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Interaction between facilitation and presynaptic inhibition at the crayfish neuromuscular junction

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Summary

Action potential-mediated calcium (Ca) entry into excitor nerve terminal boutons during presynaptic inhibition and the effects of co-activation of the inhibitor on the kinetics of muscle contraction were studied at crayfish claw opener muscle. Inhibition reduced postsynaptic excitatory junction potentials (EJPs) below the threshold to initiate contraction. Upon cessation of inhibition, EJP amplitudes immediately increased several-fold due to the build-up of presynaptic facilitation during inhibition. Consequently, muscle contraction was initiated more rapidly after a period of inhibitor–excitor coactivation.

Presynaptic inhibition reduced Ca entry into presynaptic excitor terminal boutons (range 0–50%, mean ± S.E.M.=20±1%, *N*=122 terminals; 12 preparations) and reduced the EJP amplitude (range 30–70%, mean ± S.E.M.=51±2%, *N*=27 cells). The decline in the EJP was proportional to the reduction of Ca influx raised to the power of 2.8. Since presynaptic inhibition reduces the number of Ca channels opened by an action potential, our data suggest cooperativity between Ca channel microdomains to initiate vesicle fusion at this synapse.

The amount of inhibition of Ca influx into an excitor bouton was not correlated with either the distance to the closest inhibitor bouton or the main excitor branch, although slightly more inhibition was seen for excitor boutons on tertiary *versus* secondary branches. Unlike inhibitor axon stimulation, bath application of GABA caused inhibition of Ca influx that steadily increased from proximal to distal terminal boutons on a branch. We propose a model where presynaptic inhibition causes localized shunting of an actively propagated action potential in the vicinity of release sites, which can recover its amplitude outside the shunted region.

Key words: calcium imaging, contraction, GABA, *Procambarus clarkii*.

Introduction

The crayfish claw opener (abductor) muscle is innervated by a glutamate-releasing, excitatory axon and a GABA (γ-aminobutyric acid)-releasing, inhibitory axon (Van Harreveld, 1939) (Fig. 1). There is also an inhibitory neuron common to all muscles in the limb that innervates the first 3–10 proximal fibres of the opener muscle (Wiens, 1985) and was not investigated in this study. The claw opener muscle fibres 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) (Bittner, 1968; Orkand, 1962). Unlike vertebrate skeletal neuromuscular junctions, 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 enhancement of neurotransmitter release that is essential for generating muscle contraction. The amount of enhancement produced is impressive (up to 100-fold with sustained repetitive high-frequency stimulation) and, depending on the frequency and duration of stimulation, can initiate several release-enhancing processes, including facilitation, augmentation, post-tetanic potentiation and long-term facilitation (Atwood, 1976). However, there is an inherent time delay to the initiation of contraction due to the need to stimulate enough action potentials to increase EJP amplitude sufficiently (Atwood, 1976).

GABA acts on the muscle and the excitor motor terminals through $GABA_A$-type receptors coupled to a shunting chloride conductance (Dudel and Kuffler, 1961). Most of the EJP reduction is mediated through presynaptic inhibition, with postsynaptic inhibition estimated to contribute 5–10% (Atwood and Bittner, 1971; Baxter and Bittner, 1980; Fatt and Katz, 1953). Dudel (1963) proposed a mechanism of presynaptic inhibition due to blocking of action potentials before they reached the terminals. However, this model was

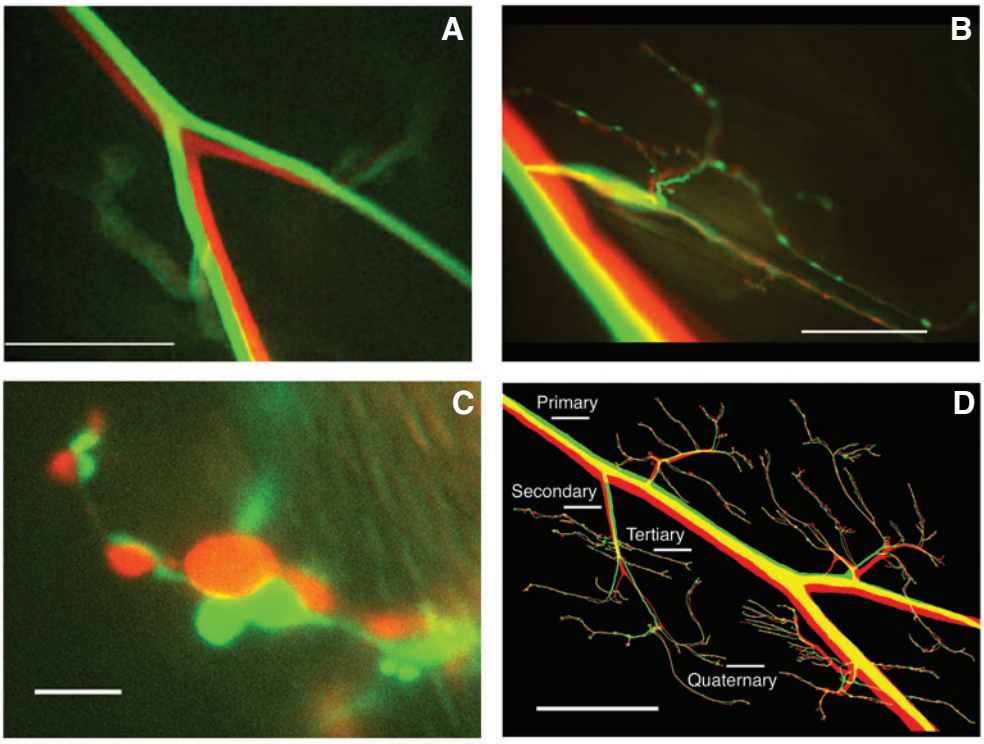


Fig. 1. Anatomical relationship between the excitor (Calcium Green-1) and inhibitor (Alexa 568; red) axons on the surface of the crayfish opener muscle. (A) The main Y branch of the excitor and inhibitor axons, showing how the axons project in parallel across the surface of the muscle. (B) The axons branch to form varicose terminal boutons. (C) The complex anatomical relationship between excitor and inhibitor terminals at the level of magnification used to image the Ca influx into excitor terminals. (D) Schematic of the anatomy of the excitor (green) and inhibitor (red) axons showing branching (overlap yellow). Scale bars: 200 μm (A,D), 50 μm (B) and 10 μm (C).

not generally supported by subsequent electrophysiological studies (Atwood and Bittner, 1971).

In the present study, we investigated the interaction between activity-dependent short-term enhancement (primarily facilitation, not augmentation or post-tetanic potentiation) and presynaptic inhibition in the excitor axon. It has been shown that facilitation develops during co-activation of the excitor and inhibitor axons, is masked by presynaptic inhibition and is unmasked within a few ms of the offset of inhibitor activity provided the excitor continues to fire (Atwood and Bittner, 1971; Baxter and Bittner, 1980; Dudel and Kuffler, 1961). We found that although presynaptic inhibition can block muscle contraction during co-activation of the excitor and inhibitor axons, it does so without eliminating facilitation. The result of this preservation of facilitation is a shortened time to initiate contraction after the offset of inhibition. We have studied the mechanism for this differential effect on release and facilitation by measuring the action potential-mediated Ca influx into excitor terminals at proximal to distal locations along transmitter-releasing axon branches in the presence and absence of inhibitor axon activity. From the pattern of changes of Ca influx during inhibition, we conclude that, although action potential amplitudes are attenuated, their propagation is maintained into small distal branches during inhibition.

Materials and methods

Animals and preparation

Crayfish, *Procambarus clarkii* Girard, 6–8 cm in length, were obtained from Atchafalaya Biological Supply (Raceland, LA, USA) and housed at 18°C in tanks with flowing, filtered water. They were maintained on a 12 h:12 h light:dark cycle, provided with black plastic ABS tubing for housing, and fed carrots, commercial fish food and occasionally each other. Experiments were performed on the first walking leg of intermoult-stage animals removed at the joint between the basipodite and ischiopodite. The leg was placed in a 35 mm Sylgard™ lined plastic Petri dish dorsal side up (Fig. 2A). 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. The meropodite, top portion of the propodite and overlying closer muscles were removed to expose the excitor and inhibitor axons and opener muscle. Motor axons were sucked into fire-polished glass electrodes to achieve a loose fit over the cut end of the nerve bundles in the meropodite. Excitor and inhibitor axons were independently stimulated using isolated (NeuroData SIU90) constant current pulses (500 μs duration) controlled by a Master-8 pulse generator (AMPI, Jerusalem, Israel).

Preparations were bathed with modified Van Harreveld's solution (Van Harreveld, 1936), comprising (in mmol l^{-1}) 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 (model 51K/J; Fluke, Mississauga, ON, Canada) 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 to the bottom of a Plexiglas recording chamber using periphery

wax and cyanoacrylate glue, leaving the dactylopodite free to move vertically over a ledge (Fig. 2A). Force measurements were obtained using a force-displacement transducer (model FT03; Grass Instruments). The dactylopodite was attached to the force transducer with #9-0 surgical silk (Deknatel). The force transducer was mounted to a 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 (low level D.C. Amplifier, 7P122; Grass Instruments, West Warwick, RI, USA), 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, respectively) were recorded with sharp (10–15 MΩ) electrodes filled with 3 mol l^{-1} KCl. Muscle fibres selected for recording were primarily fibres termed 'intermediate' by Delaney et al. (1991). These are located distal and lateral to the tight midline proximal bundle, near the major Y branch of the motor axons, and have unfacilitated EJPs in the range of 0.2–1 mV. Postsynaptic potentials were recorded with a high-impedance head stage amplifier (IR283; Neurodata, Branford, CT, USA), amplified a total of 1000-fold, filtered between 0.05 Hz and 1 kHz and digitally sampled at 4 kHz.

The time to onset of contraction in response to 20 Hz stimulation of the excitor was measured under conditions where the excitor was stimulated following a period of quiescence and compared with a paired sample *t*-test to that seen after co-activating the excitor and inhibitor axons. For co-activation, the inhibitor axon was stimulated in bursts of four action potentials at a frequency of 100 Hz, and the excitor axons were stimulated at 20 Hz with the final inhibitor stimulus in the burst preceding each excitor stimulus by 2–3 ms. The excitor and inhibitor axons were co-activated for 1.25 s (25 excitor stimuli at 20 Hz). The inhibitor to excitor stimulus delay was adjusted to produce maximal inhibition and was determined at the beginning of each experiment. Five to 10 trials were averaged for each condition with each animal.

Enhancement (facilitation and augmentation) was calculated by dividing the amplitude of the second EJP by each successive EJP.

Calcium imaging

To measure the effect of inhibition on Ca influx, a different stimulation paradigm from that used for the muscle contraction experiments was employed to avoid muscle movement; three excitor stimuli were delivered at 50 Hz and compared with excitor stimuli delivered together with inhibitor activation (Fig. 3). Studies of presynaptic inhibition are typically not conducted at high stimulation rates to avoid muscle movement (Govind et al., 1995), but we wanted to study presynaptic inhibition at a more physiologically relevant frequency for the muscle so we used a short train of three stimuli. Ca influx was measured in terminal boutons located on intermediate fibres in the vicinity of the main Y branch of the axon.

The excitor axon was penetrated distal to the main Y branch

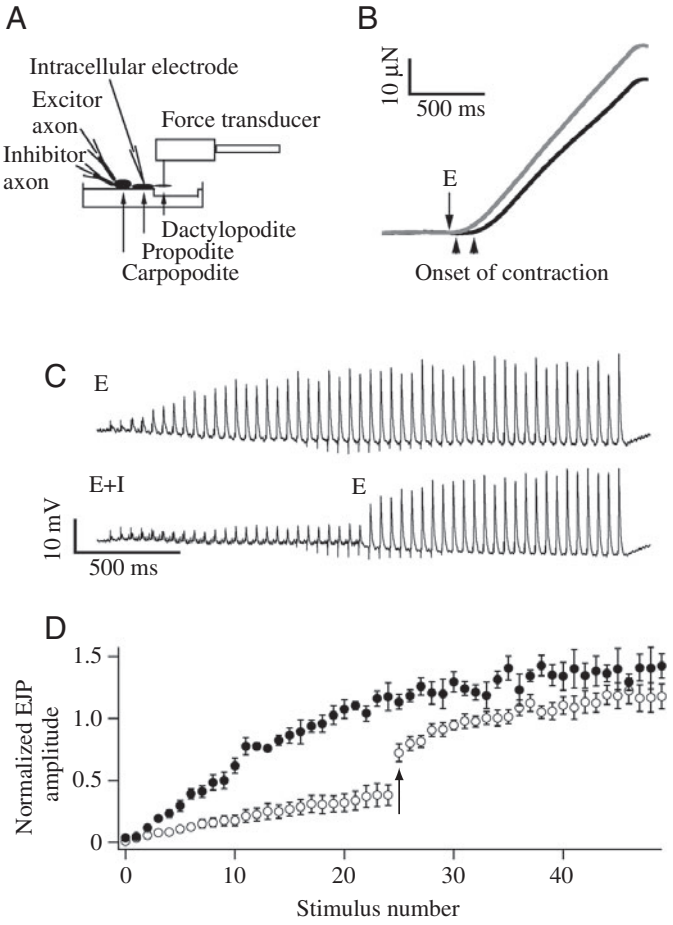


Fig. 2. Effect of previous co-activation of excitor and inhibitor axons on the time to onset of muscle contraction. (A) The apparatus used for force transduction experiments. (B) Force measurements show that the time to onset of contraction was reduced when a period of co-activation of the excitor and inhibitor axons (1.25 s) preceded excitor stimulation alone (grey) compared with excitor stimulation from rest (black). E (arrow) indicates either the start of stimulation for the control or the first stimulus after inhibition for the test condition. The onset of contraction under the two conditions is indicated by the arrowheads. (C) The excitatory junction potentials (EJPs) produced by excitor axon stimuli alone (E,top) or by a period of co-activation of the inhibitor and excitor axons (E+I) followed by the excitor axon alone (E,bottom). (D) Data pooled from several experiments where EJP amplitude (normalized to the first EJP) is plotted *versus* stimulus number (mean ± S.E.M.; control, filled circles; with preceeding inhibition, open circles). After the period of co-activation of both axons (arrow), the amplitude of the first EJP is significantly larger than the previous, inhibited EJP (paired *t*-test, $P<0.001$, $N=6$ preparations).

with a sharp electrode, containing 15 mmol l^{-1} membrane-impermeable Calcium Green-1 (Molecular Probes, Eugene OR, USA), 300 mmol l^{-1} KCl and 1.0 mmol l^{-1} Hepes with a final resistance of approximately 40 MΩ. The axon was filled with Calcium Green-1 by applying 5–10 nA of negative, continuous current to a total of approximately 250 nA min^{-1}. Terminals on the surface of the muscle in the intermediate fibre region were imaged using a custom-made epifluorescence

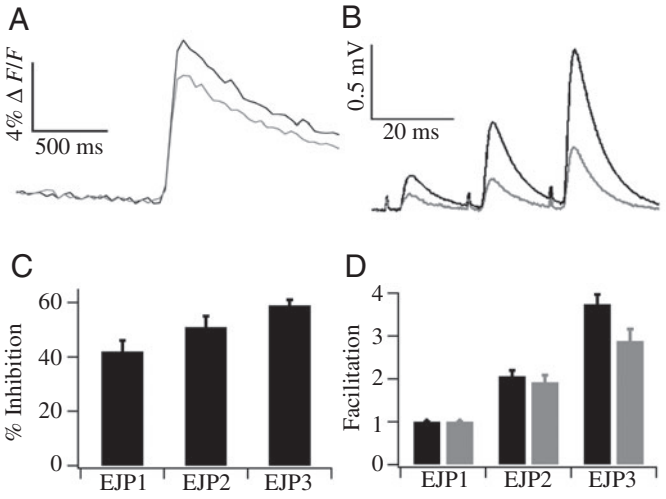


Fig. 3. Effect of presynaptic inhibition on Ca entry and transmitter release. (A) The relationship between Ca entry (observed as a change in fluorescence), during stimulation of the excitor alone (black) or the excitor and inhibitor (grey). Presynaptic inhibition caused a significant reduction in the fluorescence transient (average 20±1%) in excitor terminals (paired *t*-test, $P<0.001$, 122 terminals from 12 animals). (B) An example of excitatory junction potentials (EJPs) recorded from a muscle fibre when stimulating only the excitor (black) or the excitor and inhibitor together (grey). (C) 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). (D) Facilitation of EJPs resulting from stimulation of the excitor alone (black) or the excitor with the inhibitor (grey) was calculated by dividing the amplitude of the second and third EJP by the amplitude of the first. There was significantly less facilitation of the third EJP with inhibition (paired *t*-test, $P<0.01$, $N=27$ cells from 18 preparations).

microscope with a 60×, NA 0.9 water-immersion lens (Leica, Richmond Hill, ON, Canada). The preparation was illuminated using a 75 W Xe arc lamp coupled to a switching monocrometer (Polychrome II; T.I.L.L. Photonics, Pleasanton, CA, USA) to select excitation wavelengths. A CCD camera (Imago PCO, Madison, WI, USA) and image acquisition system (T.I.L.L. Photonics) were used to record images of fluorescent transients controlled and synchronized to the electrophysiological recordings. The image of the terminal bouton was binned on-chip prior to readout by a factor of four in the vertical and horizontal dimensions. To visualize the position of inhibitory terminal boutons relative to excitor boutons at the end of the experiment, the inhibitor axon was filled with a pipette containing 1 mmol l^{-1} Alexa 568 (Molecular Probes, Eugene, OR, USA) dissolved in distilled water using approximately 300 nA min^{-1} of 10 nA, negative continuous current. Green excitor and red inhibitor terminals were simultaneously imaged using a dual FITC/Texas Red dichroic mirror, a 510–550 nm bandpass and 600 nm long-pass emission filter (51006; Chroma Optical, Rockingham, VT, USA) with sequential 488 and 564 nm excitation. For most experiments, the change in fluorescence in the terminal boutons was measured while stimulating the excitor axon alone three times at 50 Hz or with the excitor stimulation preceded by inhibitor stimuli at 100 Hz. The stimulation protocol was as follows: four inhibitor stimuli, an excitor, two inhibitor stimuli, an excitor and finally two more inhibitor stimuli and an excitor (for a total of eight inhibitor stimuli and three excitor stimuli). Each trial comprised 55 sequential 20-ms exposure time images (each image required an additional 8–11 ms to readout for a total frame time of 28–31 ms, depending on the size and position of the CCD readout region). Twenty to 40 trials were averaged, with alternating excitor stimulation and excitor plus inhibitor stimulation. A 10-s delay was incorporated between each stimulation trial to prevent a build-up of short-term synaptic plasticity. Several terminals were imaged at a time, typically within a 60×60 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 the inhibitor axon.

Fluorescence transients were analyzed, using TILLVision™ software, by drawing a polygonal region of interest (ROI) around a varicosity from which an average level of fluorescence intensity for the pixels enclosed by the ROI 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 by subtracting the average resting fluorescence (F_r) of the terminal, calculated from the 10 frames prior to the stimulus, from the fluorescence at time t (F) and correcting for background fluorescence using the equation $[(F–F_r)/(F_r–F_b)]$ where F_b is the background fluorescence including camera offset, measured from a region of muscle adjacent to the terminals. The fluorescence transient resulting from stimulating the excitor alone was then compared with the excitor plus inhibitor transient to determine the reduction in Ca entry into terminals caused by presynaptic inhibition. The fractional change in fluorescence produced by three action potentials was ~5%, consistent with previous estimates for changes of about 10 nmol l^{-1} Ca per action potential (Delaney et al., 1991). Changes of this magnitude from resting Ca are small enough that fluorescence changes should be linearly related to Ca concentration within the accuracy of our measurements. To verify this, we confirmed for each experiment that the fluorescence change resulting from six action potentials at 50 Hz was virtually twice that produced by three action potentials, allowing reductions in fluorescence transients during inhibition to be directly equated to reductions in Ca influx without needing to convert fluorescence into an estimate of Ca concentration.

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 fibres were recorded concomitantly. Similarity between the two data sets indicated that presynaptic and postsynaptic data could be pooled from different experiments for comparison.

The amplitude of the fluorescence transient of the inhibited condition was compared with that of the control, non-inhibited,

condition for each terminal bouton with a paired *t*-test. Postsynaptically, the amplitudes 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 alone using *t*-tests.

In order to test whether failure along the length of a branch or at branch points would occur with increased inhibition, the imaging and electrophysiological experiments described above were repeated with a longer period of 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.

To test the effect of global activation of GABA receptors on Ca influx, the preparation was exposed to bath application of 10–50 μmol l^{-1} GABA for at least 10 min before recording began. The excitor action potential was recorded with an intracellular electrode in the main axon trunk distal to the Y branch to ensure faithful following of the proximal axon to stimuli. Action potential propagation, or lack thereof, into small distal branches and terminals was inferred from Ca imaging. After examining the effect of GABA on Ca influx in terminals, the preparation was rinsed with Ringer (modified Van Harreveld's solution; Van Harreveld, 1936) and a final control recording was taken. Most experiments measured the change in Ca when it had plateaued between 4 and 8 s of continuous stimulation at 5 Hz. The change in fluorescence was ~20–40% at this time, which was sufficiently large enough to measure without averaging trials and had the advantage that the muscle did not contract. Two experiments were conducted with the standard protocol used to test presynaptic inhibition: three excitor stimuli at 50 Hz.

Anatomical analysis of presynaptic inhibition

Images of the excitor axon (Calcium Green-1) were overlaid on images of the inhibitor axon (Alexa 568), and the following measurements were made: distance of excitor terminal boutons from the nearest branch, distance of excitor boutons from the nearest inhibitor bouton and whether the bouton was on a secondary, tertiary or quarternary branch (Fig. 1). The amount of inhibition at each terminal bouton was then compared with these anatomical measures by correlation analysis and paired sample *t*-tests to observe possible trends.

Results

Kinetics of muscle contraction

Stimulating the excitor axon at 20 Hz produced a contraction of the opener muscle commencing 158±22 ms after the first stimulus (Fig. 2B). If the inhibitor axon was stimulated at the same time, in 100 Hz bursts with every fourth inhibitor stimulus preceding an excitor action potential by 2–3 ms, no contraction was observed. If stimulation of the excitor axon was continued after the offset of inhibitor activity then muscle contraction occurred with a delay of 38±15 ms. Thus, a period (1.25 s) of co-activation of the excitor and inhibitor axons primed the nerve–muscle connection, allowing it to achieve the conditions required to initiate contraction significantly faster (paired *t*-test, $P<0.001$, $N=6$ preparations).

Intracellular recordings were obtained from individual muscle fibres in the proximal region during stimulation sufficient to produce contraction, and an example of data from one typical fibre is depicted in Fig. 2C, with data from six preparations summarized in Fig. 2D. In the control portion of the sample trace (Fig 2C, top), an initial rapid build-up of the EJP amplitude occurred for the first 10–12 stimuli followed by a small slower increase in EJP amplitude. The rapid build-up of EJP amplitude is characteristic of facilitation, while the slower increase may include a component of augmentation. With inhibition, the EJP amplitude was greatly reduced, but increased with each successive stimulus. The first EJP produced 50 ms after the offset of inhibitor activity showed a marked increase (~48%; paired *t*-test, $P<0.001$, $N=6$ preparations), achieving an amplitude greater than those of EJPs that were coincident with the onset of contraction when no preceding period of inhibition was applied. Although, the first EJP following the cessation of inhibition did not immediately reach the same amplitude that it would have attained in the absence of preceding inhibitor activity, it rapidly achieved full amplitude with further stimulation.

Imaging and electrophysiology

We investigated the effect of presynaptic inhibition on Ca entry into excitor terminal boutons with short trains of three stimuli at 50 Hz. For technical reasons, 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 fibres were recorded at the same time. In this series of experiments with both imaging and electrophysiology, the Ca transient was reduced by 18±2% (mean ± S.E.M., $N=60$ terminals from four preparations). In imaging-only experiments, the Ca transient was reduced by 21±2% (mean ± S.E.M., $N=62$ terminals from eight preparations). Thus, overall, co-activation of the inhibitor axon reduced Ca entry into terminals by 20±1% (mean ± S.E.M., range 0–50%, paired *t*-test, $P<0.001$, $N=122$ terminals from 12 preparations; Fig. 3A). One experiment with typical fluorescence intensities and fractional fluorescence changes was analyzed in detail to determine the accuracy of an estimate of the percent inhibition for a particular terminal. Where the inhibition was greater than 20%, a significant difference between the control and the inhibited fluorescence transients was observed with only five trials averaged (paired *t*-test, $P<0.05$). With an increased number of trials (20–30), we were able to observe a significant difference between the control and the inhibited fluorescence transients of ≥6% (paired *t*-test, $P<0.05$).

Data from one muscle cell (Fig. 3B) illustrates a reduction in the amplitude of the EJPs caused by the inhibitor.

Normalized and pooled data for 27 proximal cells from 18 experiments (Fig. 3C), revealed inhibition for the first, second and third EJPs of 42±4%, 51±4% and 59±2% (mean ± S.E.M.), respectively. The amplitudes 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 slight 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, which is addressed below. As most experiments were done with just electrophysiology, results from experiments with both imaging and electrophysiology were compared and found not to be significantly different. 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 ± S.E.M., $N=5$ cells from four preparations) *versus* 40±4%, 50±5% and 60±3% (mean ± S.E.M., $N=22$ cells from 14 preparations) for experiments without imaging.

The 20% decrease in Ca entry resulted in, on average, a 51% decrease in the amplitude of the EJP (Fig. 3). From these data, we calculated the exponent for the non-linear relationship between Ca entry and transmitter release. It has been demonstrated that 5–10% of the inhibition measured as a reduction in the amplitude of the EJP is mediated directly on the muscle fibre (Atwood and Bittner, 1971; Baxter and Bittner, 1980; Fatt and Katz, 1953). Thus, we subtracted this from the total inhibition to estimate the effect of the presynaptic component (41–46%). The relationship between Ca entry and transmitter release can be expressed as: $k[Ca^{2+}]^{(n)}=R$, where a constant, k, multiplied by the concentration of Ca to the power n equals release, *R*. We measured Ca entry as the fluorescence transient, and release as the EJP. For our experiment, we first measured this value with excitation alone and then with inhibition where: $k[Ca^{2+}]_{Inh}^{(n)}=R_{Inh}$. The constant k and power n are the same values as the previous equation. We then compared these two values and expressed the result as a ratio: $R_{Inh}/R=k/k([Ca^{2+}]_{Inh}/[Ca^{2+}])^{(n)}$. As the constant k cancels out, we are left with measured values of *R* and $[Ca^{2+}]$ and can estimate n. With this form of the equation relating changes in Ca to changes in release, 20% inhibition of the Ca transient is expressed as 80% of the normal influx. We then calculated release resulting from a given value of n for each terminal and averaged the result. We found that 41–46% inhibition of the EJP (presynaptically mediated) corresponded to an n value of 2.8±0.3.

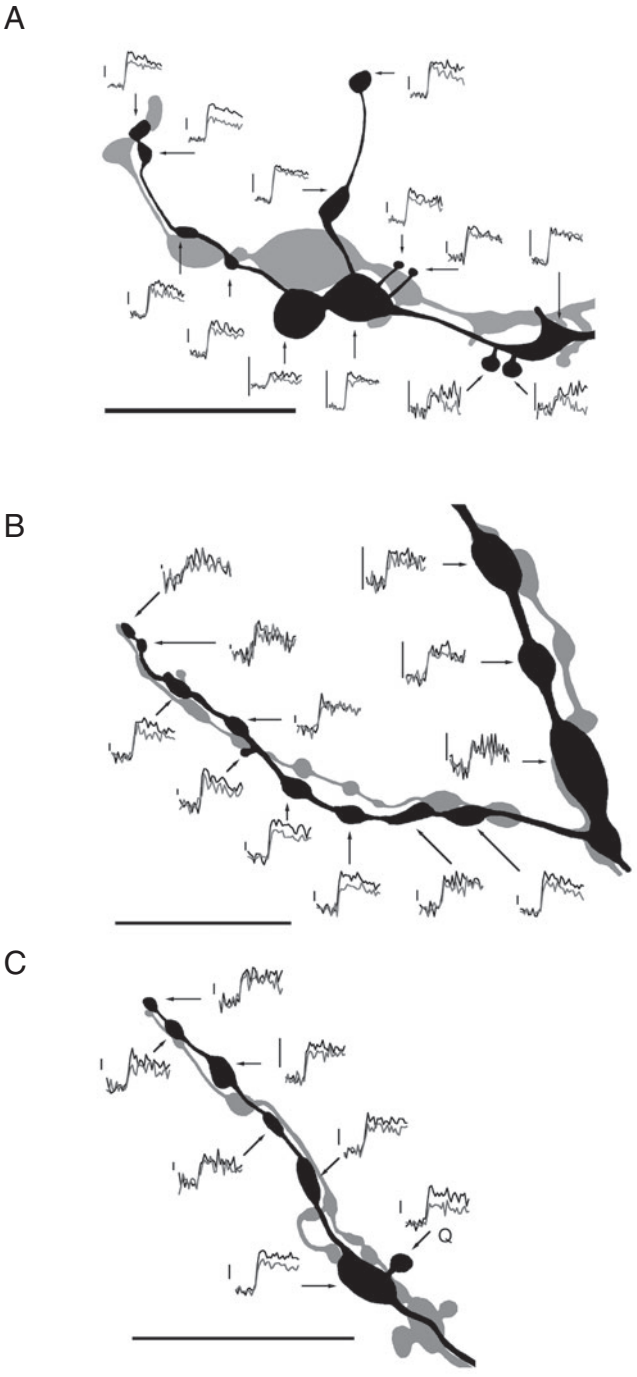


Fig. 4. Effect of presynaptic inhibition on Ca entry into terminal varicosities. (A) The complex anatomical relationship between excitor (black) and inhibitor (grey) axons and variation in the amount of inhibition between terminals. Ca entry was reduced by 0–40% at different terminals with inhibition. (B) Greater inhibition was not observed along a string of terminal boutons. (C) Greater inhibition at a terminal on a quaternary branch compared with those on tertiary branches. Inhibition at the six terminals on the tertiary branch ranged from 12 to 28%, while the terminal on the quarternary branch (Q) experienced approximately 40% inhibition. Inhibitor terminals are located in the vicinity of the bottleneck structure forming the branch of the excitor axon. Overall, there was slightly more inhibition at terminals on quaternary branches (24±2%, $N=36$ terminals) than on those on tertiary branches (18±1%, $N=86$ terminals, two-sample *t*-test, $P<0.05$). Fluorescence transients measured from the excitor axon are depicted next to the terminals that they represent (arrows). Scale bars indicate 2% $\Delta F/F$ (vertical) and 50 μm (horizontal). The black trace resulted from stimulating the excitor alone, while the grey trace resulted from stimulating the excitor and inhibitor together. The action potential was conducted from the right to the left in all of the images.

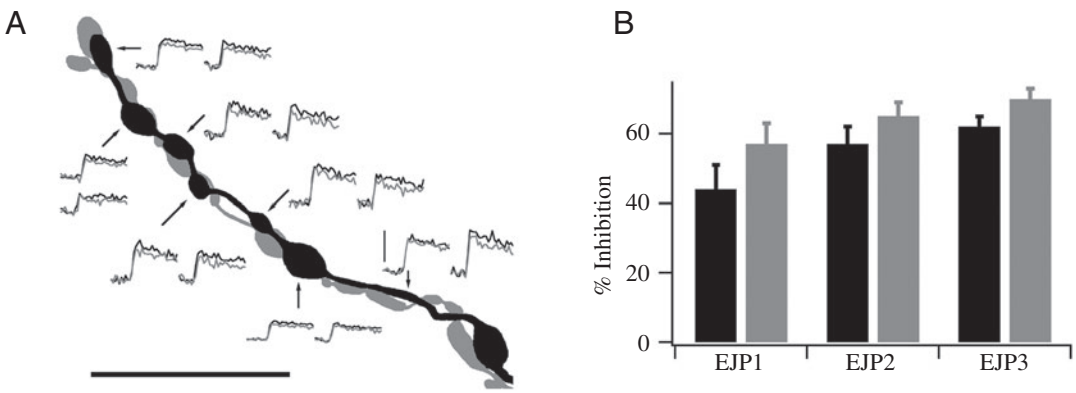


Fig. 5. Effect of increased inhibition on Ca entry and the excitatory junction potentials (EJPs). (A) More inhibitor stimuli caused slightly greater inhibition of Ca entry (excitor black, inhibitor grey). With the inhibitor stimulated in the standard manner (left-hand traces; eight inhibitor stimuli at 100 Hz), inhibition was 13±2% (mean ± S.E.M.), and with more inhibitor stimuli (right-hand traces, 24 stimuli at 100 Hz) inhibition was slightly greater: 22±2% (paired *t*-test, $P<0.05$, $N=14$ terminals from two preparations). Fluorescence transients measured from the excitor terminals, using Calcium Green-1, are depicted next to the terminals (arrows). Black traces resulted from stimulating the excitor alone, while grey traces resulted from stimulating the excitor and inhibitor together. The action potential was conducted from the bottom right to the top left. Scale bars indicate 5% $\Delta F/F$ (vertical) and 50 μm (horizontal). (B) Increased stimulation of the inhibitor axon resulted in greater inhibition (~10%) of the second and third EJP (paired *t*-test, $P<0.05$, $N=10$ cells from three preparations). Black bars represent eight inhibitor stimuli, while grey bars represent 24 inhibitor stimuli.

Anatomical effects on presynaptic inhibition

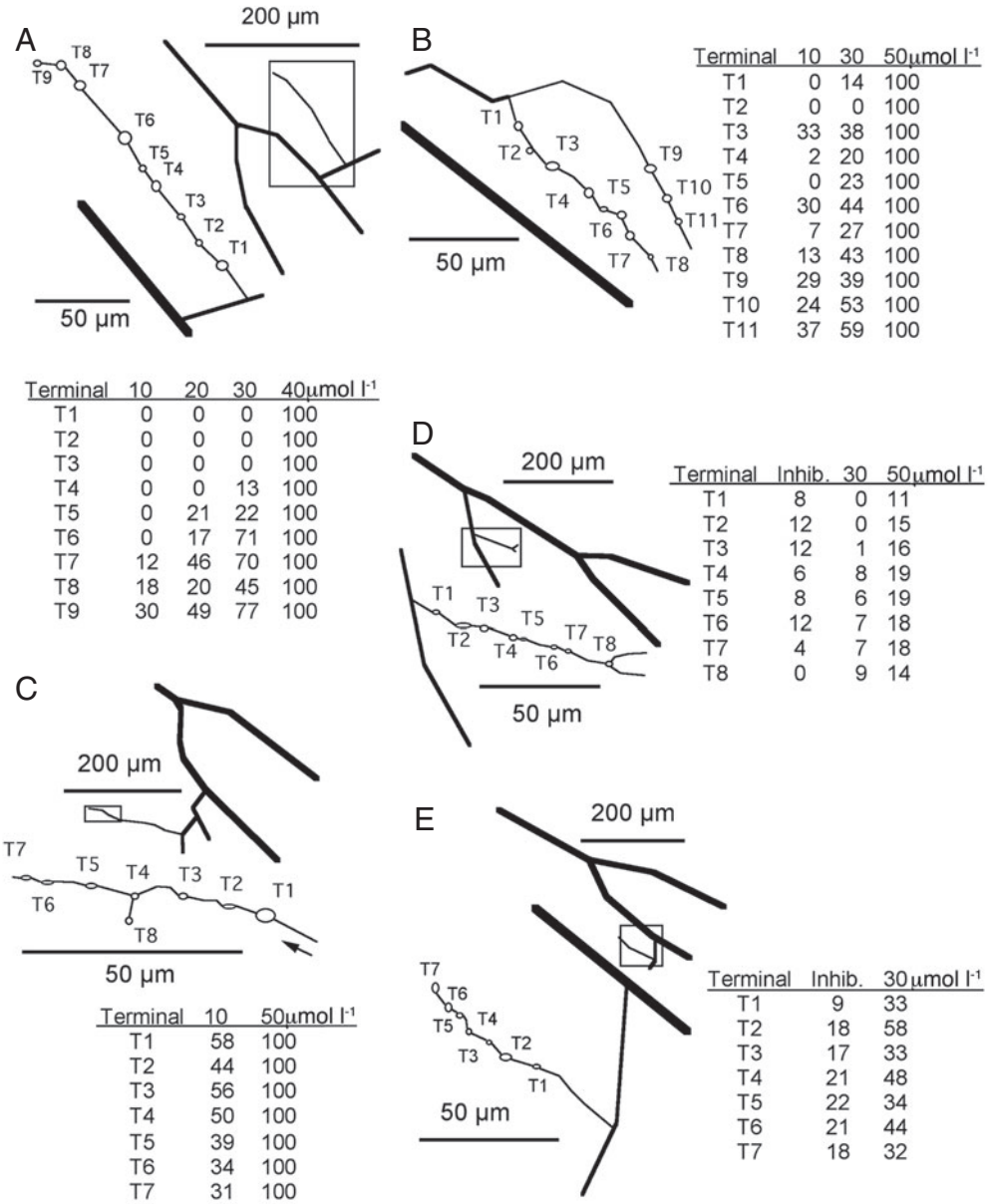
Images of the excitor and inhibitor axons were overlaid in order to analyze the effects of anatomy on inhibition. Variation in the amount of inhibition at different terminal boutons was seen (Fig. 4A). However, evidence for a consistent trend of increasing inhibition from proximal to distal along long branches of terminal boutons was never observed (Fig. 4B). We did find evidence for slightly more inhibition in terminal boutons on quaternary branches (24±2%, $N=36$ terminals) *versus* those on tertiary branches (18±1%, $N=86$ terminals, two-sample *t*-test, $P<0.05$; Fig. 4C). The distance separating an excitor bouton from the nearest inhibitor bouton was compared with the amount of inhibition observed, and no correlation was detected ($r^2=0.001$, $N=95$ terminals). Similarly, we found no correlation between the distance of the excitor bouton to the nearest main branch and the amount of inhibition of the Ca transient ($r^2=0.04$, $N=92$ terminals). Failure of propagation of the action potential did not seem to occur because we always observed Ca entry into all terminal boutons, even at the end of long (>150 μm) branches. A limitation of this analysis was that the exact location of axo-axonal synapses within large boutons or along thin axons was not known. Others have observed with electronmicrographic studies that excitor and inhibitor terminal boutons are 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 later in trains

The facilitation of the second and third EJPs in the train was calculated relative to the amplitude of the first EJP (Fig. 3D). In addition to reducing EJP amplitude, there appeared to be a small but 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). The same effect was seen in the longer, 20 Hz trains used to initiate contraction (Fig. 2). Although facilitation clearly builds during the repetitive stimulation of the excitor in conjunction with the inhibitor, the total enhancement was less than predicted from trains in which the excitor is stimulated alone.

To test the possibility that the inhibitor was not fully facilitated when four stimuli were applied prior to the onset of excitor stimulation, as in the imaging and electrophysiological experiments, 20 inhibitor stimuli were delivered to more fully develop inhibitor facilitation. A slightly greater reduction of Ca influx was observed with an increased number of inhibitory stimuli (paired *t*-test, $P<0.05$, $N=14$ terminals from two preparations). For these experiments, inhibition stimulated in the standard manner ranged from 8 to 26% (13±2.3%, mean ± S.E.M.), while more inhibitor stimuli preceding excitor activation caused a 13–35% (22±1.8%, mean ± S.E.M.) reduction in Ca influx (Fig. 5A). Faithful propagation of an action potential to terminals was inferred from the fact that we always observed a Ca transient in the excitor terminals. Thus, although there was greater inhibition of Ca entry with increased inhibitor stimulation, neither increasing inhibition along the length of branches nor branch point failure were observed. Even with more sustained trains of inhibition, the action potential was still able to propagate to distal terminals.

Although 20 action potentials are sufficient for inhibitor facilitation to substantially reach a plateau, there was still ~10% greater inhibition of the second and third EJPs compared with the first (paired *t*-test, $P<0.05$, 10 cells from three preparations; Fig. 5B). Inhibition mediated by $GABA_B$ receptors has been reported (Parnas et al., 1999) but we could find no inhibitory effect of baclofen (30–180 μmol l^{-1}; $N=4$) on excitatory transmission, and baclofen did not occlude inhibition of the EJP by the inhibitor axon nor remove the reduced build-up of facilitation during short trains. Therefore, our data suggest that $GABA_A$-mediated presynaptic inhibition



Terminal	10	30	50 µmol l^{-1}
T1	0	14	100
T2	0	0	100
T3	33	38	100
T4	2	20	100
T5	0	23	100
T6	30	44	100
T7	7	27	100
T8	13	43	100
T9	29	39	100
T10	24	53	100
T11	37	59	100

Terminal	10	20	30	40 µmol l^{-1}
T1	0	0	0	100
T2	0	0	0	100
T3	0	0	0	100
T4	0	0	13	100
T5	0	21	22	100
T6	0	17	71	100
T7	12	46	70	100
T8	18	20	45	100
T9	30	49	77	100

Terminal	Inhib.	30	50 µmol l^{-1}
T1	8	0	11
T2	12	0	15
T3	12	1	16
T4	6	8	19
T5	8	6	19
T6	12	7	18
T7	4	7	18
T8	0	9	14

Terminal	10	50 µmol l^{-1}
T1	58	100
T2	44	100
T3	56	100
T4	50	100
T5	39	100
T6	34	100
T7	31	100
T8	42	100

Terminal	Inhib.	30 µmol l^{-1}
T1	9	33
T2	18	58
T3	17	33
T4	21	48
T5	22	34
T6	21	44
T7	18	32

Fig. 6. Bath application of GABA reveals cumulative effects of inhibition along branches. Examples from five experiments are shown. The most striking effect of increasing inhibition along a branch is demonstrated by A, but a general trend of increasing inhibition along extended branches or of greater inhibition for terminals at the end of a long branch far from the main axon can be seen in other preparations. In D and E, the standard inhibitor protocol of eight inhibitor stimuli and three excitor action potentials was performed for comparison.

has a small inhibitory effect on facilitation of the excitor, which is not expected from its minor reduction of Ca influx.

Application of GABA

We attempted to relate inhibition produced by activation of the inhibitor axon to bath application of GABA (Fig. 6). Experiments were performed by stimulating the excitor at 5 Hz with and without GABA while recording the fluorescence transient. We measured the change in fluorescence once it reached a nearly steady state of ~20% above basal between 4 and 8 s after stimulus onset, which was still small enough that we did not correct for partial dye saturation effects. In two experiments, we were also able to compare GABA application to inhibition delivered by inhibitor axon stimulation (standard eight inhibitor and three excitor stimuli). Bath application of GABA was capable of producing much greater inhibition than was possible with inhibitor axon stimulation. In four of five preparations, 40 or 50 µmol l^{-1} GABA was sufficient to completely block Ca influx into terminals, but at this concentration the action potential was observed to fail at the recording site in the main axon trunk. The action potential and Ca influx could be restored within a few minutes by rinsing with normal saline. Intermediate levels of inhibition were seen with 10–30 µmol l^{-1} GABA, and the amount of inhibition varied widely between the different experiments. *Post hoc* analysis suggested that with bath application, unlike inhibitor axon stimulation, there was often increasing inhibition with distance along tertiary branches if the branch was sufficiently long (e.g. Fig. 6A,B,D) or greater sensitivity to GABA for terminals located at a distance from the main axon (e.g.

Fig. 6B,C *versus* Fig. 6D). A similar distance-dependent effect on inhibition was reported in a preliminary study by Tank and Delaney (1988).

Discussion

Presynaptic inhibition of excitatory transmission occurs in both vertebrates (Eccles, 1964; Nicoll and Alger, 1979) and invertebrates (Dudel and Kuffler, 1961; Tse et al., 1991). We studied how presynaptic inhibition at the claw opener neuromuscular junction might contribute to motor function in the crayfish and provide a model for how inhibition could potentially interact with frequency-dependent facilitation at other synapses.

Presynaptic inhibition at the crayfish neuromuscular junction can cause relaxation of the muscle in the face of ongoing excitor axon activity. In this instance, relaxation is produced by the addition of inhibitor axon activity rather than the reduction of excitor axon firing. Added inhibitor axon activity during sustained excitor axon firing is not entirely wasteful since processes that facilitate release are maintained and can be expressed immediately after the inhibition is removed when the inhibitor is silenced. Thus, although the muscle is relaxed when the inhibitor is co-activated with the excitor, the time to onset of contraction is shortened if the excitor continues to be activated after a period of excitor and inhibitor co-activation, as predicted by Atwood and Wojtowicz (1986). This phenomenon could be behaviourally significant for motor control in the crayfish because it increases the kinetics of contraction. In central synapses in other nervous systems, such a mechanism could be used to regulate the latency, and thus the timing, of information transfer at specific gating points and could underlie some priming or attentional phenomena.

With co-activation of the inhibitor axon, we measured that a moderate (20% average) reduction in Ca entry into presynaptic excitor axon terminal boutons is associated with a 51% reduction in the amplitude of the postsynaptic EJP. This is consistent with a relationship between Ca entry and transmitter release proportional to approximately the third power. Presynaptic inhibition most likely reduces Ca entry into excitor motor terminals by decreasing the amplitude of the action potential by 10–20 mV (Baxter and Bittner, 1981), thus activating fewer voltage-gated Ca channels. A non-linear relationship between a change in presynaptic Ca entry and neurotransmitter release, with a reduced number of open Ca channels, indicates a requirement for overlapping Ca microdomains to elicit neurotransmitter release (Zucker, 1999). Thus, this synapse only requires a small reduction in Ca entry to have a large effect on neurotransmitter release.

Although short-term or ‘paired-pulse’ facilitation at the crayfish neuromuscular junction and other synapses requires Ca influx (Katz and Miledi, 1968), it is not as sensitive to reduced Ca as release itself (Magleby, 1987; Zucker and Regehr, 2002). We observed a 50% reduction in release. If this were due to loss of Ca influx (and thus transmitter release) in half of the terminals with little or no reduction in the other half then we would expect to see a 50% reduction in facilitation. We observed an average 20% reduction of Ca influx in all terminals, which, based on the other studies cited, is consistent with our observation that facilitation is minimally affected. Thus, the model, where most or all terminals continue to experience near normal Ca influx during inhibition, is supported by our measurements that reveal a moderate reduction of Ca influx into most terminals with no evidence for complete failure of influx into any terminals during inhibition.

The amount of inhibition at a given terminal could potentially depend on the morphology of the excitor and inhibitor axons and the physical relationship between their terminals (Atwood and Wojtowicz, 1986). We measured inhibition in a large number of terminals (122) over many locations (18) to test for morphological trends. Although the amount of inhibition at individual terminal boutons varied, trends of increasing inhibition along a branch were not observed. Slightly greater inhibition was observed at terminal boutons on quaternary branches, as would be predicted by anatomical models (Atwood et al., 1984), but complete blockade of the action potential was never observed. Furthermore, although increasing the number of inhibitor axon action potentials that preceded the excitor action potentials (from 4 to 20) did result in slightly greater inhibition, the action potential still appeared to propagate actively, as evidenced by a relatively consistent reduction of Ca influx along branches. Therefore, we 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 build-up of facilitation is essential to elicit contraction of the opener muscle in the crayfish neuromuscular junction. Because it was observed that the majority of facilitation was maintained during inhibition, it follows that 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, thus electrically isolating entire branches of the motor axon, facilitation would be blocked in groups of terminals.

Although the predominant effect of presynaptic inhibition is to reduce release while preserving facilitation, we saw some evidence that facilitation was slightly inhibited. During sustained 20 Hz trains, the first one or two EJPs stimulated after cessation of inhibition were not as large as they would have been had the inhibition been absent (Fig. 2D). In addition, we observed that with three high-frequency (50 Hz) stimuli, the build-up of paired-pulse facilitation was slightly reduced (Fig. 3D). This contrasts to the results of Baxter and Bittner (1981), who reported no decrease in facilitation during

inhibition. The stimulation paradigm used in our experiments differed from their study in that they stimulated with a short train (11 excitor stimuli at 100 Hz) while we used a longer, lower-frequency train (25 excitor stimuli at 20 Hz). An explanation for the difference between the results of our study and their work may be that during inhibition we had some loss of augmentation, which may have normally contributed during the longer stimulus trains we used. Some loss of augmentation during presynaptic inhibition is expected because it is dependent on the build-up of free Ca concentration in these terminals (Delaney and Tank, 1994), which is reduced. However, a reduction of augmentation would not apply for our three-stimulus/50 Hz paradigm, where we clearly saw a small reduction of facilitation. Another explanation could be activation of a $GABA_B$ receptor. $GABA_B$ receptors have been indicated to inhibit transmitter release from crayfish excitor motor terminals (Fischer and Parnas, 1996a,b; Parnas et al., 1999). The apparent persistence of inhibition for a few hundred milliseconds after the offset of inhibitor axon firing (Fig. 2D) would be consistent with the decay of a $GABA_B$-mediated contribution to inhibition. However, we were unable to observe any inhibitory effect of high concentrations of baclofen on excitatory transmission, nor did we observe occlusion of the inhibitor axon-mediated inhibition by baclofen. Perhaps changes in the distribution of Ca in the active zone associated with the reduction in the number or duration of Ca channel openings caused by the reduced action potential amplitude have a small inhibitory effect on facilitation.

Bath application of GABA produced increasing amounts of inhibition of Ca influx along a branch in several preparations. In some instances, with 10–30 μmol l^{-1} GABA, the inhibition between proximal and distal terminals ranged from 0 to >60%. Increasing GABA to 40 or 50 μmol l^{-1} blocked action potentials entirely. A similar result was seen in preliminary work by Tank and Delaney (1988). However, inhibition induced by 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, perhaps from activation of $GABA_B$ receptors or by creating a spatially extended inhibition through activation of extrasynaptic $GABA_A$ receptors that cannot be reproduced by electrical stimulation of the inhibitor axon. The fact that a qualitatively differential inhibitory response profile was observed with bath application of GABA *versus* standard presynaptic inhibition demonstrates the spatial and temporal specificity of presynaptic inhibition at the crayfish neuromuscular junction.

The crayfish excitor–inhibitor motor pair is an example of a system where a single inhibitory axon delivers inhibition to virtually all of the output synapses of its target. Local regulation of synaptic output has been demonstrated to be possible in transmitter-releasing dendrites of olfactory mitral cells that receive inhibitory GABA synaptic input from ~20 interneurons at sites distributed along their length. Localized inhibition of action potential-mediated Ca influx (and thus localized reduction of transmitter release) in dendrites of mitral cells has been demonstrated with localized release of caged GABA and by local stimulation of presynaptic interneurons (Chen et al., 2000; Lowe, 2002). Thus, localized shunting inhibition can be used to selectively reduce output from some but not all synapses by reducing Ca influx without causing complete failure of action potentials. Presynaptic inhibition of transmitter release has also been shown to contribute to gating transmission by a command neuron to the appropriate phase of activity of a rhythmic pattern generator (Hurwitz et al., 2004). Thus, the spatial and temporal specificity of action that can be achieved with fast presynaptic inhibition at crayfish neuromuscular junction represents a strategy utilized by other synapses to achieve task-specific control of transmission in neuronal networks.

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