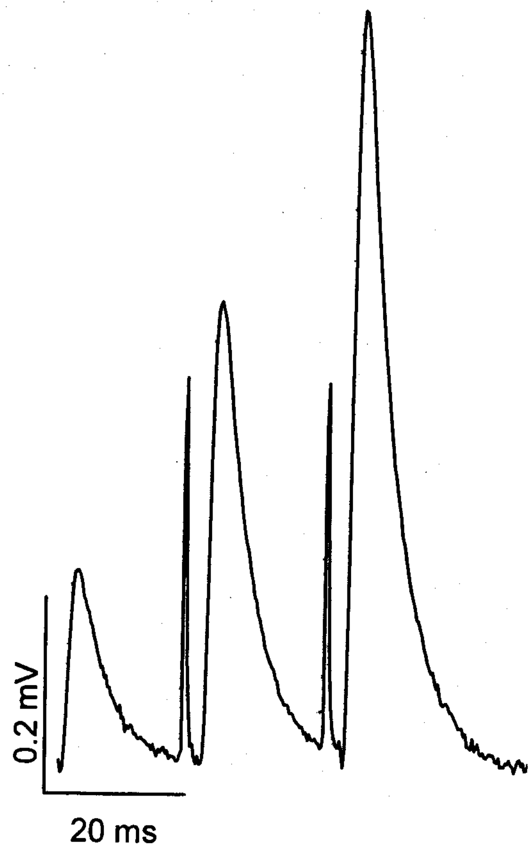
B



C



D



Discussion

Presynaptic inhibition reduced calcium entry into presynaptic terminal boutons by 20 %, resulting in a mean 51 % reduction in the amplitude of the postsynaptic EJP. Many studies have shown a non-linear relationship between calcium influx and transmitter release proportional to the power n > 1 (Dodge and Rahamimoff, 1967; Llinas et al., 1981; Charlton et al., 1982; Augustine et al., 1985; Zucker and Fogelson, 1986; Delaney et al., 1991; Zucker et al., 1991). Consistent with this, my experiments estimated the relationship between calcium influx and transmitter release to be proportional to the third power. The effect of presynaptic inhibition is most likely to reduce the number of open calcium channels and not the flux of calcium per channel. This result is indicative of a synapse that utilizes overlapping calcium micro-domains (Simon and Llinas, 1985) from closely clustered calcium channels to elicit neurotransmitter release (Zucker and Fogelson, 1986).

Experiments investigating the relationship between calcium influx and transmitter release have taken two forms: either changing calcium influx through calcium channels, or changing the number of calcium channels activated. Experiments modulating calcium entry through calcium channels are conducted by changing the concentration of extracellular calcium. These experiments find a highly non-linear relationship between calcium entry and transmitter release ranging between the fourth and fifth power (Augustine et al., 1985; Zucker et al., 1991). Vesicles located near calcium channels experience a change in calcium micro-domains resulting from a change in the calcium influx and thus a change in the probability of release (Figure 15a). This change in the probability of release is proportional to the fourth or fifth power presumably because four or five calcium ions must co-operatively bind to the secretory trigger molecule to elicit neurotransmitter release (Atwood and Wojtowicz, 1986; Zucker, 1999).

Conversely, the number of calcium channels activated can be modulated by changing the amplitude or duration of the stimulus (action potential) or

pharmacologically blocking some of the calcium channels. Modulating the amplitude of the depolarization will affect the number of calcium channels activated because voltage-dependent calcium channels require the co-operative action of multiple voltage-dependent subunits to open (Llinas et al., 1981). The flux of calcium through activated calcium channels will not be affected because the concentration of calcium has not been changed. Also, the majority of calcium entry occurs after the peak of the action potential when calcium channels have been opened by depolarization and the electrical driving force on calcium (E_m - E_{Ca2+}) is increased due to membrane repolarization. Most transmitter release is observed during the tail calcium current (Wright et al., 1996). Thus, a moderate reduction in the amplitude of the action potential (as with presynaptic inhibition) will not reduce the flux of calcium through a channel. These experiments show a distribution of co-operativity from low to high at different synapses, depending on how a particular synapse uses overlapping calcium micro-domains to elicit release. If the synapse does not use extensively overlapping calcium micro-domains to elicit release, then reducing the number of calcium channels activated will reduce transmitter release in a linear manner. This is because the calcium influx from one calcium channel would release one vesicle (Figure 15b). A subtle exception to this rule would be if an enhanced stimulus led to multiple openings of the same calcium channel which would increase the probability of release of the vesicle located near the channel resulting in a non-linear relationship slightly greater than 1. However, if a synapse had closely clustered calcium channels and required the co-operative activation of multiple channels to form overlapping calcium micro-domains in order to elicit release, then one would expect a non-linear relationship between calcium influx and transmitter release (Figure 15c). The calcium micro-domain experienced by the vesicle will be reduced due to reduction of overall calcium influx for the vesicle. Experiments of this sort have demonstrated a relationship between calcium influx and transmitter release ranging from the first to third power (Llinás et al., 1981; Charlton et al., 1982; Augustine et al., 1985; Zucker et al., 1991, Mulligan et al., 2001).

Presynaptic inhibition does not reduce calcium entry by decreasing the influx of calcium through a channel, but rather by reducing the number of

channels activated. A small, 5 to 10 %, reduction in the amplitude of the action potential through chloride shunting reduces the number of voltage-gated calcium channels activated. As calcium enters through a reduced number of calcium channels, there will be less overlap between calcium micro-domains, which will drastically reduce vesicle fusion and result in reduced amplitude EJPs. Thus, my calculation for a third power relationship between reduction in presynaptic calcium and the reduction in the postsynaptic EJP demonstrates the co-operativity between calcium channel micro-domains from closely clustered calcium channels to initiate release (Zucker and Fogelson, 1986). Anatomical data at the crayfish NMJ shows 10 to 20 membrane associated large particles (putative calcium channels) per active zone (Cooper et al., 1996; Atwood et al., 1997), thus corroborating experimental data.

Both release involving a single calcium domain from a single calcium channel (Stanley, 1993; chick ciliary ganglion) and release requiring multiple overlapping calcium domains from an estimated 60 simultaneously open calcium channels (Borst and Sackman, 1996; calyx of held) has been shown. Release involving single calcium domains would be advantageous because it would limit calcium entry into the cell, which is potentially toxic, and would require less energy to restore resting levels of calcium (Stanley, 1997). The relationship between the requirement for overlapping micro-domains and release at a synapse may change with activity. An unconditioned synapse may require overlapping micro-domains to elicit release, but with stimulation, the building of facilitation may reduce the amount of overlap necessary for release (Stanly, 1997). From my experiments, it is clear that the crayfish NMJ requires co-operativity between calcium channel micro-domains from multiple calcium channels to elicit release.

While an average of 20 % inhibition of calcium influx was observed in excitor motor terminal boutons, it varied at a given terminal from 0 to 50 %. This variability is masked when measuring the EJP, because it is the sum of activity of all of the synapses on the muscle fiber. The regional variation in the amount of inhibition at a particular terminal may give insight into how

presynaptic inhibition functions with respect to the anatomical relationship of the excitor and inhibitor axons.

The fact that the percent inhibition was greater for the second and third EJP could reflect the use of short stimulus trains. The inhibitor was fired four times before the first excitor stimulus and twice more for each additional excitor stimulus (Figure 4b). Because facilitation builds in inhibitor terminals in the same manner as in excitor terminals, it is possible that the output from the presynaptic axo-axonal inhibitory synapses was greater on the second and third excitatory stimuli due to a build up of facilitation. It may have been more appropriate to have built facilitation in the inhibitor axon to a maximum level before the first excitor stimulus was delivered in order to have ensured equal inhibition for each stimulus. This experiment is presented in chapter 5.

The model produced to determine the effectiveness of temporal summation for the opener muscle demonstrated that temporal summation is ineffective at increasing EJP amplitudes over useful stimulus frequencies. Facilitation is essential in this system for increasing the amplitude of the EJP to the threshold for contraction, and allows varying tension over multiple stimulus frequencies.

The observation that less facilitation occurred during the inhibited condition is intriguing. From previous studies, one would not have expected facilitation to be affected and further experiments with other stimulation paradigms will be needed to resolve it. These might include a more detailed analysis of the effect of presynaptic inhibition on facilitation with macropatch recording from individual terminals. A single excitor action potential could be delivered, in order to obtain a better estimate for the non-linear relationship between calcium influx and transmitter release. It would also be useful to conduct imaging experiments with longer trains of stimulation to better corroborate these data to the muscle contraction data (Chapter 3), however a method to prevent muscle movement would be needed. There are many antagonists for postsynaptic glutamate receptors, but most are not particularly effective with the exception of aga-toxins extracted from the venom from the

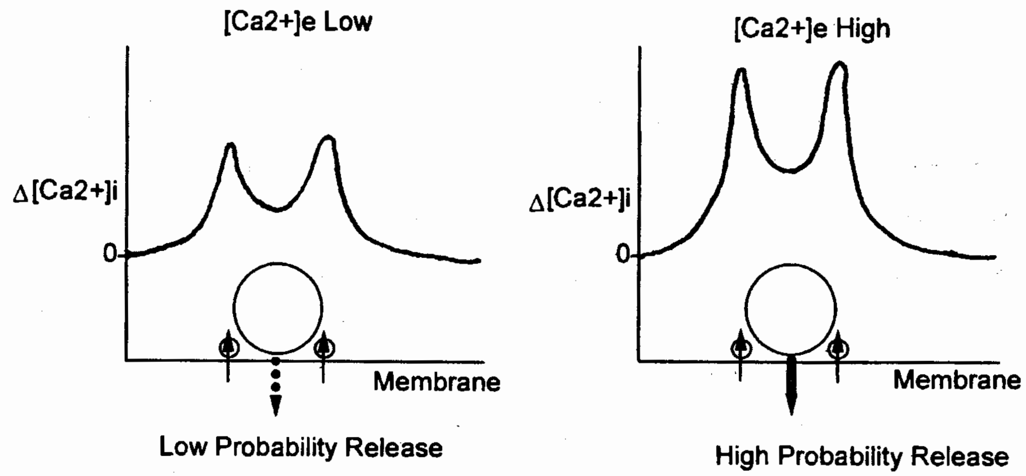
spiders *Pamphobetus platyomma and P. soracabae* (Araque et al., 1992). This venom was demonstrated to exert an effect on the muscle with no secondary presynaptic effects.

The crayfish NMJ permits experiments in which both pre- and postsynaptic activity can be observed. By reducing calcium entry into presynaptic terminal boutons with presynaptic inhibition, I observed a non-linear relationship between the decrease in presynaptic calcium entry and the reduction of the postsynaptic EJP. From this result I conclude that the crayfish NMJ utilizes overlapping calcium micro-domains to elicit neurotransmitter release. Therefore, a moderate decrease in calcium entry with presynaptic inhibition will result in a larger reduction in neurotransmitter release.

Figure 15 Schematic for the co-operative nature between calcium entry and transmitter release.

(A), shows how experiments that modulate external calcium show a non-linear relationship between calcium entry and neurotransmitter release, indicative of the number of calcium ions that bind to the trigger molecule (4 or 5). Calcium enters through activated calcium channels (small circles), forming calcium micro-domains (shown on graph as $\Delta[Ca^{2+}]_i$), causing vesicle release (large circle), with some probability. The probability of release is increased by increasing the influx of calcium through activated channels by raising the concentration of external calcium. This shows that release is proportional to $\Delta[Ca^{2+}]_i$ to the fourth or fifth power. (B) shows a synapse that only requires the activation of one calcium channel to trigger the release of one vesicle. There is a high probability of secreting the vesicle 'β' from calcium influx through one calcium channel (solid arrow) forming the calcium micro-domain indicated (solid line). Increasing the stimulus, causing the activation of more calcium channels (dotted arrow), will form calcium micro-domains (dotted line), likely causing the release of all three vesicles: α, β, and γ. Changing the number of calcium channels activated at this synapse, will result in a linear relationship between $\Delta[Ca^{2+}]_i$ and transmitter release. (C) shows a synapse that requires overlapping calcium micro-domains to elicit neurotransmitter release. With a normal stimulus (solid lines), there is a high probability of releasing vesicle β, a low probability of releasing α, and a very low probability of releasing γ. Increasing the stimulus results in activating more calcium channels, resulting in greater $[Ca^{2+}]_i$ (dotted lines). This will lead to an increase in the probability of release of all of the vesicles in a non-linear manner. Figure adapted from Zucker, 1993.

A



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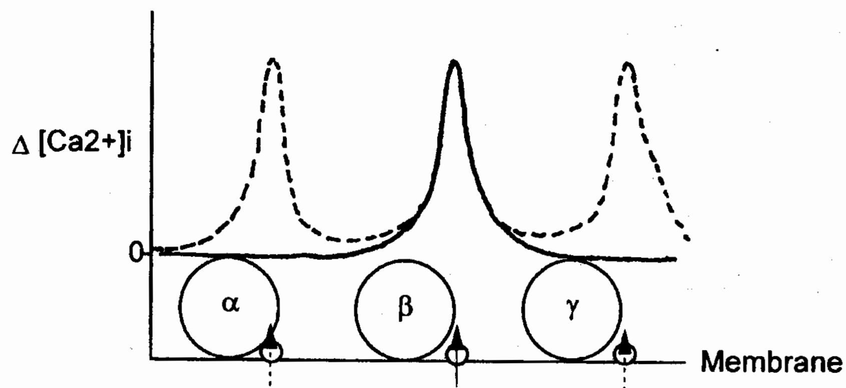

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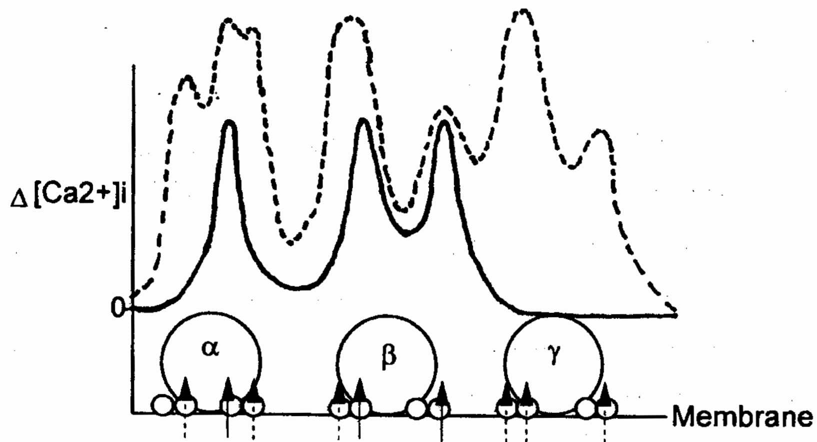

Chapter 5:
Anatomical Effects

Introduction

It is possible for the amount of inhibition exerted at a given terminal to be dependant on the morphology of the excitor and inhibitor axons and the physical relationship between their terminals (Atwood and Wojtowicz, 1986). As described previously in chapter 4, in a large number of terminal boutons (122), over many locations (18), variation was observed in the amount of inhibition of calcium entry. Measurements were made to see if the morphological relationship between the excitor and inhibitor axons affected the amount of inhibition experienced by a terminal bouton.

The majority of axo-axonal synapses are found on terminal boutons (Atwood et al., 1984), but some occur at constricted regions of the excitor axon, termed bottlenecks (Atwood and Morin, 1970; Jahromi and Atwood, 1974). It has been proposed that presynaptic inhibition at these vulnerable regions could effectively block the propagation of an action potential to entire branches of terminals of the excitor axon (Dudel and Kuffler, 1961; Dudel, 1962; Florey and Cahill, 1982; Atwood and Tse, 1988), and this phenomenon has been modeled (Atwood et al., 1984; Segev, 1990). In practice branch point failure is rarely seen physiologically, but as most experiments have been conducted at low frequencies to avoid movement, it has been suggested that branch point failure may occur at high frequencies during prolonged periods of stimulation (Govind et al., 1995). I conducted my experiment at high, physiologically relevant frequencies and evaluated the extent to which specific geometry of axonal branching influenced the efficacy of presynaptic inhibition at different excitor axon terminal boutons.

Results

Anatomical effects on presynaptic inhibition

Images of the excitor and inhibitor axons were overlaid in order to analyze for effects of anatomy on inhibition. Complex morphologies did reveal variation in the amount of inhibition experienced by a terminal bouton (Figure 16). Trends of increasing inhibition along long branches of terminal boutons were not observed (Figure 17). I found that significantly more inhibition occurred at terminal boutons on secondary branches (24 ± 2 %, n = 36 terminals), versus those on the main varicose structure (18 ± 1 %, n = 86 terminals, two sample t-test, $P < 0.05$, Figure 18). The distance from an excitor bouton to an inhibitor bouton was compared to the amount of inhibition experienced and no correlation was observed ($r^2 = 0.001$, n = 95 terminals, Figure 19a). Similarly, the distance of the excitor bouton from the nearest main branch was compared to the amount of inhibition observed and no correlation was found ($r^2 = 0.04$, n = 92 terminals, Figure 19b). Failure of propagation of the action potential did not occur because I always observed calcium entry into all terminal boutons measured, even at the end of long, 150 µm, branches.

A limitation on this analysis is the unknown locations of axo-axonal synapses. Others have observed that excitor and inhibitor terminal boutons were sometimes closely associated, without any axo-axonal synapses, while sometimes excitor terminals received numerous axo-axonal synapses from several different converging inhibitor terminals (Atwood et al., 1984).

Increased inhibition

The possibility that the inhibitor was not fully facilitated, with the 8 stimuli used for the imaging and electrophysiological experiments, was tested by delivering 24 inhibitor stimuli and measuring the pre- and postsynaptic response. Presynaptically, significantly greater reduction of calcium influx was observed with increased inhibition (paired t-test, $P < 0.05$, n =14 terminals from 2 preparations). Inhibition delivered in the standard manner ranged from 8 to

26 %, while increased stimulation of the inhibitor caused a 13 to 35 % reduction in calcium influx (Figure 20). Although there was greater inhibition of calcium entry with increased inhibitor stimulation, branch point failure was not observed. Even with maximum inhibition, the action potential was still able to propagate to distal terminals.

Postsynaptically, 10 % greater inhibition was observed for the second and third EJPs (paired t-test, $P < 0.05$, 10 cells from 3 preparations, Figure 21). It is interesting to note that proportionally more inhibition was observed with each successive EJP (Table 2), as was the case with the standard protocol (3 excitor stimuli and 8 inhibitor stimuli). It does appear that the inhibitor was not fully facilitated, and that increasing the number of inhibitor stimuli increased inhibition, but did not induce branch point failure.

Table 2 Percent inhibition of postsynaptic EJP with standard and increased inhibition (mean ± SEM, n = 10 cells).

Experiment	First EJP	Second EJP	Third EJP
Standard Protocol (E + 8 I APs)	44 ± 7 %	57 ± 5 %	62 ± 3 %
Increased Inhibition (E + 24 I APs)	57 ± 6 %	65 ± 4 % *	70 ± 3 % *

* Significant, t-test, $P < 0.05$.

Application of GABA

Bath application of GABA produced results which differed from the standard electrical stimulation of inhibition via the axon. Experiments were performed by stimulating the excitor 100 times at 5 Hz with and without GABA and recording the fluorescent transient. The amount of inhibition produced was measured by comparing the reduction in the calcium transient after it had reached a steady state. When GABA was applied in a moderate concentration, 30 μM, it was common to observe increasing inhibition along a branch (Figure 22). In these cases, proximal terminal boutons experienced no inhibition, while

100 % inhibition was observed in terminal boutons at the end of a branch. Increasing the concentration of GABA increased the amount of inhibition experienced at a given terminal bouton (Table 3). Effects of GABA were reversible with perfusion of the preparation with normal saline.

Table 3 Increasing GABA concentrations increases inhibition and affects distal terminal boutons more than proximal terminal boutons. Terminals proximal to distal (Figure 22). Percent inhibition of calcium transient shown.

[GABA] μM	T1	T2	T3	T4	T5	T6	T7	T8	T9
10	0	0	0	0	0	0	12	18	30
20	0	0	0	0	21	17	46	20	49
30	0	0	0	19	22	71	70	45	77
40	100	100	100	100	100	100	100	100	100

At high concentrations of GABA (40 to 50 μM), complete block of the action potential occurred when measured from the excitor axon distal to the main 'Y' branch. The action potential could be restored within a few minutes by rinsing with normal saline.

Most GABA experiments were conducted using long, low frequency trains of stimulation to produce a large transient that did not require averaging to accurately measure. This differed from other experiments which used short, high frequency, trains for stimulation. Thus, an experiment was performed with both techniques to allow comparison. In this experiment, loss of conduction of the action potential was not observed in the region tested and inhibition with GABA was comparable between the following conditions tested: standard electrical stimulation of the inhibitor axon, short trains of excitor stimuli (3 @ 50 Hz) with GABA, or long trains of excitor stimuli (100 @ 5 Hz) with GABA (Table 4).

Table 4 Comparison of various methods of inhibition. Terminals proximal to distal (Figure 20). Percent inhibition shown.

Experiment	T7	T6	T5	T4	T3	T2	T1
Standard Electrical Stimulation	9	18	17	21	22	21	18
Short train with GABA (30uM)	44	21	33	12	23	44	35
Long train with GABA (30uM)	33	58	33	48	34	44	32

Figure 16 Presynaptic inhibition varies at different excitor motor terminal boutons.

Shows the complex anatomical relationship between excitor (green) and inhibitor axons (red) and variation in the amount of inhibition occurring at a given terminal bouton. The calcium transient, thus calcium entry, was reduced from 0 to 40 % at different terminals with inhibition. Fluorescent transients, measured from the excitor axon using Calcium Green-1, are depicted next to the terminals that they represent (arrow). Scale bars represent a 2 % $\Delta F/F$. The green trace resulted from stimulating the excitor alone while the red trace resulted from stimulating the excitor and inhibitor together (see Figure 4b). There were no obvious trends of increasing inhibition along the axon which would have indicated branch point failure. The action potential was conducted from the right to the left of the image.

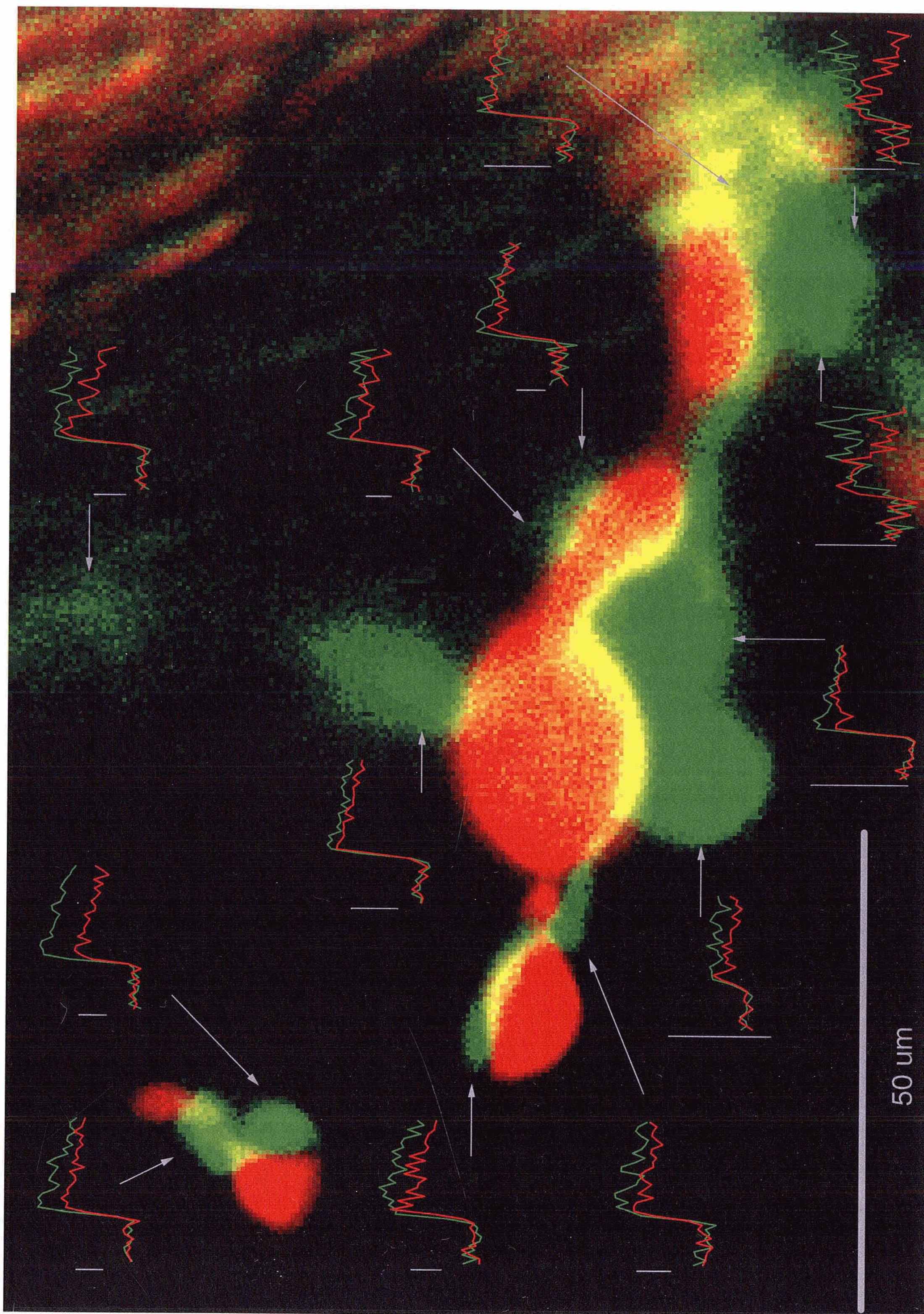
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Figure 17 Effect of presynaptic inhibition on a long branch of terminal boutons.

Shows that greater inhibition is not observed along a string of terminal boutons (excitor green, inhibitor red). No trend of increasing inhibition was observed in more distal terminals which would have been expected if the action potential was failing or inhibition was accumulating. It would be predicted that during inhibition, the action potential (or substantial depolarization) was reaching all of the terminals. Fluorescent transients, measured from the excitor axon using Calcium Green-1, are depicted next to the terminals that they represent (arrow). Scale bars represent a 2 % $\Delta F/F$. The green trace resulted from stimulating the excitor alone while the red trace resulted from stimulating the excitor and inhibitor together (see Figure 4b). The action potential was conducted from the top right to the left.

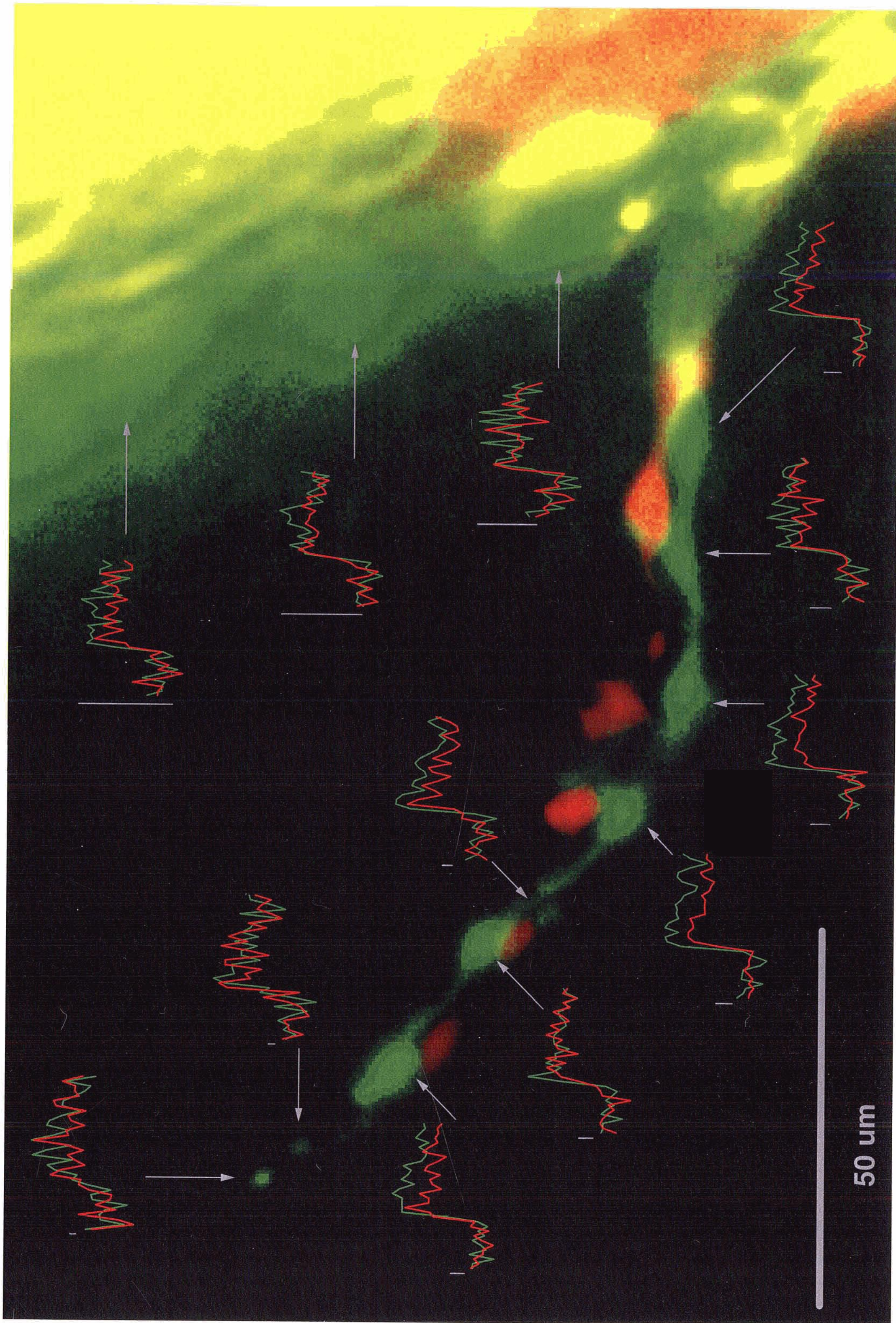

50 um

Figure 18 Effect of presynaptic inhibition on terminal boutons on secondary branches.

Shows increased inhibition at a terminal bouton on a secondary branch (see inset) from the main varicose structure (excitor green, inhibitor red). Inhibition at the six terminals on the main branch ranged from 12 to 28 %, while the terminal on the secondary branch experienced approximately 40 % inhibition. Inhibitor terminals are observed in the vicinity of the bottleneck structure forming the branch. Fluorescent transients, measured from the excitor axon using Calcium Green-1, are depicted next to the terminals that they represent (arrow). Scale bars represent a 2 % $\Delta F/F$. The green trace resulted from stimulating the excitor alone while the red trace resulted from stimulating the excitor and inhibitor together (see Figure 4b). The action potential was conducted from the bottom right to the left. The inset indicates superficial terminal boutons imaged.

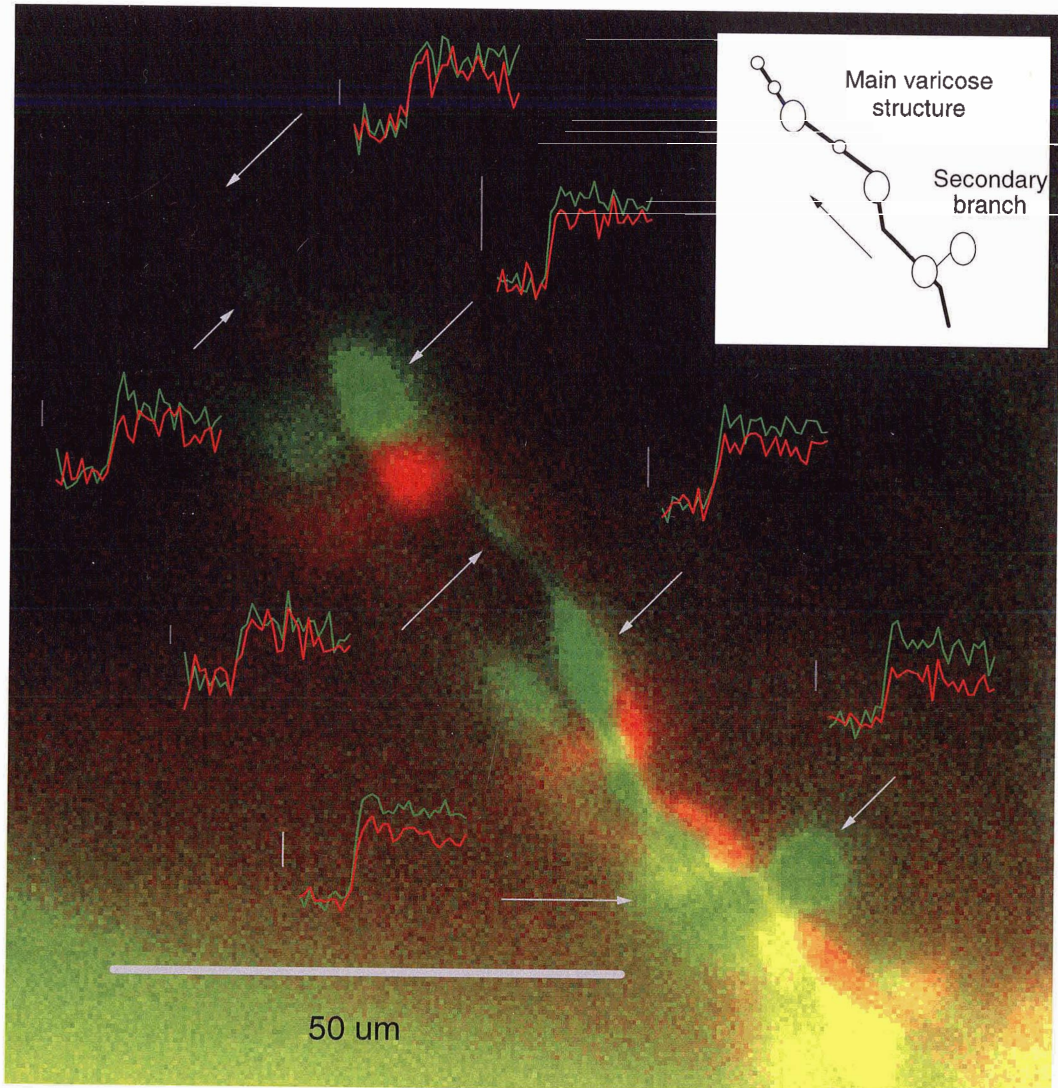
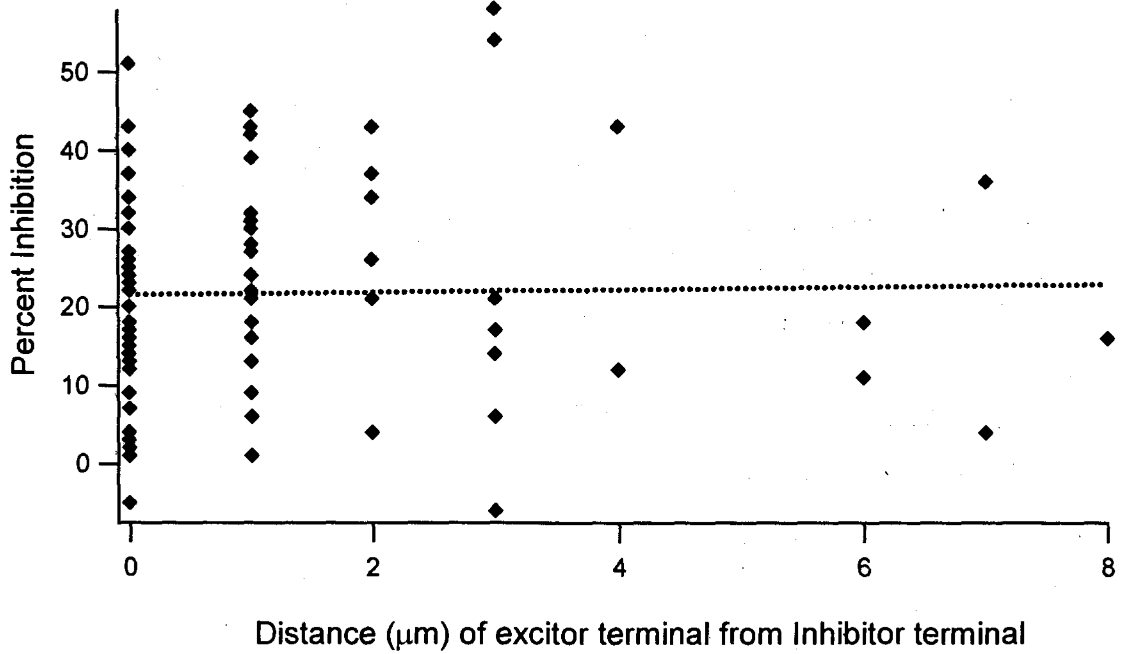

Main varicose structure
Secondary branch
50 um

Figure 19 Anatomical analysis of presynaptic inhibition

Detailed anatomical analysis revealed no correlation between the distance from an excitor terminal bouton to the nearest inhibitor terminal bouton and the amount of inhibition experienced by the excitor bouton (A). Nor was a correlation between the distance of an excitor bouton from the main branch and inhibition (B), found. Increasing inhibition with distance would have been expected if the action potential was reduced to a passive depolarization by branch point failure.

A



B

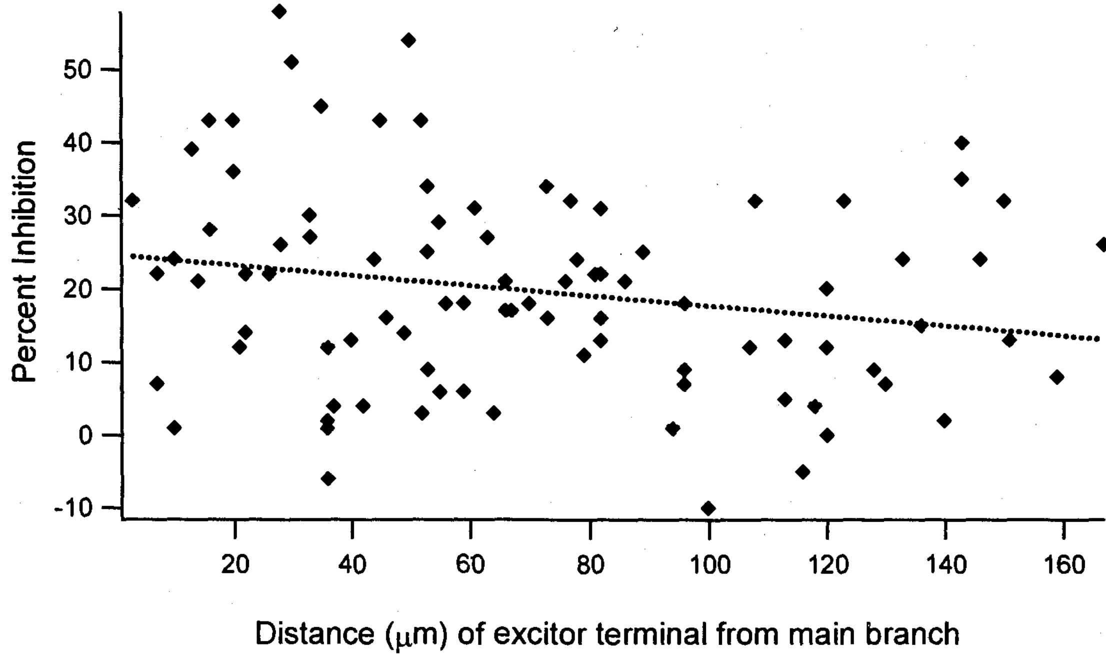


Figure 20 Effect of increased inhibition on the presynaptic calcium transient.

Shows that increased stimulation of the inhibitor axon increases the inhibition of calcium entry (excitor green, inhibitor red). With the standard stimulation protocol, inhibition ranged from 8 to 26 %, but with increased inhibitor stimulation, inhibition ranged from 13 to 35 %. This difference was significant (paired t-test, $P < 0.01$, $n = 14$ terminals from 2 preparations). Importantly, branch point failure was not observed for experiments with increased inhibitor stimulation. Fluorescent transients, measured from the excitor axon using Calcium Green-1, are depicted next to the terminal boutons that they represent (arrow). The green trace resulted from stimulating the excitor alone, while the red trace resulted from stimulating the excitor and inhibitor together. For each terminal bouton, the set of traces on the left represent standard inhibition, 8 inhibitor stimuli at 100 Hz, while the traces on the right represent increased inhibition, 24 inhibitor stimuli at 100 Hz (see Figure 4b). The action potential was conducted from the bottom right to the top left.

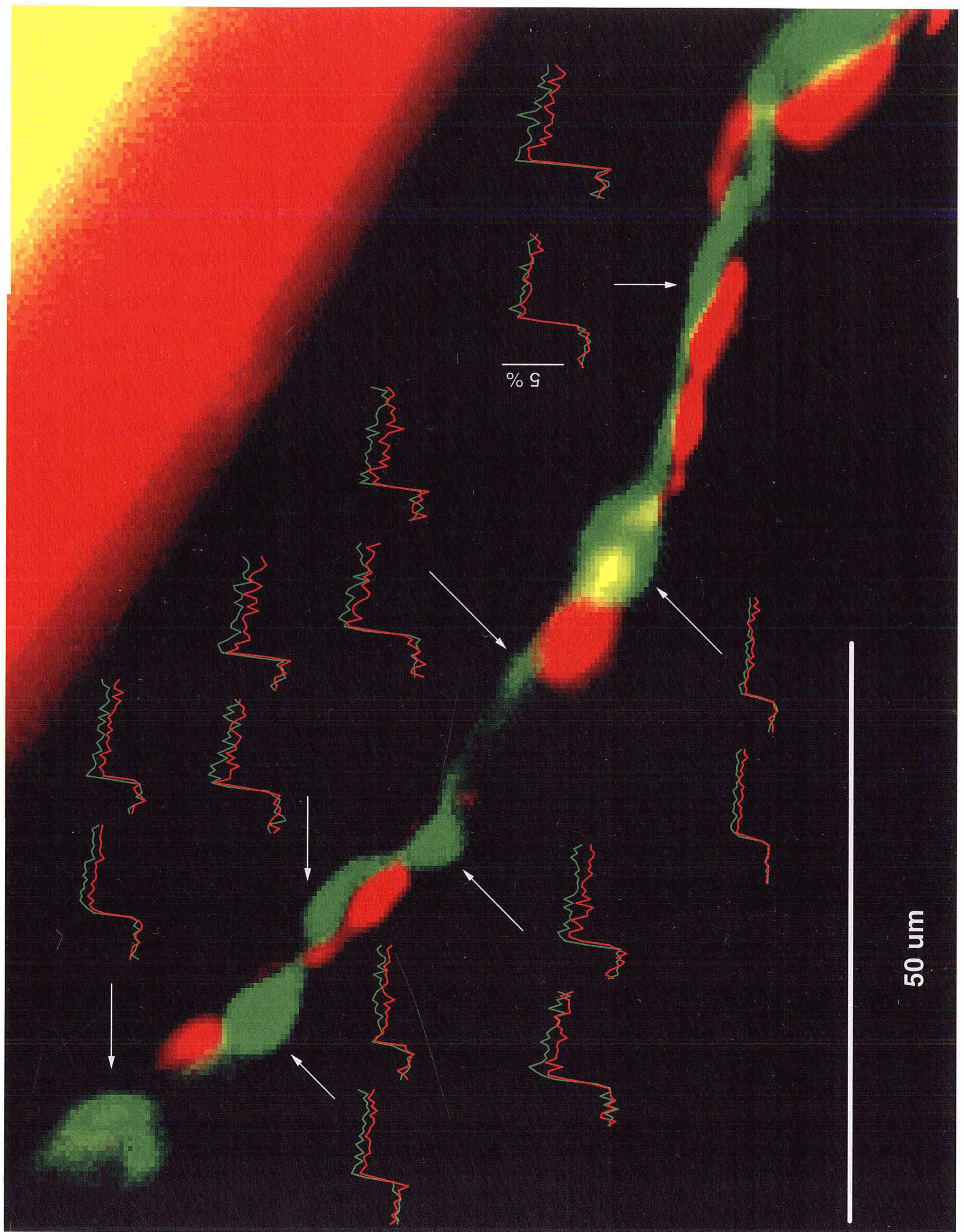

5 %
50 um

Figure 21 Effect of increased inhibition on the EJP.

Shows that increased stimulation of the inhibitor axon resulted in greater inhibition (approximately 10 %) of the second and third EJP (paired t-test, $P < 0.05$, $n = 10$ cells from 3 preparations, see table 2). Black represents the amount of inhibition with standard inhibition and red with increased inhibition (3 excitor stimuli at 50 Hz with 24 inhibitor stimuli at 100 Hz). Greater inhibition was observed for each successive EJP even with the long train of inhibitor stimuli.

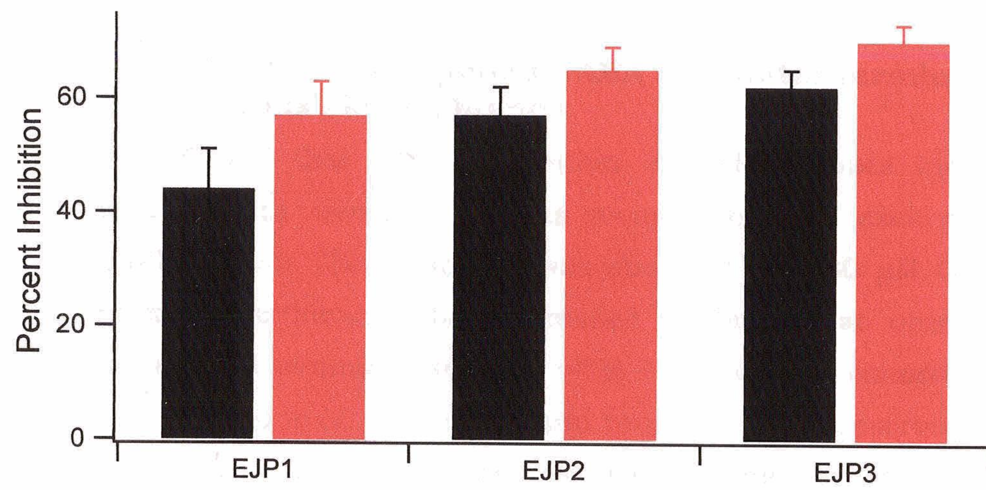
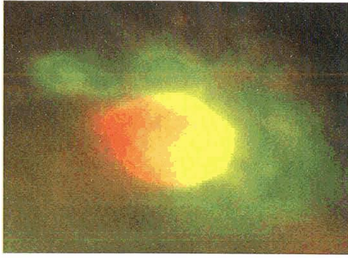
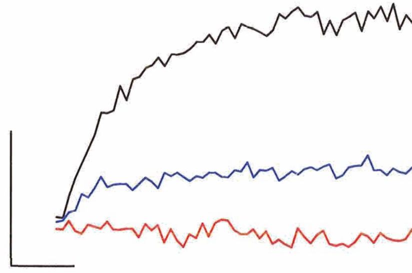
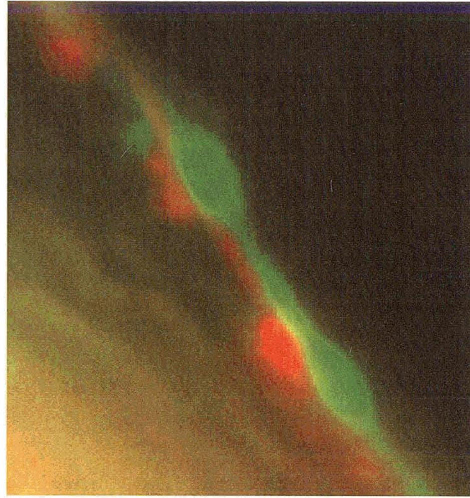

Percent Inhibition
60
40
20
0
EJP1
EJP2
EJP3

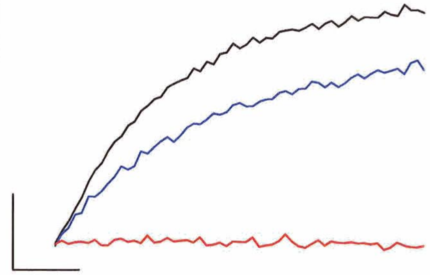
Figure 22 Effect of bath applied GABA on excitor terminal boutons along a branch.

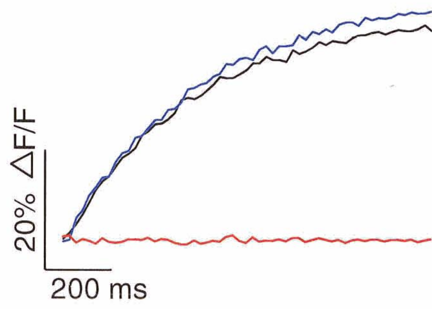
Shows that bath application of GABA causes increasing inhibition in terminal boutons along a branch (excitor green, inhibitor red). No inhibition was observed with 30 µM GABA at proximal terminals, while increased inhibition was observed at more distal terminals (see also table 3). With high concentrations of GABA, 40 to 50 µM, no calcium transients were observed in any terminals. Activity was restored with rinsing. Traces represent fluorescent transients from terminals indicated (arrow), which resulted from stimulation of the excitor axon (5 Hz; black, normal ringer; blue, 30 µM GABA; red, 50 µM GABA). The action potential was conducted from the bottom right to the top left.

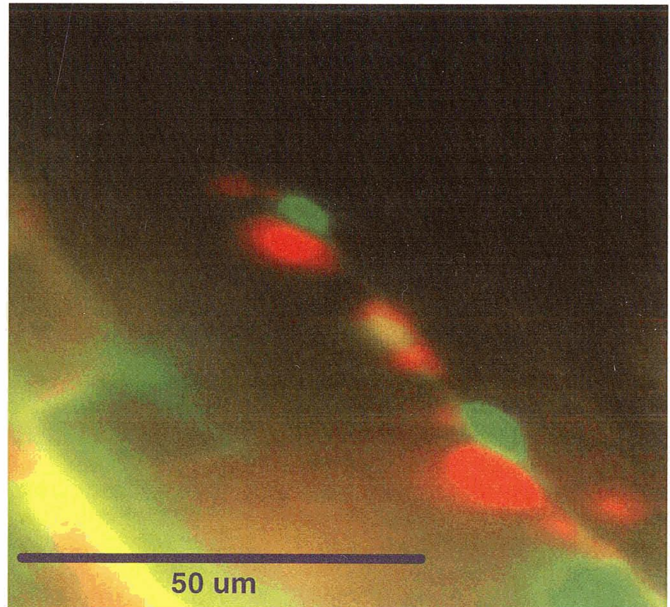









20% ΔF/F
200 ms


50 um

Discussion

Consistent with a model in which release but not facilitation is reduced at all or most terminal boutons, I did not observe complete failure of the action potential evoked calcium influx at either distal versus proximal synaptic terminal boutons or on small side branches with co-activation of the inhibitor axon. I did observe slightly more inhibition of calcium influx at terminal boutons on secondary bottlenecks versus those on the main varicose structure, which agrees with the conceptual model proposed by Atwood et al. (1984). They predicted that the efficacy of presynaptic inhibition would be greatly enhanced by bottlenecks, with smaller diameter constrictions exerting a greater effect. The terminals are arranged in a varicose structure connected by bottlenecks less than one micron in diameter. On the primary terminal boutons there are often secondary terminal boutons, which are connected by bottlenecks with an even smaller diameter. This morphological feature presents a condition which may be particularly vulnerable to electrical shunting from inhibition because it has a low safety factor for propagation of the action potential. Thus, the action potential may be more severely shunted at this location resulting in enhanced inhibition. However, the fact that terminal boutons, distal to a site where strong inhibition of calcium influx was observed, could show little inhibition, indicates that the action potential is generally capable of recovering its amplitude between sites of inhibition.

In general, anatomical analysis of my results supports a model where the action potential propagates actively with a reasonable safety factor, and presynaptic inhibition causes local shunting of the action potential, which then recovers outside the region of the shunt. I propose that the action potential is propagated actively. Others have proposed that the action potential propagates passively in this system (Dudel, 1965), however Zucker (1974) showed that it was possible to stimulate action potentials directly from terminals indicating active propagation. The most extreme example of a varicose axonal arbor is found in mammalian, unmyelinated, CA3 pyramidal cells, which are among the thinnest axons in the nervous system with minimum diameters of 170 nm

(Shepherd and Harris, 1998). A recent study has shown that the action potential propagates faithfully in this system with frequencies of up to 50 Hz (Raastad and Shepherd, 2003). Thus, the varicose nature of the axons in the crayfish NMJ should not necessarily prevent active propagation of the action potential.

It is likely that presynaptic inhibition probably exerts its effect at specific terminals rather than on entire branches, because axo-axonal synapses are more commonly found on varicosities than on bottlenecks (Atwood et al., 1984). This is contrary to the result observed by Dudel (1965) indicating that presynaptic inhibition sometimes works by blocking the action potential at a branch. However, perhaps this is not the typical situation. If facilitation is important to this synapse, which was demonstrated with the force transduction experiments (Chapter 3) then the majority of synapses must receive a moderately reduced action potential during presynaptic inhibition, in order to maintain facilitation. Terminal boutons must be sufficiently depolarized for intracellular calcium to remain at an elevated level, thus allowing the expression of facilitation after inhibition (Atwood and Wojtowicz, 1986). If presynaptic inhibition worked by electrically isolating entire branches of terminals, then facilitation would be strongly affected. Therefore, I propose that for normal operation of this system facilitation is necessary, and that in order for facilitation to be maintained during presynaptic inhibition, a moderately reduced depolarization must reach most of the terminals.

An interesting result of bath application of GABA was the observation of increasing inhibition along a branch: a phenomenon not observed when presynaptic inhibition was delivered with electrical stimulation of the inhibitor axon. I propose that, with bath application of GABA, the action potential is shunted to the point where it can no longer actively regenerate, and thus propagates in a passive manner down the rest of the branch. In fact, I observed that inhibition accumulated along a branch until no transient was observed at all. Due to the non-linear voltage dependence of calcium channels (Llinas et al., 1981), it is not long before calcium entry is no longer observed. Increased inhibition from bath application of GABA could also be produced if $GABA_B$

receptors were activated. Indeed, $GABA_B$ receptors have been pharmacologically demonstrated at this synapse (Blundon and Bittner, 1992; Fischer and Parnas, 1996 a, b; Parnas et al., 1999). Further experiments are needed to explore this issue.

With a high enough concentration of GABA (40 to 50 μM), complete loss of conduction of the action potential in the excitor axon, measured intracellularly from the secondary branch distal to the main Y branch, was observed. This may point to the existence of extra-synaptic GABA receptors. Blundon and Bittner (1992) demonstrated the specific $GABA_B$ agonist, baclofen, depressed the amplitude of action potentials in the excitor axon. This may indicate that $GABA_B$ receptors are located on the excitor axon and would explain the loss of conduction of the action potential in the axon (recorded distal to the main Y branch) with high concentrations of GABA that I observed. Further study with either antibody labeling or local puffing of GABA would be useful. Another explanation for this phenomenon could be that the shunt produced from excessive concentrations of GABA at axo-axonal synapses was so great that it affected the main branch of the axon. The electrical effect of a shunt produced from an axo-axonal synapse affects a region of membrane around the synapse. Thus a large enough shunting current could knock out an action potential some distance proximal to the synapse.

Chapter 6: General Discussion and Conclusions

Presynaptic inhibition of excitatory transmission occurs in both vertebrates (Eccles, 1964; Nicoll and Alger, 1979) and invertebrates (Dudel and Kuffler, 1961). This study examined how presynaptic inhibition may function specifically at the claw opener neuromuscular junction of the crayfish, and provides a model for how it could interact with frequency dependent facilitation at other synapses.

I found that inhibitor axon activity could effectively block contraction of the opener muscle induced by stimulation of the excitor axon. The effectiveness of the inhibition was dependent upon the time interval between the occurrence of inhibitor and excitor action potentials, as previously described by Dudel and Kuffler, (1961). This is consistent with a predominantly presynaptic mode of action, where a brief shunting chloride current reduces the amplitude of the action potential, resulting in reduced neurotransmitter release. Although the muscle was relaxed when the inhibitor was co-activated with the excitor, the time to onset of contraction was shortened if the excitor was fired after a period of excitor and inhibitor co-activation, as predicted by Atwood and Wojtowicz (1986). This result indicates that facilitation of release from excitor motor terminals is maintained during presynaptic inhibition. This is important for the crayfish because it allows 'priming' of motor terminals, where facilitation can be built up but not expressed until needed. Because a build up of facilitation is necessary to elicit muscle contraction in this system (Figures 7 and 14), a mechanism which allows facilitation to build in anticipation of muscle contraction, would be functionally beneficial to the crayfish.

With co-activation of the inhibitor, I measured a moderate, 20 %, reduction in calcium entry into presynaptic excitor axon terminal boutons associated with a greater, 51 %, reduction in the amplitude of the postsynaptic

EJP. This is consistent with a relationship between calcium entry and transmitter release proportional to the third power. Presynaptic inhibition most likely reduces calcium entry into excitor motor terminals by decreasing the amplitude of the action potential, thus activating fewer voltage-gated calcium channels. A non-linear relationship between a change in presynaptic calcium entry and neurotransmitter release, resulting from reducing the number of activated calcium channels, indicates a requirement for overlapping calcium micro-domains to elicit neurotransmitter release (Zucker, 1999). This result corroborates anatomical data showing 10 to 20 putative calcium channels per active zone (Cooper et al., 1996; Atwood et al., 1997). Thus, this system only requires a small reduction in calcium entry to have a large effect on neurotransmitter release.

Imaging the effect of presynaptic inhibition on calcium entry into branches of excitor motor terminal boutons provided information on the fundamental mechanism of presynaptic inhibition for the crayfish opener muscle. Although the amount of inhibition at individual terminal boutons varied, trends of increasing inhibition along a branch were not observed. Greater inhibition was observed at terminal boutons on secondary branches, as would be predicted by anatomical models (Atwood et al., 1984), but complete blockage of the action potential was never observed. Furthermore, increased stimulation of the inhibitor axon did result in greater inhibition, observed both pre- and postsynaptically, but the action potential was still observed to propagate actively. Therefore, I propose that the action potential propagates actively in the excitor motor axon and that presynaptic inhibition reduces the amplitude of the action potential at specific terminals. The action potential would then recover between the electrically shunted regions, resulting from inhibitory axo-axonal synapse activity. This hypothesis is supported by anatomical data where inhibitory axo-axonal synapses are typically found on the excitor axon a few microns proximal to sites of excitatory transmitter release (Atwood and Morin, 1970; Jahromi and Atwood, 1974).

The buildup of facilitation is essential to elicit contraction of the opener muscle in the crayfish NMJ. Because it was observed that the majority of

facilitation was maintained during inhibition, an actively propagated action potential must reach the majority of the terminals. If presynaptic inhibition worked by blocking the action potential at branch points or constrictions, electrically isolating entire branches of the motor axon, facilitation would be greatly reduced. Calcium is necessary to produce facilitation, as evidenced by the fact that facilitation is reduced by toxins that close calcium channels (Zengel et al., 1993), and by intracellular injection of calcium buffers (Dittman and Regehr, 1996; Delaney et al., 1991). Therefore, to reduce facilitation while reducing release, presynaptic inhibition should reduce calcium influx by a moderate amount at all the synapses on a muscle fiber, rather than reduce it a small amount at some synapses and eliminate it entirely at others. An actively propagated action potential, reduced at each terminal with presynaptic inhibition, would result in moderately reduced calcium entry into excitor motor terminals.

I propose the following model for the rapid transition from relaxed to contracted muscle observed following a period of inhibitor and excitor axon co-activation. Presynaptic inhibition shunts the action potential in the excitor axon through the opening of a $GABA_A$ mediated chloride conductance (Dudel and Kuffler, 1961), reducing the amplitude of the action potential by 10 to 20 mV near the site of transmitter release (Baxter and Bittner, 1981). This reduces voltage gated calcium channel opening probability and thus calcium influx. The third power relationship between calcium influx and neurotransmitter release results in severely attenuating the EJP. However, since calcium influx is only reduced an average of 20 %, short-term facilitation develops and is maintained in the excitor axon terminals during presynaptic inhibition (Baxter and Bittner, 1981; Magleby and Zengel, 1982). As a result, within a few milliseconds of cessation of inhibitor axon firing, inhibition disappears while facilitation that has accumulated is expressed. The resulting facilitated EJP immediately increases to an amplitude sufficient to initiate contraction. This phenomena could be behaviorally significant for motor control in the crayfish because it increases the kinetics of contraction.

Inhibiting excitor terminal boutons with bath application of GABA induced increasing inhibition along a branch until the action potential could no longer propagate actively and decayed to the point where no transient was observed in distal terminals. This result appears to fit well with modeling experiments which predict that the action potential would be reduced to a passive form where inhibitory axo-axonal synapses form at branches and constrictions of the excitor axon (Atwood et al., 1984). However, experimental data with inhibition induced through stimulation of the inhibitor axon did not produce this result. This indicates that bath application of GABA affects the excitor motor axon in a different way than presynaptic inhibition, most likely resulting from activation of $GABA_B$ receptors (Fischer and Parnas, 1996 a, b; Parnas et al., 1999). Activation of $GABA_B$ receptors may explain the delay in the EJP amplitude reaching control levels after a period of co-activation of the excitor and inhibitor axons (Chapter 3). The fact that a differential response was observed with bath application of GABA versus standard presynaptic inhibition demonstrates the profound spatial and temporal specificity of presynaptic inhibition at the crayfish NMJ.

In conclusion, this study demonstrated fundamental principles of presynaptic inhibition using the crayfish NMJ as a model. Firstly, it demonstrated the functional consequence of the maintenance of facilitation in excitor motor terminals during presynaptic inhibition. This mechanism, essential to the crayfish, may also be useful for other synapses that utilize facilitation and presynaptic inhibition. Secondly, this study demonstrated that presynaptic inhibition, in normal operation, exerts its effect at individual terminals rather than by electrically isolating entire regions of the motor axon. Finally, this study added evidence that the crayfish NMJ relies on overlapping calcium micro-domains to induce neurotransmitter release. This mechanism is essential in permitting facilitation to be maintained during presynaptic inhibition because it results in a non-linear relationship between calcium entry and neurotransmitter release. The specific nature of presynaptic inhibition and its interaction with facilitation provides the crayfish with a highly versatile neuromuscular system with a limited number of neurons.

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