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Differential facilitation of high- and low-output nerve terminals from a single motoneuron

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Crider, Misty E., and Robin L. Cooper. Differential facilitation of high- and low-output nerve terminals from a single motoneuron. *J. Appl. Physiol.* 88: 987–996, 2000.—In the crayfish opener neuromuscular preparation, regional differences in synaptic transmission are observed among the terminals of a single motoneuron. With a single stimulus, the high-output terminals of the proximal region of the muscle produce a larger excitatory postsynaptic potential than do the low-output terminals of the central region of the muscle. We tested the hypothesis that the low-output terminals exhibit more facilitation than do high-output terminals for twin-pulse, train, and continuous-stimulation paradigms. Previous studies have not employed several stimulation paradigms to induce facilitation among high- and low-output terminals of a single motoneuron. We found that the high-output terminals on the proximal fibers facilitate more than the low-output terminals on the central muscle fibers, in contrast with previous studies on similar muscles. The difference in measured facilitation is dependent on the stimulation paradigm. These results are important because ultrastructural differences between these high- and low-output terminals are known and can be used for correlatation with physiological measurements. Short-term facilitation is a form of short-term memory at the synaptic level, and the processes understood at the crayfish neuromuscular junction may well be applicable to all chemical synapses.

crayfish; neuromuscular junction; synapse

TO INDUCE CONTRACTION OF MUSCLES involved in locomotion of crustaceans, tonic motoneurons fire in bursts or trains rather than continuously firing at low frequencies. This type of neurotransmission can result in a facilitated postsynaptic response (38, 45). Facilitation of synaptic transmission is an enhancement of the postsynaptic response and is known to occur when there is an increase in electrical activity at the synapse. When the synaptic properties of neuromuscular preparations are discussed, use of facilitation protocols is advantageous because they closely mimic the physiological conditions of motoneurons compared with isolated stimuli. In addition, the use of various facilitation protocols allows one to assess more thoroughly the physiological mechanisms underlying synaptic transmission among different types of motoneurons or within a given motoneuron that shows synaptic differentiation among its terminals. Because the basic mechanisms of chemical transmission are similar for all chemical synapses among the various animal preparations investigated, the use of identified neurons in a well-defined system proves to be beneficial. The opener muscle in the crayfish is a preparation in which one can study regional synaptic differentiation. This is because the entire muscle is innervated by a single excitatory tonic motoneuron, and the postsynaptic responses are substantially different depending on the muscle fiber innervated. Synaptic differentiation for single neurons is known in other systems, such as in the cat spinal cord (56), Mauthner neurons in goldfish (47), and the cricket central nervous system (28), but only in a few cases has the physiology and terminal morphology been examined for a single neuron that shows synaptic differentiation (46, 64).

The opener muscle in crayfish has provided a great deal of insight into the basic mechanisms of synaptic transmission (1–4, 8, 12, 32, 39, 51–53, 61–63, 65, 72–74). The differentiation in responses that arise from the single motoneuron measured in well-defined regions on the opener muscle has led to many postulations as to the underlying mechanisms (1, 13–14, 33–36, 44, 51, 59, 73, 77). It is now known that the majority of the differences are due to local presynaptic alterations in synaptic structure determined by direct correlations in physiology and structure (5–8, 20–22, 25).

In adult crayfish, the central region of the opener muscle contains nerve terminals arranged in long chains of varicosities (swellings), whereas in the proximal region the nerve terminals are grouped into clusters of varicosities (6). By ultrastructural analysis it has been shown that the varicosities contain the majority of the synaptic contacts (22, 40). It has further been shown that synaptic transmission decreases along the length of a single terminal (21). The varicosities on the proximal muscle fibers are very high in synaptic output in comparison to varicosities in the central region. A direct structural correlate that accounts for the differential physiology is that the high-output varicosities along a terminal contain more active sites per synapse than do the low-output varicosities. The low-output varicosities contain a high percentage of synapses with only one or no active sites. This indicates that there are more silent synapses in the low-output varicosities, which may be a developmental regulation because the most distal varicosities are the newest formed along the

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length of the terminal for all regions on the muscles. The differences in the synaptic structure among the varicosities may in part explain the differences measured in the Ca^{2+} flux during stimulation at various frequencies (20). This synaptic complexity, as well as the number of varicosities per muscle fiber, accounts for ~80% of the difference in the excitatory postsynaptic (EPSP) amplitudes between the two fiber regions (23).

The two types of facilitation described at the crayfish neuromuscular junction are long-term facilitation and short-term facilitation (STF) (10, 31, 58, 69). The mechanism of STF is generally thought to be due to a buildup of residual Ca^{2+} within the presynaptic terminal. The residual buildup of Ca^{2+} leads to an increase in neurotransmitter release (10, 25, 29, 30). The time course of the STF may be affected by the removal of free Ca^{2+} in the presynaptic cell (51, 52, 67). Recent refinements of past concepts for facilitation are being put forth (17, 25, 62). To ascertain whether differences in facilitation are observed, by using the two regions of the opener muscle in which synaptic structural differences are known, one must define the conditions of stimulation because of their influence on the facilitation index (FI). For example, it was shown that regions vary in facilitation depending on the delay used for twin-pulse paradigms. As the delay between stimuli is increased, the distal muscle fibers begin to show more facilitation than do the central muscle fibers (48). When making a comparison between the distal and proximal muscle fibers of the claw opener, Parnas and colleagues (54) found that the distal region facilitated more than the proximal region when twin-pulse facilitation was measured. These studies have used various forms of facilitation to characterize synaptic differences, and the dorsal compared with ventral surface of the muscle was examined. Because the experimental conditions give rise to different FI values, the conclusions of synaptic differentiation and regional differences in facilitation are still open for interpretation (see Refs. 15, 48, 54, 70).

The hypothesis driving this study, based on previously reported results (7, 13), is that the lower output terminals will show a greater degree of facilitation than the high-output terminals for twin-pulse, train, and continuous-stimulation paradigms. The rationale for this hypothesis is that previous studies have indicated that the low-output terminals show a greater degree of facilitation. However, these studies used only one stimulation paradigm. We have assumed that other forms of inducing STF would induce the low-output terminals to facilitate more than the high-output ones. The importance of this study is that we have found that the differences in facilitation measures are dependent on the stimulation paradigm and that one should use a variety of stimulation paradigms to characterize synaptic efficacy. In addition, we have found that high-output synapses facilitate more than low-output terminals for most of the stimulation conditions commonly utilized. We have demonstrated these findings by comparing FIs among the high- and low-output terminals arising from a single motoneuron. Because these terminals have previously been defined for structural differences, these new observations provide one with a more complete understanding of the processes involved in synaptic differentiation and plasticity, on which can be based future correlations of structure and functional significance. This basic knowledge will likely be applicable to all chemical synapses.

MATERIALS AND METHODS

General. All experiments were performed by using the first and second walking legs of freshly obtained crayfish, *Procambarus clarkii*, measuring 6–10 cm in body length (Atchafalaya Biological Supply, Raceland, LA). Animals were housed individually in an aquatic facility and fed dried fish food, chicken eggshells, and carrots. Dissected preparations were maintained in crayfish saline, a modified Van Harreveld's solution (in mM: 205 NaCl, 5.3 KCl, 13.5 $CaCl_2 \cdot 2H_2O$, 2.45 $MgCl_2 \cdot 6H_2O$, and 5 HEPES adjusted to pH 7.4). Crayfish were induced to autotomize the first or second walking leg by forceful pinching at the merus segment. During the dissections of the opener muscle, the bathing medium was exchanged every 20 min with chilled saline at 14°C until the dissections were completed. No particular animal care procedures are required for crustaceans by the National Science Foundation, although we maintain the crayfish in laboratory conditions that promote growth, molting, and reproducibility of viable young.

Physiology. Electromyograms (EMGs) recorded from the opener muscle in situ were performed as previously described for the leg extensor muscle (18). In brief, two thin insulated copper wires were placed in two small holes that were drilled through the cuticle on the dorsal surface of the propus and held in place with Super Glue. The differentially recorded potentials were amplified with a Grass alternating-current preamplifier (model P-15) and recorded on an on-line computer by use of a MacLab 4s analog-to-digital converter (ADInstruments). As the animal voluntarily moved the propus-dactylus joint, the EMG records were able to be correlated with particular movements. Instantaneous frequency measures of the EMG activity are calculated by measuring the time between two subsequent events and calculating the corresponding frequency, whereas the average frequency is the rate for each five events to occur.

To elicit an evoked response, the excitatory axon was selectively stimulated by placing a branch of the leg nerve (from the merus segment) into a suction electrode connected to a Grass stimulator (38). Intracellular recordings were performed with 30- to 60-MΩ-resistance microelectrodes filled with 3 M KCl. EPSPs were recorded at 10 kHz. The intracellular muscle potentials were amplified by a microelectrode preamplifier (Almost Perfect Electronics, Basel, Switzerland) and were observed by using a Hameg analog-digital oscilloscope. Electrical signals were recorded on VHS tape (Vetter 400) and on-line to a Power Mac 9500 via a MacLab/4s interface.

The induction of STF was obtained using three methods. The first was to apply two stimulus pulses relatively close together. This procedure is referred to as twin-pulse facilitation. The time between stimulus pulses was varied from 10 to 60 ms, and the frequencies of stimulations used were 0.1, 0.5, and 1 Hz. The second procedure consisted of giving a train of 10 pulses at various intervals (every 1, 2, or 10 s). The frequency of stimulation within the train varied (30 and 50 Hz). A third method for inducing facilitation involved giving continuous stimulation at a constant frequency (1, 10, or 30 Hz) and allowing the membrane potential to plateau.

Analysis. To determine the FI for the twin-pulse stimulation, the amplitude of the last pulse is compared with the amplitude of the first pulse. Similarly, train facilitation involves subtracting one of the preceding pulses from the last one in the train. This ensures that if there is no facilitation present (if the amplitudes of the first and last responses are the same), the FI will be zero. To analyze continuous-stimulation experiments, initial EPSPs and final EPSPs were compared to determine the amount of increase, after a plateau was reached. In addition, the amplitudes of the plateau EPSPs were compared with those produced at different stimulation frequencies.

The amplitudes of the EPSPs were measured by using the MacLab/s v3.5 program's scope or chart. The signals recorded on the computer were calibrated to the raw traces observed on the oscilloscope. An average of 20 responses was obtained to reduce noise before amplitude measurements were taken. The averaged responses were also used for illustrative purposes in figures. The software MacLab/s v3.5 (ADInstruments) was used to average the traces.

Statistics employed were either the Student's *t*-test or a Wilcoxon paired rank-sum test, which is a nonparametric test. All experiments contained a minimum of five experimental preparations. If a consistent trend is shown among five preparations, then P is <0.05 by this nonparametric test.

RESULTS

The opener muscle of the first walking leg is easily divided into two regions: central and proximal (Fig. 1). These two regions show distinct differences in the amplitudes of EPSPs for single stimuli (Fig. 2*A*) as well as for trains of stimuli. There are also readily observable differences in the degree of facilitation within a region produced by twin, train, and continuous-stimulation paradigms (Fig. 2, *B–D*).

Twin pulse. Twin-pulse facilitation occurs when two identical stimulus pulses are given within a short amount of time, resulting in a larger EPSP amplitude after the second stimulus pulse (Fig. 2*B*). Figure 3 shows the FI for the central and proximal regions when measured at a set stimulation paradigm: 0.1-Hz frequency with a 20-ms delay between pulses. The results indicate substantial variation among preparations when the FI is measured, as well as a large variation in the difference between FI in the central and proximal regions. However, in 10 of the 11 preparations reported here, the proximal region facilitated more than the central region under these twin-pulse conditions ($P < 0.05$, Wilcoxon paired rank-sum test). Only *preparation 9* showed no differences between the two regions.

To determine whether different stimulation paradigms would give different measures of FI, the frequency of stimulation between twin-pulse tests was varied by using 0.1, 0.5, and 1 Hz. The FI decreased slightly for the recordings obtained in proximal fibers with an increase in stimulation frequency, as represented in Fig. 4. The change in FI when moving from 0.1 Hz (twin pulses given every 10 s) to 1.0 Hz (twin pulses given every 1 s) was not significant for grouped data, but, within an individual preparation, each of the conditions tested revealed a consistent trend for higher FI in central fibers. Because five of five preparations

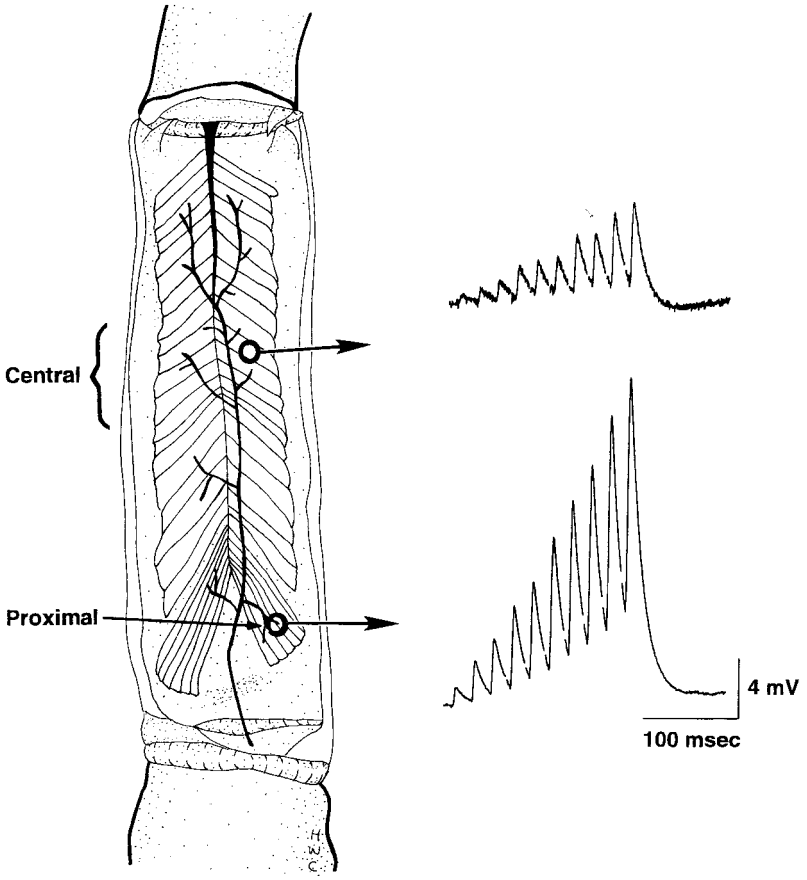

Fig. 1. Schematic of opener muscle as seen from a ventral view showing the 2 regions: central and proximal. These 2 regions have distinct differences in the diameters of the muscle fiber bundles. Excitatory postsynaptic potentials that are measured in these 2 regions are also distinctly different in their amplitudes. An average of 20 excitatory postsynaptic potentials was used to reduce noise in traces.

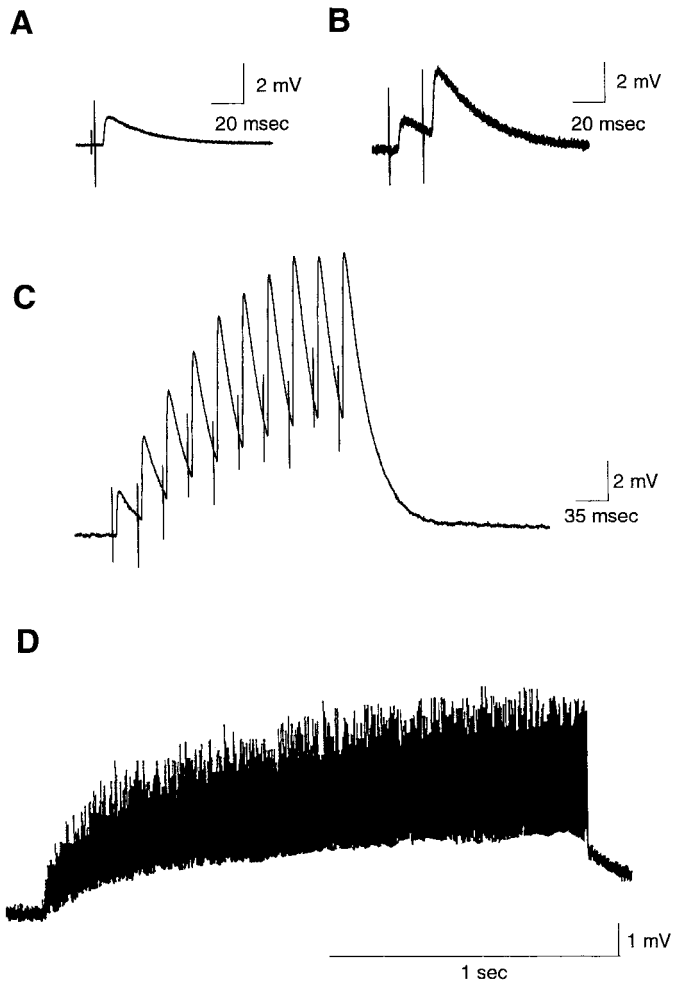

Fig. 2. Excitatory postsynaptic potentials under various stimulation paradigms: a single stimulus (*A*), identical twin-stimulus pulses (*B*), a train of 10 identical stimulus pulses at 40 Hz (*C*), and a continuous train of pulses at a given stimulation frequency (30 Hz; *D*).

showed the same shifts in the FI value there was a significant difference in the FI based on stimulation intervals between pairs of twin pulses (Fig. 5*A*; $P < 0.05$, Wilcoxon paired rank-sum test). In the proximal fibers, existence of a decreasing trend in FI was present among the five preparations only when stimulation between stimulus pairs changed from 0.5 to 1.0 Hz (Fig. 5*B*; $P < 0.05$, Wilcoxon paired rank-sum test). At every stimulation frequency, with the two regions monitored simultaneously, the proximal region facilitated more than the central region for each preparation.

In addition to variation in stimulation frequency, the delay between each pulse, in a set of twin pulses, may be altered. The delay between stimulus pulses was varied between 10, 20, 40, and 60 ms, as illustrated in Fig. 6. As the delay between identical stimulus pulses is increased, there is a trend for the FI to decrease among individual preparations ($n = 5$; $P < 0.05$, Wilcoxon paired rank-sum test), but there is substantial variation in the absolute values among preparations. When delays of 10, 20, 40, and 60 ms are used, the proximal region facilitated more than the central region.

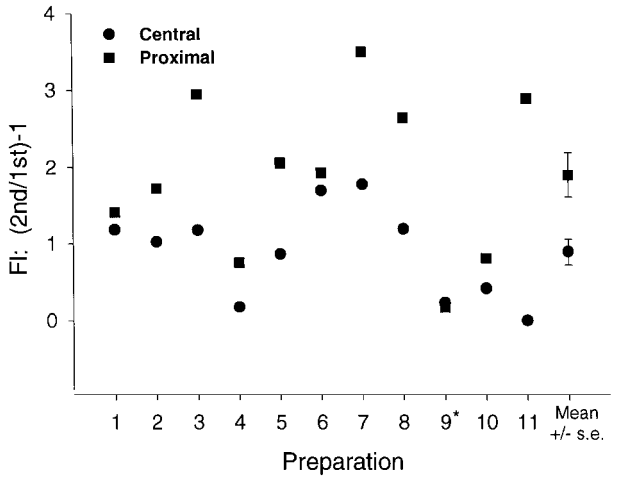

Fig. 3. Twin-pulse facilitation indexes (FIs) measured in 11 different preparations. Means ± SE for the 11 preparations are shown at *right*. Stimulation conditions were as follows: 1 set of pulses every 10 s (0.1-Hz stimulation frequency) and 20-ms delay between the 2 individual pulses. ●, Central muscle fibers; ■, proximal fibers.

Trains. Train facilitation is a less utilized method for measuring the FI of neuromuscular preparations. However, EMG recordings of the opener muscle indicate that short bursts of activity are present in the intact leg. To better understand the effect that trains of activity have on low- and high-output nerve terminals in the animal, we mimicked some of the frequencies measured in the intact leg by using trains of stimulus pulses. Recordings taken when the claw was closed revealed no activity of the opener muscle. When the animal quickly opened its claw, in a defense response to a passing shadow overhead (11), bursts of activity were present (Fig. 7*A*). Analysis of the activity revealed the instantaneous frequencies of the bursts in the EMG recording (Fig. 7*B*). The average of each five events of the burst frequency illustrated that a range of 20–100 Hz would be sufficient to mimic short trains of stimuli (Fig. 7*C*).

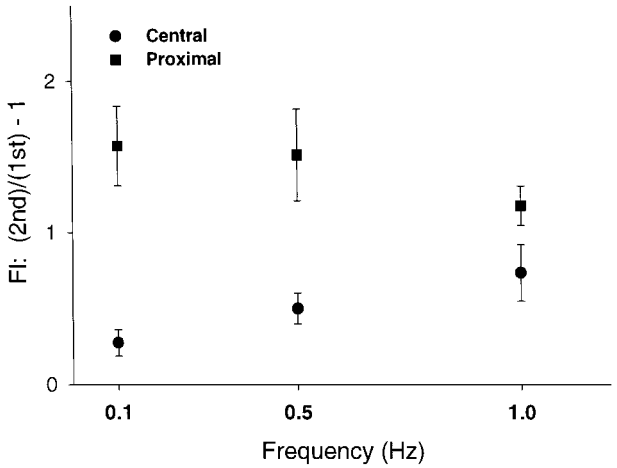

Fig. 4. Twin-pulse FIs measured at different stimulation frequencies between pairs of stimuli. Delay between the 2 pulses was kept constant at 40 ms. Values are means ± SE of a minimum of 5 different preparations. ●, Central muscle fibers; ■ proximal fibers.

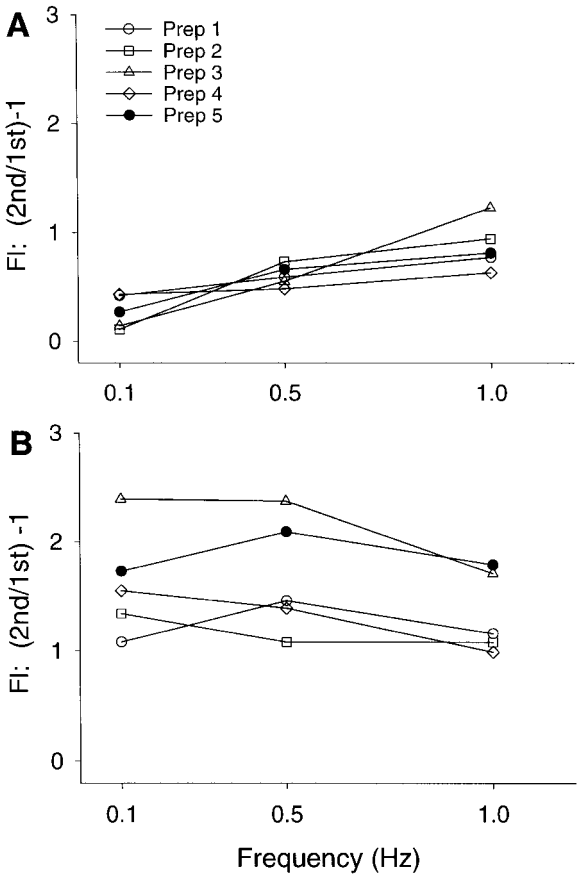

Fig. 5. Twin-pulse FIs at various stimulation frequencies in central region (*A*) and proximal region (*B*). Each data set represents a different preparation (Prep). Delay between individual pulses was held at 40 ms while stimulation frequency was varied from 0.1 Hz (twin pulses every 10 s) to 1.0 Hz (every 1 s).

Quantification of the facilitation induced by trains can be more problematic than for twin-pulse facilitation. We induced train facilitation by providing a series of 10 identical stimulus pulses within a short period of time. Because these stimulus pulses are given so close

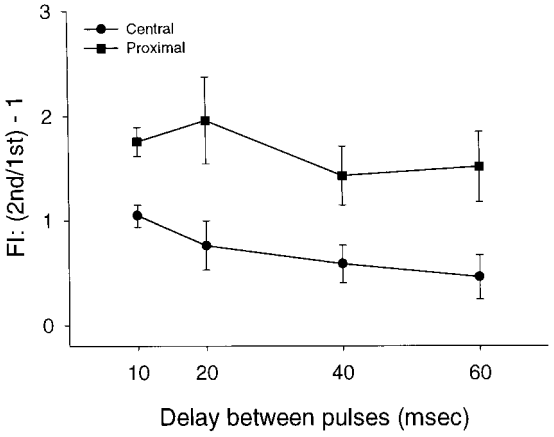

Fig. 6. Twin-pulse FIs at various delays between stimuli within a twin pair. Values are means ± SE for 5 preparations in the central and the proximal regions. Stimulation frequency remained constant at 0.1 Hz while delay between individual pulses was varied from 10 to 60 ms. ●, Central muscle fibers; ■, proximal fibers.

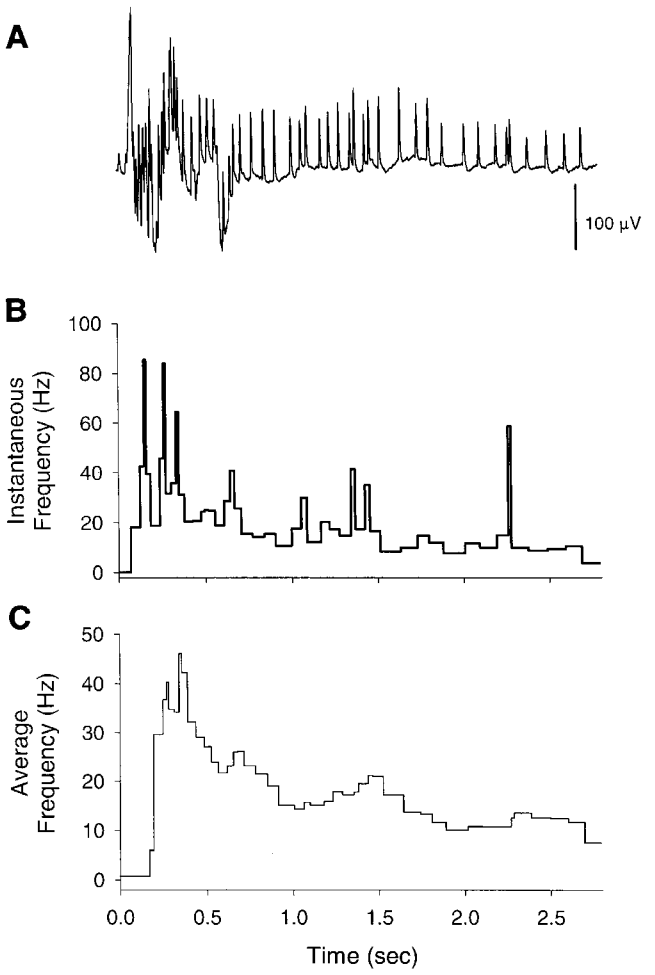

Fig. 7. Electromyographic recordings of intact crayfish claw opener muscle. *A*: muscle potentials recorded when claw is opened quickly and then maintained in open position. *B*: instantaneous frequency of motoneuron firing during opening of chelae. *C*: average frequency of events for each 5 events for the same response. Note change in frequency scale.

together, an EPSP will often rise on the decay of the previous EPSP, as illustrated in Fig. 2. This problem is especially relevant in the proximal region. This leads to difficulty in accurately determining the amplitude of the 10th EPSP. Measuring from the baseline to the peak will give too large an EPSP; measuring from trough to peak represents an EPSP that is too small. This is because the differences in the timing of the trough and the peak are not the same. Thus the real peak is actually smaller because, at the time of the peak, the amplitude of the 9th EPSP continues to decay. Therefore, to account for the discrepancies, a correction factor was used in analysis. The amplitude of the 10th EPSP was measured from baseline to peak to get the absolute difference. The time at the peak of the 10th EPSP was also determined. In a train of nine stimulus pulses, the time to the peak of the 10th EPSP, in a previous train, was measured on the decay of the 9th EPSP. The amplitude at this time was subtracted from the baseline to give the correction factor. The correction factor was then subtracted from the absolute difference

Table 1. *Short-term facilitation in central and proximal muscle fibers*

Preparation No.	FI = (10th/1st) − 1			FI = (10th/3rd) − 1		
	Central	Proximal	%Difference	Central	Proximal	%Difference
1	7.48	10.43	39.4	3.47	4.86	40.1
2	2.13	12.79	500.5	1.5	1.46	2.7
3	1.40	16.93	1,109.3	0.43	2.61	507.0
4	3.10	37.75	1,117.7	1.72	3.00	74.4
5	3.39	9.27	173.5	2.29	0.77	66.4
Mean ± SE	3.50 ± 1.06	17.43 ± 5.3*	588 ± 227	1.88 ± 0.55	2.54 ± 0.70*	138 ± 93

Short term facilitation was measured by using train stimulation paradigm. Values compare central and proximal regions by using facilitation indexes (FIs) calculated with 1st and 3rd pulses. Train rate of 0.1 Hz was used at 30-Hz stimulation frequency. Ninth pulse correction factor is used for all calculations (see MATERIALS AND METHODS). As a result of variability from central region, there was no significant difference between the 2 types of analysis, although with the use of the 1st pulse the mean is much higher. As for the proximal fibers there is a significant difference between the 2 methods utilized (* $P = 0.008$, Mann-Whitney rank-sum test).

(baseline to peak of the 10th EPSP), yielding the corrected 10th EPSP amplitude (26, 27).

Another problem in measuring the FI, as well as in comparing values provided in the literature, is that when a train paradigm is used, the low-output nature of the central region often produces very small (<1-mV) amplitudes of the first EPSPs, resulting in very large FI values, thus producing wide ranges in values among preparations. As the second and third stimulus pulses are given, however, the EPSPs facilitate and a response can readily be measured. Therefore, as an added means of comparison, the amount of facilitation was calculated by altering the equation for FI to compare the 10th EPSP with the 3rd EPSP, rather than the first EPSP. The results are outlined in Table 1. Because the first EPSP is so small in comparison to the 10th, the FI can be larger than when the third and 10th EPSPs are utilized. Each of the preceding nine EPSPs could be used to calculate an FI to determine at which event there is a plateau of the responses and to compare with other types of tonic innervated muscles. At present, beyond the scope of this initial study, future work is in progress for comparing other limb musculature for facilitation profiles.

As with twin-pulse facilitation, significant variation occurred among preparations with train facilitation. When given a 30-Hz train at an interval frequency of 0.1 Hz, the proximal region showed more facilitation than the central region, although the amount of facilitation varied, as did the difference in FI between the two regions (Table 1).

Differences in FI were also observed when the frequency interval between train sets was varied. Figure 8 illustrates how EPSP amplitude is altered when a 30-Hz train is given with interval frequencies of 0.5 and 0.1 Hz. It appears that FI decreased over time with the 0.5-Hz interval for the proximal region. This occurs because a background facilitation resulted in an increased first EPSP amplitude, thus giving rise to a lower ratio in the FI.

In addition, an increase in stimulation train frequency from 30 to 50 Hz, while maintaining the train interval at 0.5 Hz, showed a significant enhancement in the FI for both the central and proximal fibers. The central fibers produced a smaller FI than those for the proximal fibers at both stimulation frequencies (Fig. 9).

Continuous stimulation. STF is obtained quickly when a continuous-stimulation (Fig. 2*D*) paradigm is used. Three stimulation frequencies were used in this set of experiments: 1, 10, and 30 Hz with at least a 20-min recovery period between conditions within a preparation. There are a number of approaches that can be utilized for indexing facilitation when continuous stimulation is given. One method (*method A*) is to take the mean amplitude of the EPSPs at a plateau,

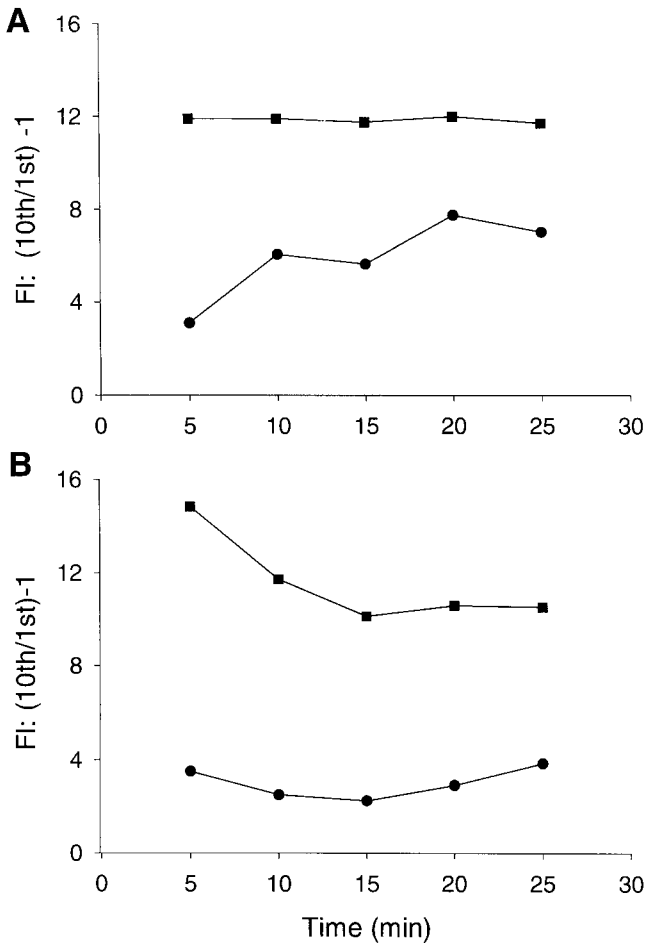

Fig. 8. Train FIs measured at different train rates. FIs are an average of 5 min of stimulation. Preparations were stimulated at 30 Hz every 10 s (0.1 Hz; *A*) and 2 s (0.5 Hz; *B*). ●, Central muscle fibers; ■, proximal fibers.

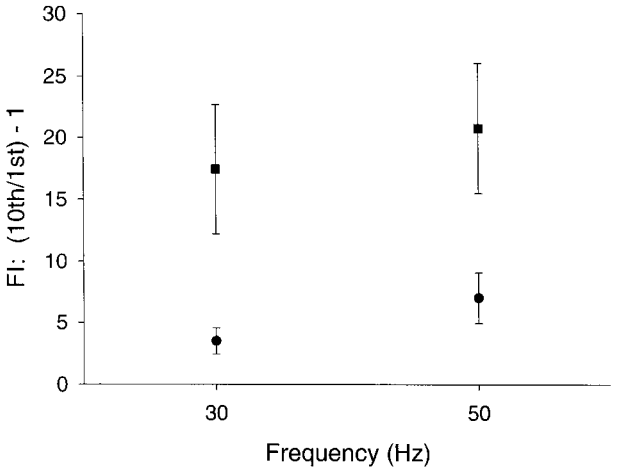

Fig. 9. Train FIs assessed at different frequencies. Train frequency was varied from 30 to 50 Hz while train rate was kept constant at 0.1 Hz. Values are means ± SE of the 5 preparations. ●, Central muscle fibers; ■, proximal fibers.

after a period of stimulation, and divide by the mean amplitude at a lower stimulation frequency [i.e., (10 Hz/1 Hz) −1]. Another approach, *method B*, is to use the EPSP amplitude at the plateau with the EPSP amplitude at the beginning of stimulation. Typical comparative values obtained by these two methods, within a single preparation, revealed that the FI was smaller in *method A* (0.80) than for *method B* (3.80) for central muscle fibers, but, for proximal muscle fibers, *method A* (6.61) produced a larger FI than did *method B* (1.46). This again demonstrated that contrasting FI values obtained for central and proximal fibers depend on the quantifying approach utilized. Differences were noted between the central and proximal regions, although there was not a consistent difference among the central and proximal regions for the continuous-stimulation paradigm, as with those obtained from trains or twin pulses.

DISCUSSION

Facilitated responses are readily observed in crustacean neuromuscular junctions (9) because the muscles do not normally conduct depolarizations via action potentials. The well-identified neuromuscular junctions of the crayfish provide accessible and viable preparations for investigating the underlying mechanisms of synaptic facilitation. In this study, we used the leg opener muscle to compare differences in facilitation responses between two regions of the muscle innervated by the same single motoneuron. Previous investigations using this preparation have examined differences between muscle regions by testing a single stimulation paradigm but not a variety of conditions. We have shown in this study that very different FIs are able to be obtained on the basis of the stimulation protocol. In addition, because the EMG activity indicates that trains of activity are normally used to produce muscle contractions, we used a range of pulse trains within physiologically recorded responses of muscle activity. The novelty of the results presented in this study are *1*) the motor nerve terminals on the proximal fibers show a greater facilitation than those on the central fibers, *2*) the degree of facilitation is dependent on the stimulation paradigm used, *3*) the FI is also dependent on the type of measurement and analysis for a given response, and *4*) the time duration between induction of train stimulations can influence background facilitation and can thus obscure results over time if not controlled.

Contrary to our initial hypothesis that the low-output terminals will show a greater degree of facilitation than high-output terminals for twin-pulse, train, and continuous-stimulation paradigms, the data presented here clearly show just the opposite. (Nonetheless it is quite interesting that when the low- and high-output regions of the crayfish opener motoneuron are compared, differences in the degree of facilitation are inherent among the terminals of this single neuron.) When twin-pulse facilitation at a given set of stimulation conditions (20-ms delay, 0.1-Hz frequency) is examined, the high-output proximal region displayed a larger enhancement of EPSP amplitude and a larger FI compared with the low-output central region. In twin-pulse experiments, as the frequency of paired stimulation is increased, the amount of facilitation increased. Facilitation decayed when the delay was increased from 10 to 60 ms. When train facilitation was used, the proximal region again facilitated more than the central region, and both regions showed an increase in FI as the train frequency was increased from 30 to 50 Hz. The train rate is important as well. When the rate was increased (from 0.1 to 0.5 Hz), the high-output proximal region began to decrease in facilitation. Facilitation evoked by continuous stimulation showed quite a bit of variation among the central and proximal fibers. However, the facilitation was proportional to the frequency: the higher the frequency, the larger the EPSP amplitudes at the plateau.

An earlier report (13) examined the differences among EPSP amplitudes of the distal and central fibers of the opener. This past study used the dorsal superficial aspect of the muscle compared with the ventral superficial approach. The ventral aspect, as used in our study, allows one to examine the very proximal fibers, which show even greater differences in responses compared with the central fibers. The most dorsal distal fibers behave in a similar manner to the ventral proximal fibers in that they produce a larger EPSP than do the central fibers in either the dorsal or ventral side. Only with the ventral approach can one distinguish the very proximal grouping of fibers that are strikingly different from the central fibers because their diameter is much smaller and the bundles are tightly packaged together (22). Linder (48), using the dorsal approach to the opener muscle, compared twin-pulse facilitation values between the distal (higher output terminals than central) and the central fibers. He did show that at short intervals (<30 ms) the lower output terminals facilitated more than the distal fibers, but as the interval between stimuli was increased, the higher output terminals demonstrated a greater FI. Similarly, in our study, the fibers (distal) in which a larger EPSP is recorded

with a single pulse results in a larger facilitation for twin pulses. Unfortunately, Linder did not try multiple-stimulation paradigms for a fuller comparison in FIs between the central and distal fibers on the dorsal surface. The ventral proximal grouping of fibers can easily be missed because of their pinnate arrangement, as illustrated in Fig. 1 and in reports that show the stained nerve (20, 22). The very proximal tuft of muscle fibers should be used in the anatomic description for the "proximal" muscle fibers. In attempts to clearly differentiate variations in responses that were being observed in synaptic current recordings, Parnas et al. (54) made recordings from terminals on the very proximal tuft of fibers and central fibers (see Fig.1 in Ref. 54). They recorded the synaptic currents with a focal macropatch electrode placed over a discrete region of the terminals in the two regions. Because the terminals were not visualized with the recent advent of vital dyes (8, 49), the electrode had to be placed blindly along the fibers until responses could be measured. The results of the Parnas et al. study showed overlap among preparations in the degree of twin-pulse facilitation at the Ca^{2+} concentration of 13.5 mM used in our study for the central and proximal fibers. In their study, they called the fibers "distal" to represent the fibers not in the proximal grouping but those that "cover most of the inner surface of the muscle" (54). In fact, these actually contain mostly central fibers and some truly distal fibers, as described in this study (Fig. 1) and other studies for the ventral surface of the opener (20–22). Because of the use of vital dyes to localize varicosities along the nerve terminals on proximal and central fibers, it has been shown that large differences in the synaptic efficacy occur along the length of single terminals (21–23, 25). This means that in blindly placing a focal electrode one does not know the location on the terminal at which the recording is made. It appears that too many variables have not been controlled for in previous studies to characterize differences in FIs among terminals on the proximal and central muscle fibers. This points to the necessity for multiple-stimulation paradigms to be used and anatomic locations to be similar before results of earlier studies can be used for comparison.

To explain the facilitation results, we must look at the mechanisms underlying STF. Vesicular release is due to the influx of Ca^{2+} into the nerve terminal via voltage-gated channels clustered near release sites (55). The amount of neurotransmitter released into the synaptic cleft is proportional to the length of depolarization and therefore to the amount of Ca^{2+} that has entered the cell (61–63, 68). Once the action potential has occurred, there are several mechanisms for returning the nerve terminal to basal Ca^{2+} concentrations. Ca^{2+} is stored intracellularly, bound by Ca^{2+}-binding proteins, and extruded from the cell via Ca^{2+} pumps (51). Therefore, if the kinetics of the action potential are changed, the amount of Ca^{2+} influx is altered, and thus the degree of synaptic transmission is also altered (10). When the time between pulses is short, there is not enough time to allow Ca^{2+} extrusion mechanisms to return the terminal to basal concentration levels. Therefore, the subsequent action potential begins on the decay of the preceding Ca^{2+} current (19, 75, 76, 78). The increase in residual Ca^{2+} will allow more neurotransmitter to be released, and therefore larger EPSPs will be produced. Thus STF is dependent on the time it takes for the removal of Ca^{2+} from the nerve terminal (52). The structural data obtained from serial sections of the terminals between the two regions has shown that the higher output terminals contain more synapses with multiple active zones (sites where vesicles are released) than do synapses on low output terminals (9, 21, 22, 25, 41). This structural difference in the synapses may account for the greater release with single pulses as well as with repeated stimuli, thus resulting in a greater facilitation than for the synapses with relatively few active zones.

The continuous-stimulation protocol indicates that the high-output proximal terminals release large amounts of neurotransmitter initially, but, as the frequency of stimulation is increased, FI is increased up to a point after which a saturation is reached for the EPSP amplitudes. EPSP amplitudes only obtain a maximum amplitude in the range of 10–15 mV before a plateau is reached (see Fig. 2*C*). Similarly, the central fibers showed saturation as well. Because the plateau it is not due to the lack of a driving gradient, given that the resting membrane potentials of the fibers are in a range of -60–70 mV and given that the glutamate-induced currents have a reversal of $\sim$0 mV, this means that there is still plenty of driving force for more current to enter the muscle fibers if more ligand-gated channels are opened by the presence of glutamate. When given only two stimulus pulses, this saturation is not reached. In the case of trains, this may mean that the first, second, and third pulses are still increasing, but the latter pulses begin to reach their plateau. Therefore, there is not as much difference among the 8th, 9th, and 10th EPSP amplitudes. This means that while individual EPSP amplitudes are enhanced with frequency facilitation, the difference between responses, or the ratio, at a given time is decreased. This may explain some of the varied results during the continuous stimulation.

There are several posited reasons for the saturation in synaptic transmission in the proximal region. One is the depletion in the ability to continually supply newly loaded vesicles for docking and subsequent release. Therefore, although there is an increase in residual Ca^{2+}, there may not be enough rejuvenated vesicles to respond. Additionally, the cytoplasmic proteins present at the active zone that establish the docking complex may become rate limiting (43). It is also possible that the cause is postsynaptic. The number of glutamate receptors present on the postsynaptic membrane may be inadequate to accommodate larger amounts of neurotransmitter released when the frequency of release is increased. This possibility can be examined with quantal analysis of spontaneous events (21) and kinetic states of the glutamate receptors (39). Another explanation might be that the average presynaptic Ca^{2+} reaches a plateau level (57) and that high amounts of Ca^{2+} may be buffered by mitochondria or Ca^{2+}-binding proteins (50, 60).

This report has given insights into STF. However, the exact mechanisms responsible for the differences in release under facilitated conditions are not yet all defined. Perhaps with computational analysis utilizing morphological and physiological data for these terminal regions, insights as to these mechanisms will be forthcoming (24, 67, 71).

In conclusion, regional differences in synaptic transmission observed along the terminals of a single motoneuron depend on the region of the target tissue innervated. The high-output terminals on the proximal region of the muscle produce larger EPSPs than do the low-output terminals on the central region of the muscle. Differences in STF seen between these two regions are dependent on the type of stimulation paradigm utilized. Ranges in stimulation conditions are useful to characterize possible underlying mechanisms that account for synaptic differentiation. In an attempt to explain why there are such differences among the terminals of a given neuron, we postulate that the different muscle phenotypes between the proximal and central muscle fibers of the opener (42) provide different types of contact or retrograde cues to the local terminals. This results in synaptic structural and physiological differences for terminals of the same neuron.

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