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Quantal Release at Visualized Terminals of a Crayfish Motor Axon: Intraterminal and Regional Differences

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ABSTRACT

Synaptic transmission was measured at visualized terminal varicosities of the motor axon providing the sole excitatory innervation of the "opener" muscle in walking legs of crayfish (*Procambarus clarkii* Girard). Two questions were addressed: 1) How uniform is quantal emission at different locations along terminals innervating a single muscle fiber, and 2) can differences in quantal emission account for the different excitatory postsynaptic potential (EPSP) amplitudes generated by terminals localized in defined regions of the muscle?

Extracellular "macropatch" electrodes were placed over individual varicosities, viewed after brief exposure to a fluorescent dye, and synaptic currents were recorded to determine quantal content of transmission. Along terminals supplying a single muscle fiber, nonuniform release was found: Varicosities closer to the point of origin of the terminal branch released more transmitter than those located more distally. Quantal content was higher for varicosities of the muscle's proximal region (where large EPSPs occur) than for varicosities of the central region (where small EPSPs occur). The probability of transmitter release per synapse is estimated to be greater for the proximal varicosities.

At low frequencies of stimulation, quantal content per muscle fiber is two to four times larger in the proximal region. Taken in conjunction with a twofold higher mean input resistance for the proximal muscle fibers, the difference in quantal content can account for a four- to eightfold difference in EPSP amplitude. The observed mean EPSP amplitude is at least eight times larger in the proximal region. We discuss factors contributing to differences in EPSP amplitudes.

Indexing terms: synapse, active zone, motorneuron, presynaptic, postsynaptic

For investigation of synaptic physiology, the excitatory and inhibitory axons of the "opener" muscle of the crayfish walking leg have been widely used as a general model (Dudel and Kuffler, 1961a–c; Bittner, 1968a). A single glutamatergic excitatory axon innervates all the opener muscle fibers and generates excitatory postsynaptic potentials (EPSPs) of different amplitude in the individual muscle fibers of different regions (Iravani, 1965; Bittner, 1968a; Thompson and Atwood, 1984; see Fig. 1). The larger synaptic potentials facilitate less at higher frequencies of activation (Bittner, 1968a; Atwood and Bittner, 1971). Physiological differentiation of the nerve terminals and their follower cells provides for selective recruitment of muscle fibers through different EPSP amplitudes, an important means of regulating muscle performance in neuromuscular systems with only one or a few motor axons. Physiological differentiation of synapses is found also in the central nervous systems of mammals (Pierce and Mendell, 1993; Shigemoto et al., 1996), leeches (Muller and Nicholls, 1974), and other species.

In the present study, we investigated two aspects of differential neurotransmission. 1) Given that a nerve terminal (the part of the motor axon in contact with the muscle fiber, where synapses occur) is able to release neurotransmitter at different locations along its length, is the amount released the same at all release sites on a single muscle fiber? 2) In regions of the muscle with different EPSP amplitudes, is differential release of neurotransmitter in those regions the major determinant of EPSP amplitude?

The first question has not been carefully and systematically addressed in previous studies of crustacean neurotransmission. In part, this has been due to technical limitations:

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Vital dyes permitting electrophysiological recordings from visualized, normally functioning nerve terminals have not, until recently, been available. Thus, in past work, it has often been assumed that all transmission sites derived from one axon and located on a single crustacean muscle fiber have the same physiological properties (Frank, 1973), although a few examples have been reported where this is likely not to be the case (Dudel and Kuffler, 1961b; Lang et al., 1970). In contrast, for frog neuromuscular junctions, it has been shown that the parts of the terminal nearest the contacting motor axon have a higher rate of transmitter secretion than more distal locations (Bennett and Pettigrew, 1975; Robitaille and Tremblay, 1987). With the advent of new fluorescent dyes (Magrassi et al., 1987), it is possible to visualize individual living nerve terminals with better resolution and fidelity than before. With a focal electrode, one can record from several locations along a nerve terminal and relate the recordings so obtained to the structures giving rise to them. In the present study, we found that release along a single visualized terminal is not uniform. This finding must be taken into account in assessing physiological results and in estimating quantal release for the muscle fiber as a whole.

The second question, regarding regional differentiation of EPSP amplitude, has been addressed in a number of previous studies (Atwood, 1965, 1967; Iravani, 1965; Bittner, 1968a,b; Atwood and Bittner, 1971; Sherman and Atwood, 1972; Walrond et al., 1993; Govind et al., 1994; Cooper et al., 1995a; review, Atwood and Wojtowicz, 1986). The regional differences in EPSP properties have been shown by Bittner (1968a) to have major importance in grading the tension of the whole muscle at different frequencies. In crustacean limb muscles, the muscle fibers with large EPSPs tend to have a higher input resistance than those with small EPSPs, but this factor accounts for only a modest fraction of the difference in EPSP amplitude. Presynaptic mechanisms resulting in higher levels of synaptic transmission account for more of the difference (Bittner, 1968a; Atwood and Bittner, 1971; Sherman and Atwood, 1972). Among the factors that could be involved in differential performance of a single axon's endings, those emphasized in previous investigations have been differences in morphological features, such as the presence of larger or more numerous synaptic "active zones" in high-output terminals (Atwood and Marin, 1983; Walrond et al., 1993; Govind et al., 1994), and physiological factors, including possible differences in the nerve terminal's electrical properties (Sherman and Atwood, 1972; Dudel et al., 1983) and the entry of more calcium per impulse in terminals with higher transmitter output (Atwood et al., 1994; Cooper et al., 1995a).

With the development of fluorescent dyes that can be applied to living nerve terminals without severely injuring them, morphological diversity among endings on crustacean muscle fibers has been frequently observed (Tse et al., 1991; Atwood et al., 1994; Govind et al., 1994; Harrington and Atwood, 1995). The question arises, are the morphological differences among terminals in different locations accompanied by physiological differences, and, if so, what implications would this have for attempts to define the structure and function of a neural system by taking limited samples for physiological or microanatomical analysis?

The question of whether it is possible to account fully for the physiological properties of transmission on different target cells on the basis of analysis of quantal release at individual transmission sites can now also be raised. Earlier surveys of crayfish muscle fiber innervation produced an estimate of the number of synapses per muscle fiber (Florey and Cahill, 1982), but differences among muscle fibers were not studied, and physiological information was not presented. The most complete physiological information on the crayfish (claw) opener muscle is provided by Bittner (1968a,b) and Bittner and Kennedy (1970), but there is no detailed information on nerve terminal morphology in these papers.

In the present work, we addressed these questions with regard to the crayfish (leg) opener muscle by combining observations using a vital fluorescent dye with focal macropatch recordings at single visualized varicosities of the nerve terminals. We also analyzed samples of nerve terminals, obtained from electron micrographs, of defined regions of the muscle. On the basis of these observations, we reassessed the overall comparative effectiveness of individual synaptic varicosities and synapses in two regions of the muscle where the EPSP amplitudes are markedly different. A provisional "balance sheet" to account for the contribution of pre- and postsynaptic factors to EPSP differences was drawn up. The limitations of the sampling approach are illustrated and discussed. This information will be useful both for attempts to account for physiological differentiation in more detail and for explorations of the developmental mechanisms that give rise to the definitive organization and properties of this neural system and others like it.

MATERIALS AND METHODS

Animals

Freshwater crayfish, *Procambarus clarkii* Girard, of intermediate size (6 cm rostrum to telson, weight 5–6 g) were used for most of these experiments. Larger adults (7.5–9 cm, 19–21 g) and smaller animals (3.5–4 cm, 2–2.5 g) were also used for a few comparative morphological measurements on nerve terminals (Table 1). The opener muscle of the first (or occasionally the second) pair of walking legs, prepared by standard dissection (Dudel and Kuffler, 1961a; Wojtowicz and Atwood, 1984), was set up for simultaneous fluorescence microscopy and electrophysiology in modified van Harreveld's solution (which contained 205.3 mM NaCl, 5.3 mM KCl, 13.5 mM $CaCl_2.2H_2O$, 2.5 mM $MgCl_2.6H_2O$, and 0.5 mM HEPES buffer, adjusted to pH 7.4).

Light microscopy

Fluorescence microscopy of living nerve terminals was carried out with the aid of a vital fluorescent dye, 4-[4-(diethylamino)styryl]-N-methylpyridinium iodide (4-Di-2-Asp; Magrassi et al., 1987), obtained from Molecular Probes (Eugene, OR). The use of this dye for this preparation had been evaluated in two previous studies (Cooper et al., 1995a; Harrington and Atwood, 1995). Concentrations of 2–5 μM in crayfish solution were used; preparations were immersed for 2–5 minutes and rinsed twice with dye-free solution before viewing. Preparations so treated continue to release transmitter for the duration of the experiment as in standard solution (Cooper et al., 1995a). An upright epifluorescence microscope (Nikon Optiphot model) equipped with ×20 (long distance dry; NA 0.4) and ×40 (water immersion; NA 0.55) objectives, appropriate filter blocks, and a photomicrographic attachment were used for preliminary morphological work and for simultaneous electrophysiology. For

more detailed morphological work, such as obtaining composite images for illustrating the double labelling (see Fig. 5), the fluorescence microscope was used in conjunction with a Bio-Rad 600 confocal laser microscope. Further technical information can be found elsewhere (Harrington and Atwood, 1995).

Innervation of proximal and central regions was compared: length and number of branches and number of terminal varicosities were observed after staining the preparation with 4-Di-2-Asp (see Figs. 1, 2, 4). Particular attention was given to determining the number of excitatory varicosities on selected muscle fibers, as a step in estimating the number of synapses on a muscle fiber. Most of the individual synapses occur on these varicosities (Florey and Cahill, 1982). As in previous studies (Lnenicka et al., 1986), a varicosity was defined as a region of the terminal where its diameter increased abruptly by a factor of 5 or more to produce a structure 1 μm or greater in diameter. In preparations treated with 4-Di-2-Asp, total varicosities on the exposed inner surface were counted; these counts were used to provide an estimate of the number of excitatory varicosities, after additional observations had been made (as described below), to determine what percentage of the total was excitatory and how many additional varicosities were likely to occur on the unexposed surfaces of the muscle fibers.

A second method was employed in several preparations to estimate the number of excitatory varicosities independently. The excitatory motor axon was selectively filled with horseradish peroxidase (HRP) by pressure injection (Lnenicka et al., 1991). Following HRP injection, fixation, and processing, varicosities were counted on selected muscle fibers in which the terminal innervation on the facing surface of the muscle fiber could be clearly visualized.

A third method was also employed to estimate relative numbers of excitatory and inhibitory varicosities. The excitatory or the inhibitory axon was impaled with a microelectrode filled at the tip with Lucifer yellow CH (3% in distilled water) or with 5% Texas red conjugated to 10 kD dextran (Molecular Probes) in 100 mM filtered KCl; these dyes were ejected iontophoretically and by applied pressure, respectively, to fill the axon and its regional branches. Texas red was preferred because it bleaches less rapidly with illumination. Photographs of filled branches were then taken under fluorescence microscopy. Following this, the preparation was treated with 4-Di-2-Asp to stain the branches of the unfilled axon, and the same regions were again photographed. The branches of the Lucifer yellow-filled axon were usually only faintly visible after the second staining treatment, but their locations could be readily discerned. Texas red-filled branches remained visible (see Fig. 5). Separate counts of excitatory and inhibitory boutons were then made for sampled regions. This method and the HRP method showed clearly that the numbers of excitatory and inhibitory varicosities on a muscle fiber were approximately the same, as reported previously for this muscle in another crayfish species by Florey and Cahill (1982). Low-power electron micrographs provided additional information that served to confirm the proportion of excitatory varicosities in the two muscle regions of interest (Table 1). Thus, in preparations stained with 4-Di-2-Asp, the total number of varicosities was multiplied by the observed fraction of excitatory varicosities to obtain the number of exposed excitatory varicosities on a muscle fiber. Counts of excitatory varicosities obtained by the three methods described above were similar. The most precise information was derived from electron micrographs, and this was used in estimating the percentage of excitatory varicosities per muscle fiber (Tables 1, 2).

Electron microscopy

Low-power electron microscopy of muscle fiber bundles in proximal and central regions was used to estimate the fraction of excitatory varicosities on exposed (ventral superficial) muscle fibers of the opener muscle's inner surface. In preparations made for light microscopy, we could readily count varicosities on the exposed surface of the muscle fibers, but we could not be certain the innervation seen on the unexposed dorsal (outer) surface of muscle fibers belonged to the superficial layer, which we were sampling, or to the next (subsurface) layer. The muscle fibers are too closely packed in the living (or even in fixed) preparations to permit unambiguous spatial resolution of subsurface varicosity locations. Thus, we made an estimate of additional innervation on the unexposed surface through analysis of low-power electron micrographs in which the relative number of varicosities on inner and outer surfaces of the most superficial layer of muscle fibers could be unambiguously ascertained (see Fig. 3).

For this procedure, two surveys were conducted. The first, limited to the central region, involved counting varicosities found in random sections of seven different specimens that had been prepared for previous studies (Wojtowicz et al., 1994; Govind et al., 1994). The second survey was done in isolated bundles of muscle fibers from the two regions. Four opener muscles were fixed for electron microscopy using well established procedures (Jahromi and Atwood, 1974). Identified bundles of muscle fibers (5 or 6 fibers per bundle) were dissected from proximal and central regions and embedded with their ventral surface at the top of the block. One of the central bundles and four proximal bundles (each from a different animal) were sampled systematically along their lengths. These bundles were each 1–2 mm long from cuticular to apodeme attachments. Ultrathin sections were taken every 200 μm for visualization and printing of montages. Thus, for each fiber bundle, five or six locations were sampled. The resulting photographic negatives were printed at ×12,000 in order to distinguish excitatory from inhibitory innervation by the characteristic differences in vesicle shape and synaptic membrane density (Jahromi and Atwood, 1974; reviewed in Atwood and Tse, 1993). Excitatory varicosities on exposed and unexposed surfaces of superficial muscle fibers were counted (Table 2). From these counts, the fraction of excitatory varicosities on the exposed ventral surface of the muscle fibers was determined and used as the basis for estimating the total number of excitatory varicosities on a muscle fiber (Table 3).

Excitatory postsynaptic potentials

Recording of EPSPs was accomplished with standard intracellular microelectrode techniques (Wojtowicz and Atwood, 1986). Isolation of the excitatory axon for stimulation was carried out as described by Dudel and Kuffler (1961a). Average EPSP amplitude was measured in "low-output" (central) and "high-output" (proximal) muscle fibers in several preparations by stimulating selectively the excitatory motor neuron 200 times at 1 Hz (see Fig. 1).

Electrical properties of the muscle fibers

Input resistance and diameter of muscle fibers in central and proximal regions were measured. Fiber diameters were measured with a ×10 eyepiece micrometer (×40 water-immersion objective; NA 0.55) with transmitted incandescent light. To confirm these measurements, we filled selected fibers iontophoretically with Lucifer yellow and remeasured their diameters under epifluorescence. There was no difference between the two sets of measurements.

Input resistance (R_{in}) was determined by impaling each sampled fiber in its central region with a microelectrode, 10–20 MΩ (3 M KCl) and passing current pulses of 20 milliseconds' duration within the range of −5 to +5 nA. The slope of the voltage-current plot produced a value for R_{in}.

A comparative measure of the specific membrane resistance (R_m), referred to unit surface area of the muscle fiber and without regard for the infoldings that are known to occur in crustacean muscle fibers (Selverston, 1967), was calculated from the standard cable equations:

$$R_m = 8\pi^2 a^3 R_{in}/R_i,$$

where a is the radius of the muscle fiber and R_i is the specific internal resistance. Values for R_i have been previously determined for crayfish muscle fibers by Law and Atwood (1971) and by Lnenicka and Mellon (1983a); the estimated mean values are 140–150 Ωcm and 167 Ωcm, respectively. Lnenicka and Mellon (1983b) have shown that R_i is relatively constant over a wide range of muscle fiber diameters in crayfish; we used their reported value of 167 Ωcm in our calculations. We emphasize that R_m is a relative measure used to compare the proximal and central muscle fibers and not an absolute measure, because the calculation does not take into account the fiber's true surface area (Selverston, 1967). The calculation was made to see whether it was likely that relative differences in R_m could account for differences in measured EPSPs.

We also compared the rise times of membrane potential changes induced at the impaling microelectrode by square pulses of injected current. For this comparison, we selected the time taken for the voltage change to reach 50% of its plateau level (referred to here as t_{50}; Table 4) during hyperpolarizing and depolarizing current injections 1–20 nA in amplitude and 20 milliseconds in duration. This comparison was made to ascertain whether it was likely that differences in temporal distortion of voltage changes resulting from synaptic currents could arise from muscle fiber membrane properties and thereby play a significant role in determining the observed differences in EPSPs of central and proximal regions.

Focal extracellular recording

Focal extracellular recording of synaptic currents with "*macropatch*" electrodes (Dudel, 1981) of 10–15 μm inside diameter was done with the aid of an amplifier designed for this purpose and obtained from Zeitz Instrumente Vertriebs GmbH (Augsburg, Germany). The recording electrode was filled with the standard crayfish solution. In the present experiments, the focal recordings were obtained from individual visualized varicosities of nerve terminals in the central and proximal regions of the opener muscle (see also Cooper et al., 1995a). Selected terminal varicosities were visualized under fluorescence microscopy after 4-Di-2-Asp application, allowing precise placement of the macropatch electrode. A photograph of the terminal was taken to provide an accurate record of the recording site. Drawings were later made to display the positions of the recording electrode along the nerve terminals (see Figs. 6–8).

The time constant of decay (τ) of single quantal events was measured (from peak to baseline) from current recordings obtained over the visualized terminals. Determination of quantal content (m) at low frequencies of stimulation was achieved by counting the individual quantal events. This was feasible at 1 Hz, but usually not so at higher frequencies; therefore, we used 1 Hz as the standard frequency for accurately comparing synaptic transmission in the two regions (detailed consideration and comparison of different methods for determining quantal content in this preparation is given in Cooper et al., 1995b).

Analysis of binomial parameters for this preparation has been described in detail elsewhere (Wojtowicz et al., 1994; Cooper et al., 1995a,b). In brief, binomial distributions with uniform or nonuniform probabilities of release generally provided good fits to the observations, which consisted of counts of quantal units for sequences of 1,000 or more stimuli at each site. From the best fit distribution, the quantal parameters n (number of responding elements or putative release sites) and p (the mean probability of release at responding sites) can be calculated. We used two algorithms to estimate n and p: One allows for nonuniform probability of release and uses a modified chi-square (χ^2) criterion, and the second assumes uniform probability of release and derives parameters from maximum likelihood estimation (MLE). The "bootstrap" estimate of standard errors was used to determine the accuracy of n and p estimates. The evoked responses from each reported recording site met the requirements for stationarity for the time of the experiment. For accurate statistical analyses, at least 1,000 sweeps were collected at each recording site (Smith et al., 1991; Wojtowicz et al., 1991).

RESULTS

Innervation of the opener muscle

The inner (ventral) surface of the opener muscle is supplied by two major axons, one excitatory (the "opener excitor," or OE, axon) and one inhibitory (the "opener inhibitor," or OI, axon), and by a branch of the leg's "common" inhibitory (CI) axon in a restricted part of the proximal region only (Wiens, 1989). The two major axons branch extensively over the inner surface, and in dye-treated preparations they can readily be followed to the level of terminals on the muscle fiber (Fig. 1). Each muscle fiber is typically contacted by two to four secondary branches at different locations along its length. These branches in turn give rise to varicose tertiary terminal branches or (in the proximal region) to more localized clusters of varicosities (Figs. 1, 2). The varicosities of the terminals are known to provide most of the synaptic input (Fig. 3) to the muscle fibers (Jahromi and Atwood, 1974; Smith, 1978; Florey and Cahill, 1982). The two major axons, OE and OI, are always closely associated and provide a similar number of varicosities to each muscle fiber (Figs. 2, 5). The CI axon was sometimes observed as a smaller, third axon innervating a few of the proximal fibers but was not seen in the central region; this anatomical feature is in agreement with physiological observations (Wiens, 1989).

The regional physiological differences of the opener muscle have long been known (Iravani, 1965; Bittner, 1968a;

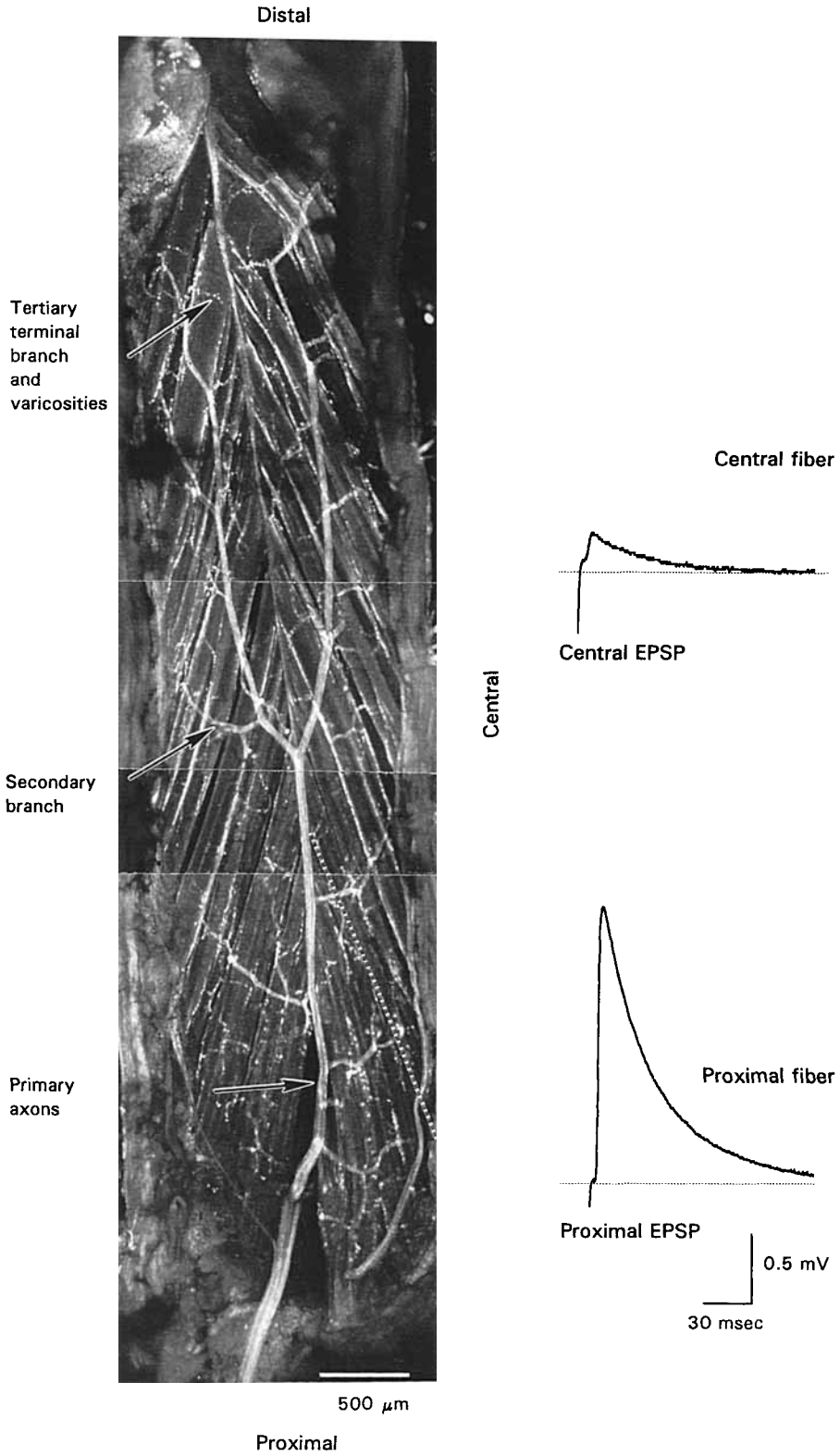

Fig. 1. General view of the inner surface of the opener muscle, showing distribution of nerve branches. A preparation stained with 4-Di-2-Asp was photographed with the confocal microscope. The primary axons, and representative secondary and tertiary structures (the latter with synapse-bearing varicosities) are indicated by arrows. A dotted line indicates the division between central and proximal regions of the right side of the muscle. Representative averaged excitatory postsynaptic potentials (EPSPs) recorded at 1 Hz from two different fibers (one central, one proximal) are shown to illustrate the range in amplitude. EPSPs of the proximal region are the largest. Scale bars = 500 μm (anatomical), 30 milliseconds, 0.5 mV (EPSP records).

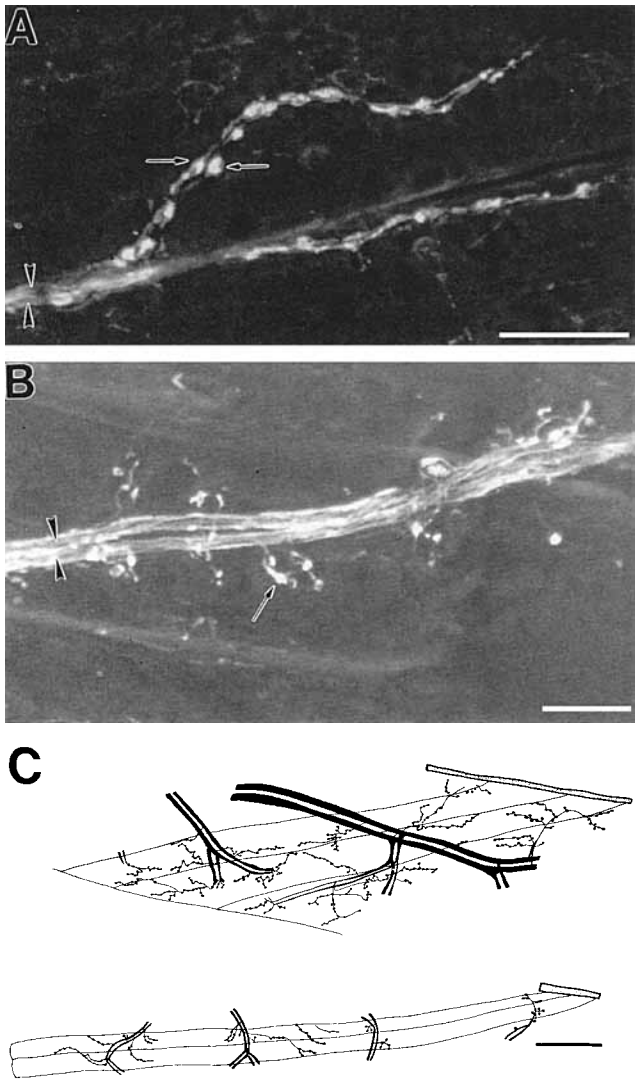

Fig. 2. Nerve terminals and synaptic varicosities of the crayfish opener muscle. **A:** Enlarged view of varicosities (arrows) of the central region in a preparation stained with 4-Di-2-Asp. The varicosities occur in "strings," with parallel excitatory and inhibitory varicosities often evident. The two axons are indicated by arrowheads in a secondary branch. **B:** General view of secondary branch and associated terminals in the muscle's proximal region (4-Di-2-Asp stain). Varicosities occur in "clusters" near the prominent secondary branch, which has two large axons in it. Arrowheads indicate the two axons; the arrow indicates one of the varicosities. **C:** Drawing of representative muscle fibers from 4-Di-2-Asp stained central (upper) and proximal (lower) regions to illustrate the comparative extent of innervation and relative numbers of visible varicosities. The proximal fibers typically have fewer varicosities on their exposed surface and less extensive tertiary strings. Scale bars = 50 μm in A, 25 μm in B, 44 μm in C.

Atwood and Bittner, 1971; Thompson and Atwood, 1984; Cooper et al., 1995a). Muscle fibers in the proximal region of the muscle's inner surface usually have a much larger EPSP than those of the more extensive central region when low frequencies of stimulation are applied to the excitatory motor axon (Fig. 1). Physiological properties of the inhibitory axon parallel those of the excitatory axon in this and other crustacean limb muscles (Atwood and Bittner, 1971).

In dye-stained preparations, morphological differences were observed in the two muscle regions. The terminals in the proximal region were generally shorter than those in the central region and often produced clusters of varicosities near the main axon branches (Fig. 2B,C). Terminal branches and numbers of varicosities on the exposed surface of the muscle were compared for the two regions. Three stained preparations were photographed to provide data on branch length and amount of branching; these data are summarized in Figure 4. Branch length was measured as the sum of all terminal segments; the first varicosity marked the beginning of the first segment. Branches after the first varicosity were considered in the branch count. *Branching factor*, defined as the number of bifurcations along a terminal, was determined also for the two regions.

In all three preparations, branching was less in the proximal than in the central region: Branching factors were two to three times smaller. In two of the three preparations, terminal length was also significantly less in the proximal region, whereas, in the third preparation, terminal length was not significantly different between the two regions due to the occurrence of a particularly long branch in the proximal region. The data indicated that branching is less and terminal length often shorter in the proximal region. A similar observation was previously made for the homologous motor neurons in a crab leg muscle: Fibers with large EPSPs had fewer sites of innervation, shorter terminals, and compact clusters of varicosities rather than long strings (Tse et al., 1991).

Counts of varicosities on muscle fibers

We first estimated the relative numbers of excitatory and inhibitory varicosities on the exposed surfaces of central and proximal muscle fibers. Differential staining of excitatory and inhibitory innervation (Fig. 5) confirmed previous reports (Florey and Cahill, 1982) that the two major axons are closely associated and provide nearly equivalent numbers of varicosities to the muscle fibers which they innervate. This observation was checked in electron micrographs (Fig. 3), in which the ratio of excitatory to inhibitory varicosities could be clearly determined for central and proximal regions. It was confirmed that this ratio was close to 50% in both regions, with excitatory varicosities slightly more frequent (Table 1). The mean values for percentage of excitatory varicosities in Table 1 were used in estimates of the number of excitatory varicosities per muscle fiber (Table 3).

Next, we estimated the number of excitatory varicosities per muscle fiber in the two regions. Varicosities on the superficial surface of the most ventral layer of fibers were stained with 4-Di-2-Asp, photographed, and counted (Table 3). The number of excitatory varicosities was then estimated from the ratio of excitatory to inhibitory varicosities reported in Table 1. Additional counts were derived from preparations in which only the excitatory axon was injected with HRP. The latter values do not have to be corrected for inhibitory innervation (Table 3).

In addition to the varicosities counted on the exposed surface of the muscle, innervation occurs on the unexposed dorsal surfaces of the fibers. An estimate of the additional innervation on unexposed surfaces was needed to account more fully for all the varicosities on a muscle fiber. Although we attempted to assess this with HRP injections and clearing of the muscle (Lnenicka et al., 1991), experimentally it still proved too difficult to accurately determine

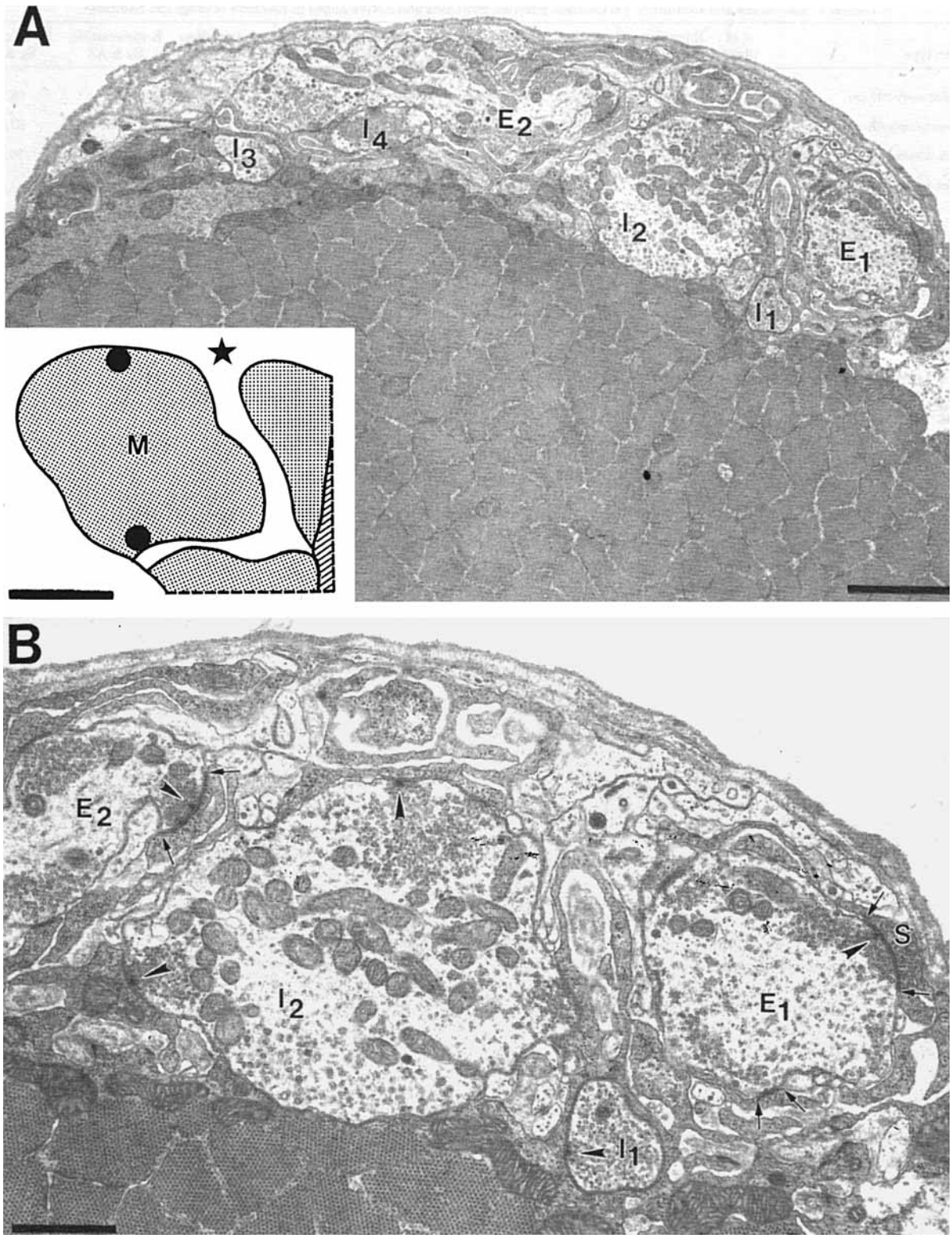

Fig. 3. Representative electron micrographs from a series made to determine the positions of varicosities on muscle fibers. **A:** Low-power view of muscle fiber from the central region. The **inset** shows the locations of two groups of varicosities (circles) on one muscle fiber: one on the exposed (inner) surface (which is designated by a star) and one on the unexposed (outer) surface. The central tendon (hatched area) is present at right. The micrograph shows a cluster of varicosities on the exposed surface of a proximal muscle fiber. **B:** Higher power views to illustrate synaptic structures and active zones (presynaptic dense bodies) in the same cluster of varicosities. For micrographs of A and B: M, muscle fiber; E_1, E_2, excitatory innervation; I_1, I_2, I_3, I_4, inhibitory innervation; arrowheads, presynaptic dense bodies (at active zones); arrows and S, synapses of the excitatory axon on the muscle fiber. E_1, E_2, and I_2 are classed as "*varicosities*" (>1 *μm in diameter*), whereas the other structures are classed as nonvaricosity "profiles" (<1 μm in diameter). Scale bars = 2 μm in A, 50 μm in inset, 1 μm in B.

TABLE 1. Excitatory and Inhibitory Varicosities, Profiles, Synapses and Active Zones in Electron Micrograph Samples[1]

Specimen type	# of fibres	E profiles: # & %	I profiles: # & %	E varicosities: # & %	I varicosities: # & %	E profiles: Sy & AZ	I profiles: Sy & AZ	E varicosities: Sy & AZ	I varicosities: Sy & AZ
Central									
1-sampled every 200 μm	8	36/61	25/61	26/45	19/45	77:11	37:6	68:11	33:5
		59.0%	41.0%	57.8%	42.2%				
2-random samples (n = 7)	39	30/53	25/53	22/41	19/41	65:9	40:8	60:9	37:8
		56.6%	43.4%	53.7%	46.3%				
Totals & % values	47	66/114	48/114	48/86	38/86	142:20	77:14	128:20	70:13
		57.9%	42.1%	55.8%	44.2%				
% values, n = 8									
Mean		57.0	43.0	53.0	47.0				
SE		4.0	4.0	9.0	9.0				
Active zones per 100 synaptic profiles						14	18	16	19
Proximal									
1-sampled every 200 μm	11	20/41	21/41	16/35	19/35	39:18	39:8	34:14	36:8
		48.8%	51.2%	45.7%	54.3%				
2-sampled every 200 μm	8	25/45	20/45	19/34	15/34	32:14	25:4	30:11	23:4
		55.6%	44.4%	55.9%	44.1%				
3-sampled every 200 μm	5	6/14	8/14	6/14	8/14	7:2	13:3	7:2	13:3
		42.9%	57.1%	42.9%	57.1%				
4-sampled every 200 μm	5	13/19	6/19	9/14	5/14	21:5	11:3	20:4	8:3
		68.4%	31.6%	64.3%	35.7%				
Totals & % values	29	64/119	55/119	50/97	47/97	99:39	88:23	91:31	80:18
		53.8%	46.2%	51.5%	48.5%				
% values, n = 4									
Mean		53.9%	46.1%	52.2%	47.8%				
SE		5.0	5.0	5.0	5.0				
Active zones per 100 synaptic profiles						39	26	34	23

"Varicosities" are structures > 1 μm in diameter. "Profiles" include in addition structures < 1 μm in diameter. "Sy," synapses; "AZ," active zones, indicated by presynaptic dense bodies; E, excitatory; I, inhibitory.

the degree of innervation on the dorsal surface of the muscle fibers. Thus, we made estimates of the percentage occurrence of varicosities on the dorsal and lateral surfaces of the superficial ventral fibers from electron micrographs (Table 2).

For the central region, we used eight samples, one of which was a bundle sectioned at five locations along its length; the rest were random sections from seven different opener muscle preparations. The results from these samples were on average quite consistent: Approximately 60% of the varicosities occurred on the exposed surface and 40% on the unexposed surface (Table 2). Variation among samples was also noted, as indicated in Table 2.

For the proximal region, the micrographs indicated more variability in the proportion of exposed varicosities (Table 2). We therefore used three different estimates from these samples (the mean value and the observed maximum and minimum values designated respectively "mean," "max," and "min" in the tables) in subsequent calculations to illustrate the range of possible outcomes for this sampling approach. It was believed that this would be preferable to performing more sampling, because the mean value so obtained would still have much fiber-to-fiber variation associated with it. The limitations of the sampling approach are exemplified in these data and will be discussed below.

To ascertain whether varicosities are added with age in this muscle, as in the American lobster (DeRosa and Govind, 1978; Govind, 1982), supplementary observations (4-Di-2-Asp method only) were made on smaller and larger crayfish (Table 3). Counts of exposed varicosities for central fibers were significantly higher than for proximal fibers in all three sizes of animals sampled. This indicates that varicosities are added as the animal grows and that not all varicosities are the same age. The ratio of proximal to central counts decreased slightly with age (by approximately 12%) in the larger, older animals.

Observations on the number of varicosities on the exposed surface of the muscle (Table 3) were combined with estimates of the percentage of exposed varicosities (Table 2) to derive an estimate for the total number of excitatory varicosities per muscle fiber (Table 3). The approach used to estimate the total number of excitatory varicosities per fiber can be illustrated for the central fibers. The mean number of varicosities on the fiber's surface (82) was obtained by direct counts from 4-Di-2-Asp-stained preparations. This value was then adjusted for the mean percent-

TABLE 2. Varicosities on Exposed and Unexposed Surfaces of Superficial Fibers Counted in Electron Micrographs

Specimen type	# of fibres	% of clusters	% of EXP clusters	% of UNEXP clusters	% of EXP varic.	% of UNEXP varic.
Central						
1-sampled every 200 μm	8	19	12/19	7/19	28/45	17/45
			63.2	36.8	62.2%	37.8
2-random samples (n = 7)	39	23	13/23	10/23	20/41	21/41
			56.5%	43.5%	48.8%	51.2%
Totals & % values	47	42	25/42	17/42	48/86	38/86
			59.5%	40.5%	55.8%	44.2%
% values, n = 8						
Mean			61.5	38.5	60.4	39.6
SE			9.0	9.0	10.0	10.0
Proximal						
1-sampled every 200 μm	11	11	9/11	2/11	30/35	5/35
			81.8%	18.2%	85.7% (max)[1]	14.3%
2-sampled every 200 μm	8	16	7/16	9/16	19/34	15/34
			43.8%	56.3%	55.9%	44.1
3-sampled every 200 μm	5	8	5/8	3/8	5/14	9/14
			62.5%	37.5%	35.7% (min)[1]	64.3%
4-sampled every 200 μm	5	6	3/6	3/6	7/14	7/14
			50.0%	50.0%	50.0%	50.0%
Totals & % values	29	41	24/41	17/41	61/97	36/97
			58.5%	41.5%	62.9%	37.1%
% values, n = 4						
Mean			59.5%	40.5%	56.8%	43.2%
SE			8.0	8.0	11.0 (mean)[1]	11.0

[1]Since there is a wide variation in the % of EXP (exposed) and UNEXP (unexposed) varicosities (varic.) in samples taken in proximal muscles, the minimum (min), maximum (max) and mean values were used to bracket further calculations concerning the number of varicosities per fiber.

TABLE 3. Mean Number of Varicosities on Exposed Surfaces of Muscle Fibers in Central and Proximal Regions Along with Estimates for Total Excitatory Varicosities Per Muscle Fiber

Size of animal	Central mean ± SE (n)[1]	Proximal mean ± SE (n)[1]	Ratio Proximal/ Central
Observed varicosities			
5–6.5 cm (intermediate)			
(a) 4-Di-2-Asp (2[2])	82 ± 8.9 (15)*	46 ± 6.5 (8)*	0.56
(b) HRP (4[2])	32 ± 2.0 (12)*	22 ± 2.1 (7)*	0.69
3.5–4 cm (small, 3[2])			
4-Di-2-Asp	30 ± 2.4 (18)*†	18 ± 2.67 (12)*†	0.60
7.5–9 cm (large, 2[2])			
4-Di-2-Asp	126 ± 4.6 (13)*†	66 ± 3.3 (10)*†	0.53
Estimated varicosities			
5–6.5 cm (intermediate)			
Estimated total excitatory varicosities per fiber			
(a) 4-Di-2-Asp[3]	77*†	mean 42*†	mean 0.55
		max 28	max 0.36
		min 67	min 0.87
(b) HRP[3]	53*†	mean 39*†	mean 0.74
		max 26	max 0.49
		min 61	min 1.15

* & †: $P < 0.05$ (t-test or Mann-Whitney test: "*" for horizontal; "†" for vertical) between central and proximal muscle fibers as well as between sizes of crayfish within the same region of the muscle. The number of muscle fibers is given by (n). Observed counts are for the exposed inner ventral surface of the muscle fibers in all animals. The innervation in small and large animals was visualized with 4-Di-2-Asp only. In the intermediate sized animals, a second estimate was obtained by filling the excitatory axon with HRP and counting all exposed varicosities on single muscle fibers.

[1]The number of muscle fibers sampled.

[2]The number of animals used.

[3]Values corrected for the % of excitatory (E) and inhibitory (I) varicosities observed as well as the % of innervation on the exposed vs. the unexposed surfaces obtained in the electron microscopic study (Tables 1 & 2). Three values (mean, min, max) were used for the proximal fibers because of the wide range in the % of exposed varicosities (Table 2).

age of exposed (60%) and unexposed (40%) varicosities for central fibers (Table 2). The value of 82 represents only 60% of the total varicosities. The adjusted total number of varicosities estimated for an entire fiber is 137. This number is corrected for the mean percentage of excitatory (56%) and inhibitory (44%) varicosities as shown from the electron microscopic study (Table 1). The value 137 then becomes 77, which represents the estimated total number of excitatory varicosities per central muscle fiber. The same procedure is used to calculate the values from the varicosities visualized by HRP axonal injection, except that no correction for inhibitory innervation is necessary.

To estimate the number of excitatory varicosities for the proximal muscle fibers, we used a range of values because of the wide dispersion in the percentages of exposed and unexposed varicosities in the electron microscopic study (Table 2). We chose to provide three estimates of the number of excitatory varicosities per fiber, using the mean, the maximum (max), and the minimum (min) percentage of exposed varicosities. These considerations were applied in Table 3 and carried over to Table 7, providing a range of possible outcomes for the calculations that can be made with currently available data. This "bracketing" approach was thought to be preferable to relying entirely on the mean value.

Another problem we encountered concerns the estimates of exposed excitatory varicosities from 4-Di-2-Asp and HRP procedures. The estimated values obtained for proximal fibers using these two methods are comparable, whereas the values estimated for the central muscle fibers differed substantially, values for the axons injected with HRP being less than those for 4-Di-2-Asp. This is most likely due to incomplete filling of all the multiple branches arising from the primary and secondary axon branches that innervate the central fibers in different locations. The proximal fibers appear to be innervated more compactly (Figs. 2, 4), and most of the terminals are likely to be filled by the injected HRP. Because we were more confident in the counts of varicosities for fibers stained with 4-Di-2-Asp, these counts were selected as the more reliable and used to estimate the parameters shown in Table 7.

Synapses

From the electron micrographs, some information on the numbers of excitatory and inhibitory synapses and active zones (indicated by presynaptic dense bodies) was obtained for the two regions (Table 1). First, it is evident that most of the synapses and active zones appeared on varicosities (structures over 1 μm in diameter in electron micrographs) rather than on the smaller structures. (For example, in Table 1, for the central region, 128 of 142, or 90%, of excitatory synapses occurred on varicosities, and 20 of 20, or 100%, of observed active zones appeared on varicosities). This observation confirms in general those of Florey and Cahill (1982). Second, excitatory synapse counts are invariably greater than inhibitory synapse counts (with a greater difference in the central then in the proximal region). This observation confirms in general those of Jahromi and Atwood (1974), Tse et al. (1991), and others. Third, the occurrence of active zones among sampled synapses is substantially greater in the proximal region, particularly for the excitatory axon. This is in accordance with previous observations of Govind et al. (1994) and Cooper et al. (1995a) and supports the proposal of a morphological correlate for synaptic efficacy in different terminals of a single neuron.

Muscle fiber properties and EPSP amplitude

We measured the electrical properties of the muscle fibers to estimate their likely effect on EPSP amplitude. Both input resistance and fiber diameter were measured directly, and relative values for specific membrane resistance (R_m) were then calculated (Table 4). Input resistance in proximal fibers was twice that in central fibers, largely due to their smaller diameter, because the calculated relative R_m was actually lower in proximal fibers. Because EPSP amplitude is eight to nine times greater in the sample of proximal fibers, the difference in muscle fiber input resistance can account for less than one-fourth of the total difference.

To determine whether the quantal currents differ in duration in the proximal and central fibers, we measured the decay time constants of individual quantal currents and averaged evoked currents in the two regions. There is not a significant difference in τ for single quantal or evoked currents (Table 4). This indicates that the glutamate receptors are probably of the same type and have the same kinetics throughout the muscle. A further check on possible effects of muscle fiber membrane time constant differences was obtained by comparing the time taken for the membrane voltage to attain 50% of its final value in response to a square pulse of current injected at one point near the middle of the fiber (t_{50}). There was no difference between proximal and central fibers. In view of the lack of difference in these various temporal measurements, no additional corrections were made for differences in duration of synaptic current or for differences in muscle fiber time constant.

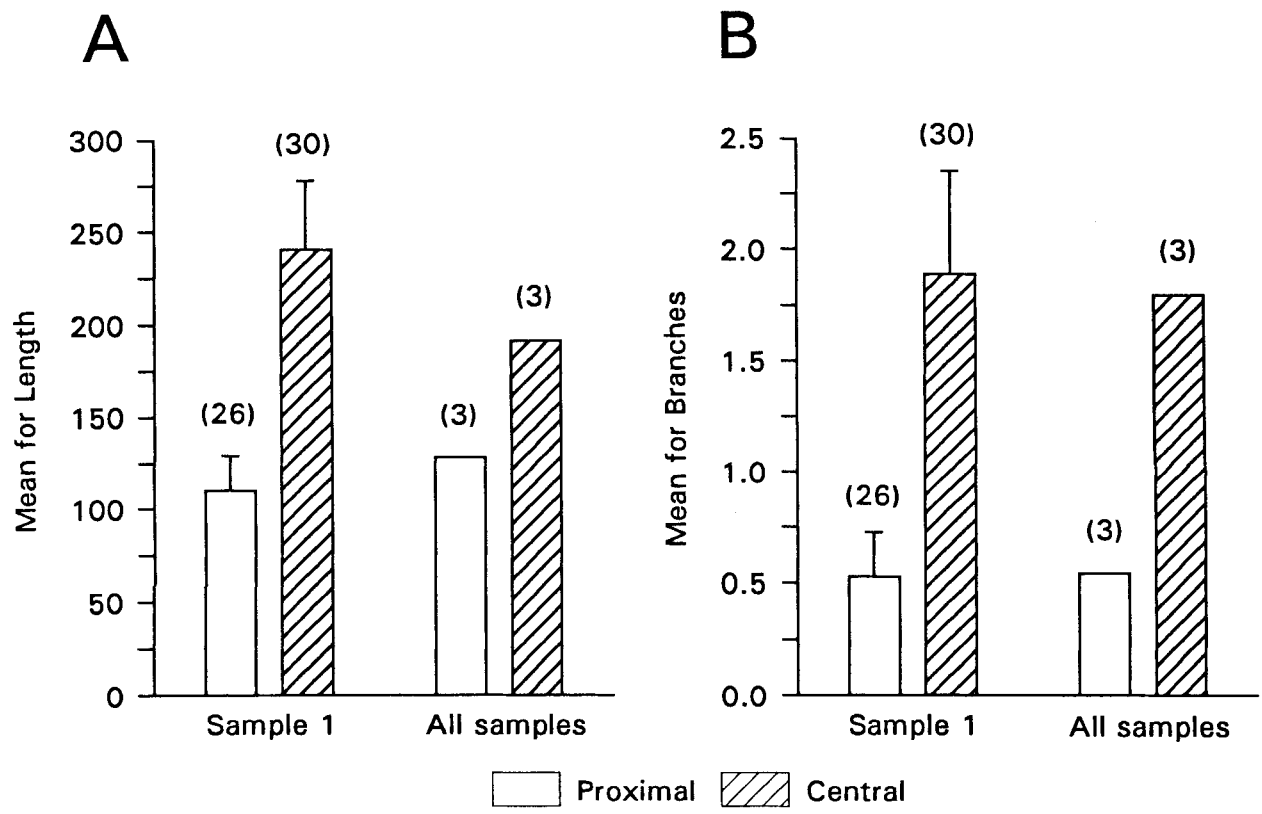

Fig. 4. Branch length and degree of branching in the proximal and central regions for one of three preparations (sample 1) in which the terminals had been stained with 4-Di-2-Asp and measured. For comparison, pooled data from all three preparations are also shown. **A:** Mean length of terminals in proximal and central regions. **B:** Mean branching (bifurcations per terminal) for the same terminals. Error bars: ±1 standard error. In sample 1, significant differences were found from the Student's t test for both length ($P = .004$) and branching ($P = .012$). Pooled samples were not compared statistically.

Quantal content along visualized terminals

Results of mapping quantal release at varicosities of known location are illustrated in Figures 6–8, which show representative examples from central and proximal muscle fibers. These figures include drawings of the photographed nerve terminals, an indication of the measured quantal content at 1 Hz, and representative focal recordings.

Overall differences between proximal and central regions are evident in these figures and in the quantal content values in Tables 5 and 6. As was observed in previous studies (Govind et al., 1994; Cooper et al., 1995a), the mean quantal content at low frequencies is invariably greater in proximal fibers.

When recordings were made at different locations along a tertiary branch ("string") attached to one muscle fiber, the quantal content was found to vary with position. In the muscle's central region, the quantal content values were lowest at the ends of a string. The relatively small varicosities located most distally from the main axons had lower quantal content than the larger, more proximal varicosities (Figs. 6, 7, Table 5). This trend was consistently observed in all strings and clusters from which more than one recording was made.

In the proximal region, recordings were made from two clusters of varicosities (Fig. 8, Table 6). The quantal content was higher than for central varicosities in all cases. Within a cluster, the highest quantal contents were obtained from relatively large varicosities close to major axon branches. The data were not sufficient to judge whether there was a relationship between the size of a varicosity and its quantal content; this could possibly be the case for varicosities along central strings, but proximal varicosities with consistently higher quantal contents were not markedly different in size from central varicosities.

Estimated quantal parameters

The discrete numbers of evoked events produced at varicosities in the two regions at 1 Hz stimulation are presented in Tables 4 and 5, along with the best-fitting distributions. From the latter, the release can be classified as uniform binomial, nonuniform binomial, or Poisson (Smith et al., 1991; Wojtowicz et al., 1994; Cooper et al., 1995b). For proximal varicosities, binomial distributions (usually nonuniform) always provided very good fits (as evidenced by comparing the observed data to those predicted from the model distributions). For central varicosities, the majority of the data sets were best fitted by binomial distributions, but several cases were best fitted by Poisson distributions. However, the fits for Poisson distributions were always considerably less good than those for the binomial distributions, as can be seen by comparing the observed and predicted values in Table 5.

Estimates of quantal parameters (n and p) indicated nonuniformity of the probability of release, which was

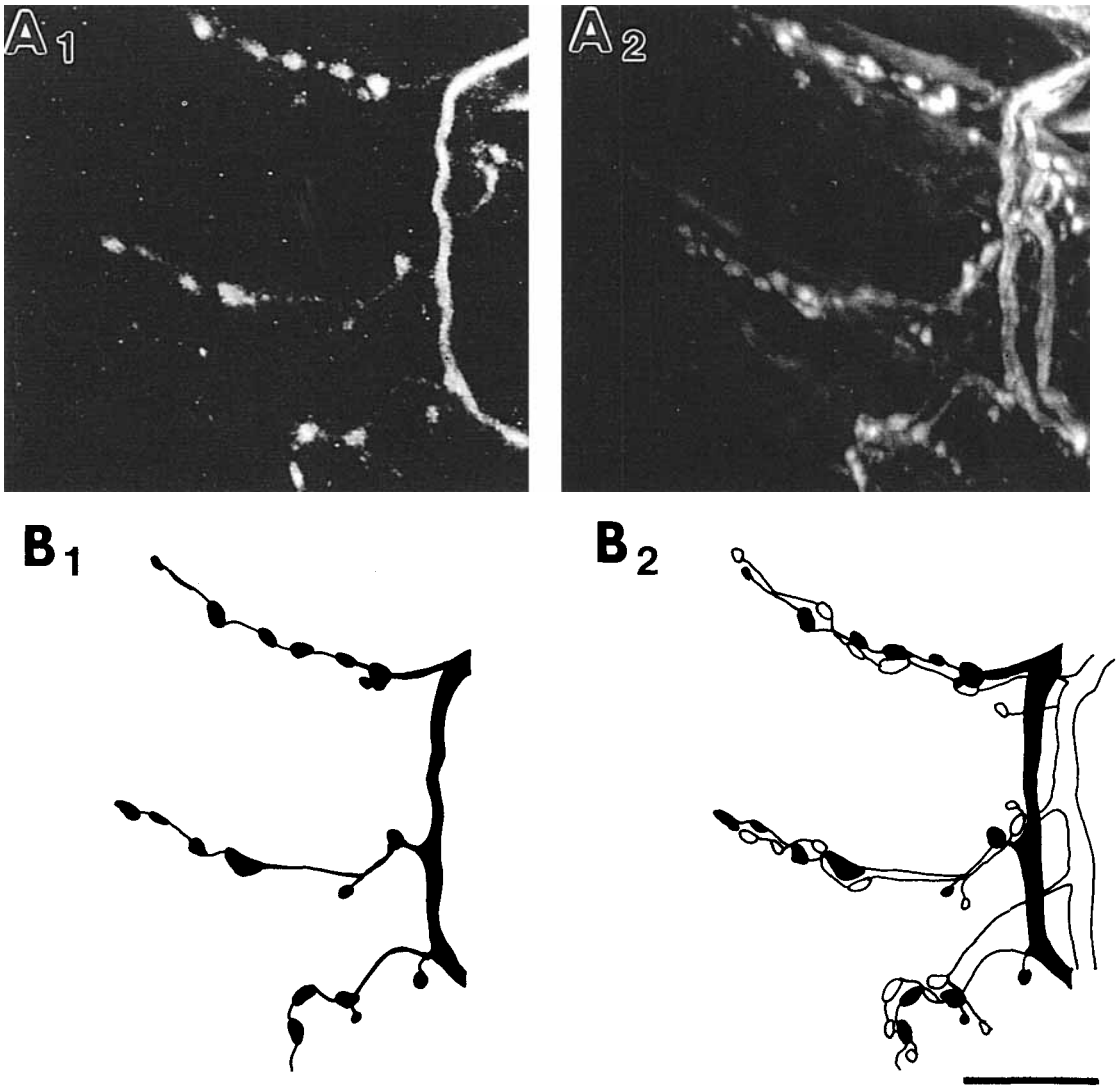

Fig. 5. Differential staining to identify visualized excitatory and inhibitory axons and varicosities. **A:** Representative secondary and tertiary branches of a preparation in which the excitatory axon was first pressure-injected with Texas red-conjugated dextran (10 kD; A_1) and then stained with 4-Di-2-Asp (A_2) to reveal also the inhibitory axon. **$B_{1,2}$:** Drawings of the same region to show more clearly the identity of the axons and varicosities (excitatory structures are shown as black, inhibitory structures as white). There are 18 excitatory and 18 inhibitory varicosities in B, excluding those to the right of the secondary axons. Scale bar = 50 μm.

position dependent (Tables 5, 6). For either of the two types of binomial distribution, the estimated mean p values of release indicate that the varicosities on proximal fibers have a greater probability of release than those on central fibers at the same stimulation frequency. For central fibers, two of the terminals showed a greater p for primary varicosities than for varicosities located more distally along it, whereas two other terminals showed the opposite result, and another terminal showed no real difference along the string of varicosities. There is no significant difference in the estimated n values of the proximal and central regions, but at the low frequencies employed in this study the values for n were small in all samples. The general picture that emerges from the statistical calculations is that probability of release is higher at low stimulation frequencies for transmitting synapses of primary varicosities in the proximal region of the muscle than for the primary varicosities of the central region. There is no evidence that the number of transmitting synapses on a varicosity (judged from calculated values of n) is significantly higher in proximal varicosities, though we cannot rule out the possibility that more extensive sampling might demonstrate a small but real difference.

DISCUSSION

The results presented herein, taken in conjunction with recent ultrastructural determinations of the number of synapses on individual varicosities (Wojtowicz et al., 1994; Cooper et al., 1995a), permit a more detailed assessment of the efficacy of synaptic transmission at the single-varicosity and single-synapse level than has previously been attempted for high-output and low-output terminals of a single axon. The data for the comparative assessment are

TABLE 4. Electrical Properties and EPSPs of Proximal and Central Muscle Fibers

Parameters	Proximal	Central	Ratio Proximal/ Central
EPSP amplitude (mV) mean ± SE	1.10 ± 0.14 (10)*	0.13 ± 0.01 (10)*	8.46
Input resistance (kΩ) mean ± SE	832 ± 91 (17)*	417 ± 21 (41)*	2.0
Fiber diameter (μm) mean ± SE	25.4 ± 1.7 (6)*	43.6 ± 2.3 (6)*	0.58
Membrane resistance (R_m)	666.4 Ω cm²	851.8 Ω cm²	0.78
t_{50} of step potential (μsec) mean ± SE	229 ± 23 (8)	244 ± 49 (11)	0.94
τ of quantal currents (msec) mean ± SE	1.41 ± 0.07 (30)	1.55 ± 0.07 (30)	0.91
τ of averaged evoked currents (msec) mean ± SE	1.26 ± 0.09 (5)	1.67 ± 0.15 (7)	0.76

*$P < 0.001$, t-test, for comparisons of central and proximal fibers. In cases in which the data were not normally distributed, the non-parametric Mann-Whitney test was used. t_{50} is the measured time for the voltage to reach 50% of its maximum amplitude with injected stepped currents. τ, decay time constant of synaptic currents. Number in brackets represents muscle fibers or varicosities sampled.

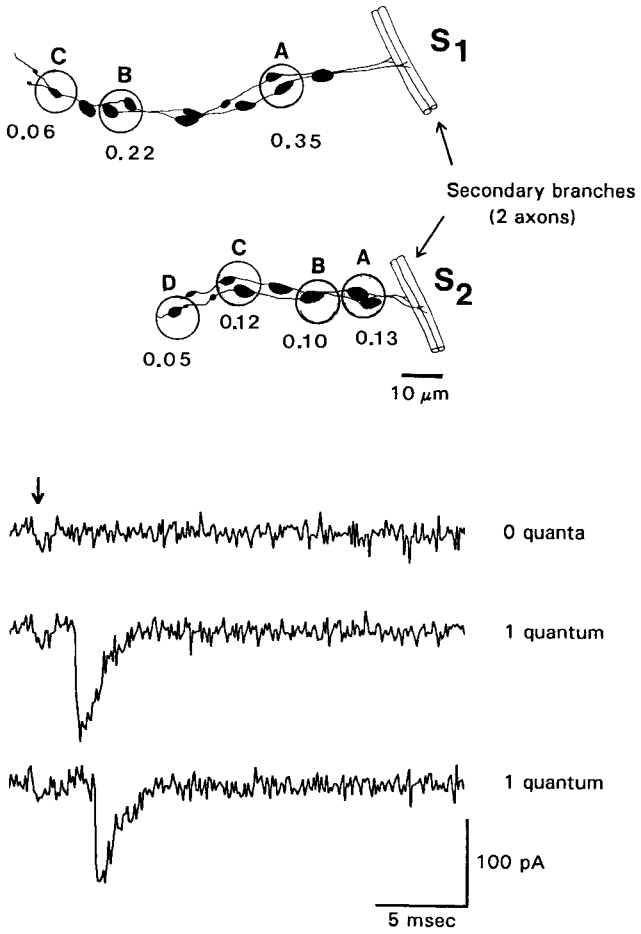

Fig. 6. Records of quantal release obtained from two representative defined "strings" (S_1 and S_2) on central terminals. Recording locations are shown in the drawings of the terminals; the circles indicate the position of the tip of the "macropatch" electrode. Three representative sweeps are included to illustrate focal macropatch recordings from one location. Values of mean quantal content (m) were determined by direct counts at 1 Hz (1,000 sweeps) for three locations (A–C) on S_1 and from four locations (A–D) on S_2; these values are indicated for each recording site. The arrow indicates the time of the stimulus artefact. Scale bars = 10 μm (anatomical); current traces = 100 pA, 5 milliseconds.

assembled in Table 7, which includes measurements from the present study, along with supplementary data on quantal content and on the number of synapses per varicosity added from a previous study (Cooper et al., 1995a). From these data, simple arithmetic calculations estimate the comparative efficacy of transmission in terms of quantal content per muscle fiber, per varicosity, and per synapse.

A limitation of this comparison is that not all types of data were collected for the same muscle fibers, and this will introduce some loss of definition. A counterargument is that less damage to muscle fibers and synapses ensues when fewer different types of measurement are attempted at the same time on one cell. In the present study, we elected to preserve physiological integrity by limiting the possible damage to sampled cells. This approach has been used in previous studies on the crayfish opener muscle (Bittner and Kennedy, 1970). Nevertheless, it is clear from the samples made to collect anatomical data that there is sufficient variability in estimates of varicosities per muscle fiber to weaken comparisons based upon overall counts; thus, future improvement in quantitative cellular comparisons for this system is more likely to result from the difficult procedure of collecting as many types of data as possible from individual cells, as in some of the studies of frog neuromuscular junctions (Nudell and Grinnell, 1983), than from taking larger separate samples of different kinds of data from the regions to be compared. The comparisons of transmission in central and proximal regions, although more detailed than previous ones, are still provisional due to variance in some of the data.

Influence of muscle fiber electrical properties

Our investigation of the ventral superficial muscle fibers of the walking leg showed at least an eightfold difference in EPSP amplitude between proximal and central fibers, as originally described by Iravani (1965). Bittner (1968a) (Table 2) surveyed the dorsal superficial muscle fibers of the claw opener muscle and found that EPSPs were up to 14-fold larger in distal than in central muscle fibers. Bittner concluded that input resistance of the muscle fibers could account for only a small fraction of the amplitude difference. This general conclusion is borne out in the present study: The difference in muscle fiber input resistance would produce on average approximately a twofold difference in EPSP amplitude, or about one-fourth of the total difference observed.

We checked whether differences in duration of synaptic currents could be a factor in the differences of EPSP amplitude. A longer synaptic current in the proximal region would result in charging of the muscle membrane capacitance over a longer period of time, which could produce a larger EPSP (Lnenicka and Mellon, 1983a). However, the decay time constants of spontaneous synaptic currents and of averaged evoked currents at high-output and low-output varicosities stimulated at 1 Hz did not reveal any differences in the time course. No obvious difference in rise time of the membrane potential for step-function currents was observed. Therefore, the possibility of a differential time course of membrane charging contributing substantially to the differences in EPSP amplitude remains unlikely.

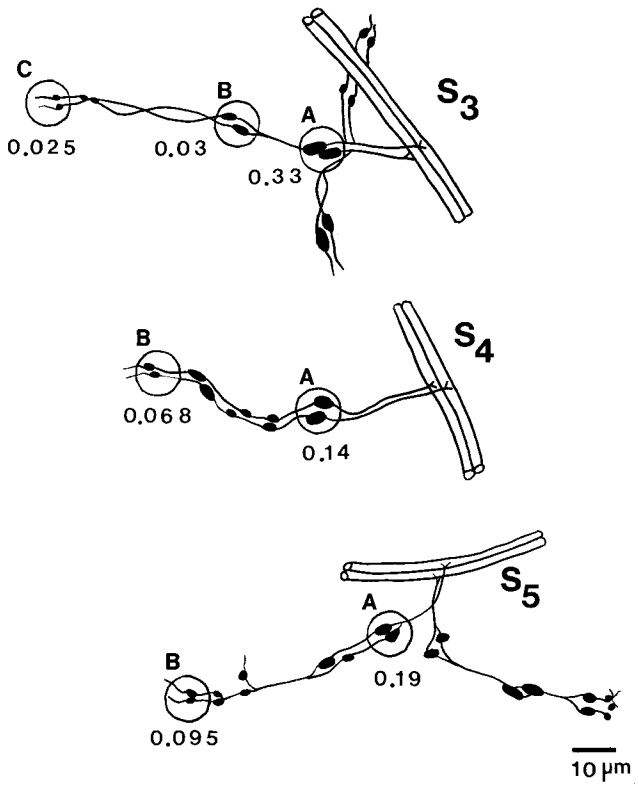

Fig. 7. Recording locations for three additional strings of the central region (S_3, S_4, S_5) in which recordings could be compared for extreme distal and proximal boutons. Mean quantal content (m) values are given next to the respective recording sites. The circles indicate the position of the tip of the macropatch electrode. Scale bar = 10 μm.

Quantal content per varicosity

The comparison of proximal and central transmission was extended through observations on physiological properties of the presynaptic terminals, using focal macropatch recordings from visualized varicosities and quantal analysis procedures. In confirmation of previous observations (Govind et al., 1994; Cooper et al., 1995a), we have shown that mean quantal content of proximal varicosities is greater than that of central varicosities. A new observation is that there is a marked difference in mean quantal content along the strings of varicosities on central muscle fibers. The varicosities closest to the primary axon on a central string resemble more closely the proximal varicosities in mean quantal content, whereas more distal varicosities along a string have substantially lower mean quantal contents and are usually smaller in diameter. Nonuniformity of synaptic transmission for a single postsynaptic element indicates the need to specify the anatomical locations of physiological samples, a procedure which has generally not been attempted in previous studies on this preparation.

Quantal content variation along the length of the terminal has been well established for the amphibian neuromuscular junction. The probability of release is highest at the site where the nerve first becomes a terminal structure and then decreases along the length of the terminal (Bennett and Lavidis, 1982; Bennett et al., 1986a,b; D'Alonzo and Grinnell, 1985). The structural correlate for this decline in

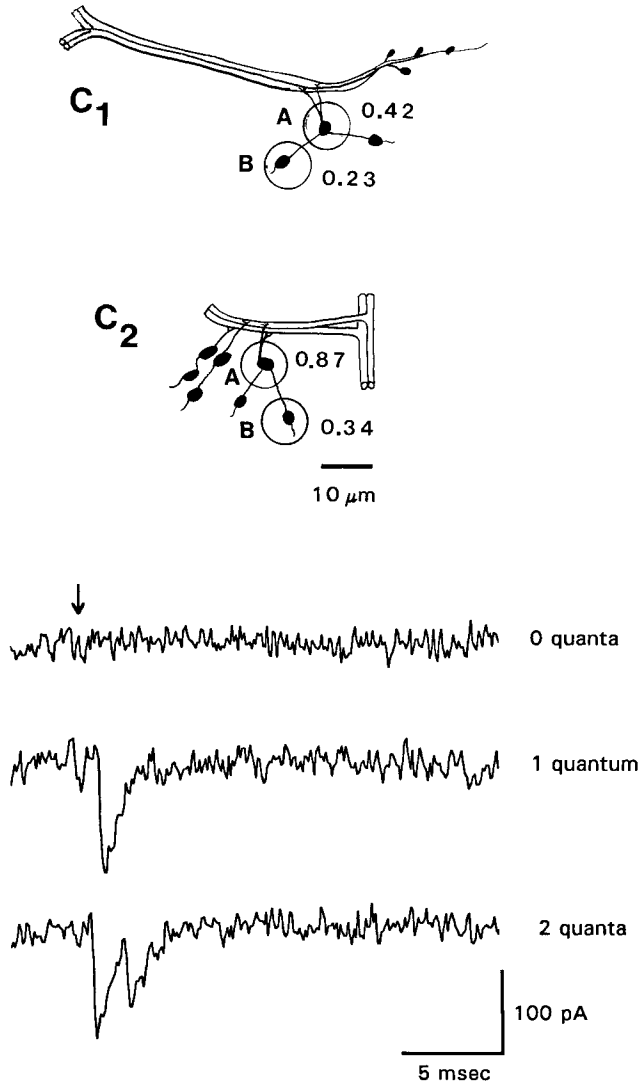

Fig. 8. Records of quantal release obtained from two representative clusters of varicosities (C_1 and C_2) of proximal terminals. Recording locations (two for each cluster, A and B) are shown in the drawings of the terminals, together with the mean quantal content values. The circles indicate the position of the tip of the macropatch electrode. Three representative sweeps from focal macropatch recordings at one location are included (arrow, stimulus artefact). Note that in the third sweep, a second delayed quantal event occurs. Scale bars = 10 μm (anatomical); current traces = 100 pA, 5 milliseconds.

output along the terminal is a progressive decrease in the synaptic contact area (Davey and Bennett, 1982; Bennett et al., 1989). With the use of scanning electron microscopy, Tremblay et al. (1989) showed that the distance between postjunctional folds is greater more distally along the terminal. While these observations suggest that structural differences along the terminal could be important in determining the probability of transmitter release, other recent observations on "strong" and "weak" amphibian neuromuscular junctions have not provided a clear-cut structure-function correlation (Pawson, 1995; Tobias et al., 1995), and differences in single-channel performance may contribute.

In attempting to arrive at an overall "balance sheet" for comparison of transmission in central and proximal re-

TABLE 5. Statistics of Evoked Quantal Release at Single Varicosities on Five Visualized Terminal 'Strings' on Individual Central Muscle Fibers, from Direct Counts of Quanta

Exp.	Distribution Evts.	Obs.	Best fit		n	p	Quantal Content (m)
STRING 1							
A (1°)	0	675	675	non-uniform Binomial	2	0.173	0.35
	1	304	304				
	2	21	21				
B (2°)	0	814	792	Poisson	high n	low p	0.22
	1	159	185				
	2	2	22				
	3	1	2				
C (2°)	0	941	946	uniform Binomial	1	0.05	0.06
	1	54	54				
	2	5	0				
STRING 2							
A (1°)	0	902	800	Poisson	high n	low p	0.13
	1	80	179				
	2	14	20				
	3	2	2				
	4	2	0				
B (2°)	0	901	901	non-uniform Binomial	2	0.051	0.10
	1	97	97				
	2	2	2				
C (2°)	0	895	837	Poisson	high n	low p	0.12
	1	96	149				
	2	8	13				
	3	0	1				
	4	1	0				
D (3°)	0	949	949	uniform Binomial	1	0.05	0.05
	1	51	51				
STRING 3							
A (1°)	0	341	340	non-uniform Binomial	3	0.111	0.33
	1	153	150				
	2	5	10				
	3	1	0				
B (2°)	0	969	969	uniform Binomial	1	0.031	0.03
	1	31	31				
C (3°)	0	975	975	uniform Binomial	1	0.025	0.025
	1	25	25				
STRING 4							
A (1°)	0	869	866	Poisson	high n	low p	0.14
	1	120	125				
	2	10	9				
	3	1	0				
B (3°)	0	932	932	uniform Binomial	1	0.068	0.068
	1	68	68				
STRING 5							
A (1°)	0	810	810	non-uniform Binomial	2	0.097	0.19
	1	186	186				
	2	4	4				
B (3°)	0	905	905	uniform Binomial	1	0.095	0.095
	1	95	95				
Mean m for initial (1°) varicosities (±SE, n)							0.228 (±0.047, 5)*
Mean m for intermediate (2°) varicosities (±SE, n)							0.106 (±0.032, 5)
Mean m for final (3°) varicosities (±SE, n)							0.06 (±0.015, 4)*

*$P < 0.05$ between 1° and 3° groupings. (Abbreviations: Exp., recording site as shown in Figures; Evts., the number of discrete events, indicated as 0-failures, 1-one's, 2-two's, etc. . . ; Obs., the observed occurrences of each event). An indication as to whether the distribution conforms best to a Poisson distribution, or to a uniform or non-uniform Binomial distribution, is included.

TABLE 6. Statistics of Evoked Quantal Release at Single Varicosities of Two 'Clusters' on Proximal Muscle Fibers, from Direct Counts of Quanta[1]

Exp.	Distribution Evts.	Obs.	Best fit		n's	p's	Quantal Content (m)
CLUSTER 1							
A	0	602	602	non-uniform Binomial	2	0.212	0.42
	1	373	373				
	2	25	25				
B	0	787	785	uniform Binomial	2	0.114	0.23
	1	199	201				
	2	14	13				
CLUSTER 2							
A	0	150	150	non-uniform Binomial	2	0.436	0.87
	1	828	828				
	2	22	22				
B	0	689	689	non-uniform Binomial	2	0.167	0.34
	1	287	287				
	2	24	24				
Mean m (±SE, n) for cluster varicosities							0.46 (±0.14, 4)

[1](Abbreviations: As in Table 5.)

gions, we included data from reconstructed and recorded varicosities from an earlier study (Cooper et al., 1995a). Thus, the entries for quantal content (m) per varicosity in Table 7 are designated as to their source. Because the data from the two studies were obtained from animals of similar size and physiological condition, they are combined to give a mean value in Table 7.

Variation in quantal content per varicosity along central strings was taken into account by assigning different values to the three anatomically defined classes in Table 5, the initial (most proximal) varicosities in a string (first degree, 1°), more distal varicosities (second degree, 2°), and terminal varicosities (third degree, 3°). These values are used in a subsequent estimation of the quantal content per muscle fiber in Table 7; quantal content for primary varicosities is accounted for separately.

The estimated number of primary (1°) varicosities (Table 7) is based on observations of approximately eight exposed tertiary terminal branches per central fiber stained with 4-Di-2-Asp. The eight exposed tertiary branches contribute at least eight exposed primary varicosities, and, because it was estimated that the surface varicosities represent only 60% of the total, there would be approximately 13 primary varicosities for the entire fiber. This figure is used to estimate quantal content for the primary varicosities of a muscle fiber in Table 7.

It is pertinent to compare the present results on quantal content per varicosity to those in a previous study in which quantal content per muscle fiber was estimated (Bittner and Kennedy, 1970). In that study, mean quantal content (m) per varicosity was measured by placing 2.5 M NaCl filled recording electrodes of 1–3 MΩ impedance over randomly located terminals. The m values of Bittner and Kennedy (1970) are very similar to those we report, despite the fact that they used the opener muscle of the large cheliped, whereas we used the opener muscle of much smaller walking legs. Bittner and Kennedy (1970) defined two types of terminals, those that facilitate at low-frequency stimulation and those that facilitate at high-frequency stimulation. Earlier work (Bittner, 1968a) had shown these types to be segregated on the superficial, dorsal surface of the claw opener muscle. The low-frequency facilitating fibers (distal on the dorsal surface) are similar to the ventral, proximal group that we have described here in that both groups exhibit larger EPSPs than the respective centrally located fibers and have similar values of m per varicosity. Bittner and Kennedy (1970) reported that the m per varicosity in this dorsal, distal group is 0.85 at 1 Hz. The average m we measured for proximal, high-output varicosities is 0.75. Varicosities of centrally located dorsal fibers in the claw (Bittner and Kennedy, 1970) and the 2° and 3° varicosities of central walking leg fibers (this study) have similar values of m: 0.14 and 0.19, respectively. Considering the differences in recording method, muscle size, and animal size, there is remarkably good agreement between the two studies.

Number of varicosities per muscle fiber

For a determination of the total m exhibited for an entire muscle fiber based on the known m per varicosity, the number of varicosities per fiber must be determined. There have been various attempts over the years to estimate the

TABLE 7. General Comparison of Quantal Release for Proximal and Central Excitatory Terminal Varicosities and Muscle Fibers[1]

Measurement	Central			Proximal	Ratio of Proximal/ Central
	1°	2–3°	All		
m per varicosity (±SE, n)[2] (data from Cooper et al., 1995a)	0.473 (0.05, 2)	0.180 (0.028, 11)		0.94 (0.13, 6)	
m per varicosity (±SE, n)[3] (Data from the present study)	0.228 (0.047, 5)	0.085 (0.02, 9)		0.46 (0.14, 4)	
Combined (Both studies) *m* per varicosity (±SE, n)	0.298 (0.057, 7)	0.137 (0.020, 20)	0.747 (0.12, 10)		
mean # of varicosities per muscle fiber	13	64	77	42 mean 28 max 67 min	0.55 mean 0.36 max 0.87 min
Calculated *m* per fiber	3.87	8.78	12.65	31 mean 21 max 50 min	2.45 mean 1.66 max 3.95 min
# of synapses per varicosity[2] (data from Cooper et al., 1995a)	31	35		29	
Calculated *m* per synapse	0.0096	0.0039		0.0258	
# of synapses with active zones[2] (data from Cooper et al., 1995a)	30	24		27	
Calculated *m* per synapse with active zones	0.0099	0.0057		0.0276	

[1]Abbreviations: *m*, mean quantal content determined by direct counts; n, number of identified varicosities recorded; 1°, primary varicosities of strings on central muscle fiber; 2°, secondary varicosities of strings on central muscle fiber.
[2]Data obtained from Cooper et al., 1995a.
[3]Data obtained in this study (Tables 5 & 6).

number of varicosities on a fiber and the quantal content per varicosity. The ratios of total spontaneous events recorded intracellularly for the whole muscle fiber to the number seen at a focal extracellular recording site has been used as a measure of the number of "sites of transmission" on a single muscle fiber (Dudel and Kuffler, 1961a; Bittner and Kennedy, 1970). Values of approximately 45–50 have typically been found from this estimate. However, such calculations suffer from at least two problems: 1) The anatomical identity of the recording sites is not known, so there is doubt about the representativeness of the selected extracellular recording sites, and 2) the concentrated electrolyte in the focal extracellular recording electrodes probably increased the frequency of the spontaneous events at the recording site. The latter effect would generate a spuriously low value for the number of sites of transmission. A third possible source of error is the failure of the intracellular recording to detect all of the released quantal units; at some recording sites, a significant number of quantal currents do not produce a measurable voltage change in the muscle fiber (Wojtowicz et al., 1994). This effect would also lower the ratio of intracellular to extracellular quantal events and decrease the estimated sites of transmission.

In an anatomical study, Florey and Cahill (1982) counted the total number of varicosities on single muscle fibers of the opener muscle in first walking legs of adult crayfish (*Astacus leptodactylus*). To visualize the varicosities, they isolated the preparation and exposed the nerves to $CoCl_2$ for 7 days to "forward-fill" the terminals. They reported a total of 152 excitatory motor neuron varicosities per muscle fiber. This value is substantially greater than the sites of transmission estimated electrophysiologically, and also more than three to four times the values we report here for the central fibers in walking legs of intermediate-sized or large *Procambarus clarkii*. Even for our large animals (Table 3), an estimate for excitatory varicosities of approximately 110 is still substantially less than the values reported by Florey and Cahill (1982). The difference in our counts and those of the earlier report could be due to at least three factors. 1) We reported only the varicosity numbers from the exposed surface of the muscle fibers and estimated the percentage of varicosities seen beneath the surface but identified as belonging to the surface fibers; 2) the species used in the two studies, and probably the sizes of animals used, were different; and 3) the long incubation times in $CoCl_2$ employed by Florey and Cahill (1982) could lead to artifactual "ballooning" along fine axon terminals, thereby generating a spuriously large number of varicosities at the light microscopic level. We have observed this type of artefact with several different staining procedures, especially when incubation times were long.

Our attempt to determine the total number of varicosities by estimating the degree of innervation on the dorsal aspects of these most superficial ventral fibers from low-magnification electron microscopy avoids the latter artefact, and the uncertainty of deciding the fiber assignment of subsurface varicosities, but raises another problem: fiber-to-fiber variation in location of varicosities (Table 2). To overcome this problem, collecting the various types of data from individual fibers would be better than attempting to derive mean values for all the measured parameters, as we and previous investigators have done. The sampling approaches based on mean values can serve to bracket the true situation but not to define it exactly.

Synapses per varicosity

Estimates of the number of morphologically defined synapses are presently available only from serial reconstructions using electron micrographs. The available studies give different estimates. In *Procambarus clarkii*, a varicosity has on average 40–45 synapses (Atwood et al., 1994; Cooper et al., 1993, 1995a). The number reported in *Astacus leptodactylus* is on average only 10 (Florey and Cahill, 1982). This indicates a fourfold overall difference in the number of morphologically defined synapses per varicosity. Species differences could be responsible for the difference. Interestingly, the total number of synapses per muscle fiber is not much different, because the smaller number of synapses per varicosity in *Leptodactylus* is offset by the larger number of varicosities.

Quantal content per muscle fiber

An approximation of the quantal content that a muscle fiber experiences was calculated (Table 7). The calculation takes into account the difference in quantal emission for primary and secondary varicosities in the central region

and provides three different estimates for the proximal region. The estimated mean quantal content per muscle fiber at 1 Hz is approximately 13 for central muscle fibers and ranges from 21 to 50 for proximal muscle fibers. The mean for proximal fibers is 31, which is 2.5 times the estimate for central fibers.

The differences in the EPSPs of the central and proximal muscle fibers are not fully explained by our measurements. Approximately 25% of the eightfold difference is attributable to the twofold difference in postsynaptic R_{in}. The calculated 2.5-fold difference in quantal content per muscle fiber based on the mean proximal value could account for an additional 30%, leaving approximately 45% of the difference unaccounted for. If the "min" value for proximal fibers is used, most of the difference in the EPSPs could be accounted for, but this value is an extreme one. The observation of "missing quanta" (Wojtowicz et al., 1994), in which not all extracellulary recorded quantal events produce an observable voltage change in the muscle fiber, could introduce a further difference. Studies of this effect have so far been made only on central fibers. Currently, we do not know whether missing quanta are more prevalent in one type of fiber than another. Therefore, at present, the factors accounting for the difference in the EPSPs of the two regions have not been fully elucidated. As was discussed above, sampling to obtain a mean value is likely not to be the best approach to this problem.

The bottleneck regions between varicosities were not included in calculations of the mean quantal content experienced for the muscle fiber. Earlier studies (Jahromi and Atwood, 1974; Florey and Cahill, 1982; Wojtowicz et al., 1994; Cooper et al., 1995a) have shown that there are indeed synapses present in bottleneck regions, but they are many fewer in number than in the varicose regions. Our data show that relatively few active zones occur in such locations (Table 1). Because we observed that the length of terminals and the degree of branching is greater on central muscle fibers, we would expect a greater number of synapses on bottlenecks of central fibers. However, in actuality, we have observed that the number of quanta recorded in regions between varicosities is very small and the number of synapses with active zones is also small (Table 1). These observations indicate a negligible contribution to quantal content for the bottlenecks.

Quantal emission per synapse

Current and previous data (Cooper et al., 1995a) permit a calculation of quantal emission per synapse in the two regions. Serial sectioning of representative varicosities provided counts of synapses included in Table 7. It has been shown (Jahromi and Atwood, 1974; Wojtowicz et al., 1994; Cooper et al., 1995a) that some synapses lack active zones, whereas others have one or more. From quantal content per varicosity in the two regions, we calculated m per synapse and m per synapse with active zones (Table 7). The calculated values indicate a 6.6-fold difference between varicosities of the proximal region and the 2° central varicosities and only a 2.7-fold difference between proximal and 1° central varicosities. These values are approximations, because only a few varicosities from which physiological information is available have been serially reconstructed (Cooper et al., 1993, 1995a; Atwood et al., 1994). Nevertheless, the calculations strongly indicate a substantial difference in overall release probability per synapse in the two regions, and this reinforces the results of the binomial statistical analyses of Tables 5 and 6, in which a difference in probability of release at responding synapses is inferred.

The statistical data of quantal evoked release presented here indicate that, at low frequencies of stimulation, there is not a significant difference in the number of release sites (n) but there is a difference in the probability of release (p) between proximal and central terminals. The results are in agreement with those of an earlier study (Cooper et al., 1995a). The proximal varicosities have a mean p of 0.46, whereas for 1° central and (2° plus 3°) central varicosities the mean values are 0.13 and 0.06, respectively (from Tables 5 and 6). It is known that there are more identified active zones within a recorded varicosity than the values of n (putative number of active sites) calculated at low frequencies from the current statistical models based on binomial distributions (Atwood et al., 1994; Cooper et al., 1995b). Earlier studies have also reported that release at crustacean neuromuscular junctions is often non-Poisson (Atwood and Parnas, 1968) and that release at the crayfish neuromuscular junction can be described by binomial distributions (Bittner and Harrison, 1970; Johnson and Wernig, 1971; Zucker, 1973). The distribution profile appears to be quite variable from low-output recording sites of central fibers: Even along a given string of varicosities, the distribution can vary from Poisson to binomial at low frequencies (Table 5). Recently, we have observed that, when the frequency of stimulation is increased, almost all recording sites along low-output terminals exhibit binomial distributions of quantal release, even when a Poisson distribution occurred at low frequency (Msghina et al., 1995). Thus, the binomial description is likely more relevant for consideration of underlying physiological mechanisms. A current hypothesis based on the binomial distribution is that some active zones are not releasing quanta at low frequencies, because n is much lower than the morphologically defined active zones on a varicosity. At higher frequencies of stimulation, more synapses may be recruited: Values for n increase with frequency, along with values for p (Atwood et al., 1994; Wojtowicz et al., 1994). Viewed in this light, the large number of synapses on a varicosity may provide a mechanism for increasing the short-term plasticity of the nerve terminal, especially frequency facilitation.

In conclusion, we have provided answers to some of the questions raised in the Introduction. First, we have shown that the amount of transmitter released by a nerve terminal onto a single target cell is not the same at all points along its length. In the crayfish, as in the frog, the more distal locations of a terminal release less transmitter at low frequencies. Second, we have confirmed that the amounts of transmitter released by different endings of the same neuron onto defined target cells are different, and we infer that this difference arises mainly from the probabilities of release at responding synapses and not from different numbers of synapses or active zones. In the samples we analyzed, calculations based on mean values indicated that the difference in R_{in} accounts for approximately 25% of the difference in EPSP amplitude, with a further 30% being accounted for by the difference in quantal content. For the quantal content contribution, wider limits are possible (20–50%). In estimating this contribution, we were forced to conclude that sampling to obtain mean values for physiological and morphological parameters is an imperfect vehicle for addressing differences in synaptic performance of two populations of target cells, even in this relatively simple system.

The reasons for the difference in probability of release at synapses of the two regions have yet to be fully elucidated. Electrical differences in the terminals and higher incidence of active zones in the proximal region (Table 1) are possible underlying causes.

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