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CHANGES IN BINOMIAL PARAMETERS OF QUANTAL RELEASE AT CRUSTACEAN MOTOR AXON TERMINALS DURING PRESYNAPTIC INHIBITION

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SUMMARY

1. The effects of presynaptic inhibition on quantal release of transmitter were investigated at neuromuscular junctions of the motor axon supplying one of the limb muscles of a crab (*Pachygrapsus crassipes*).

2. Binomial analysis of transmitter release recorded at selected neuromuscular junctions with an extracellular 'macro-patch' electrode indicated high probability of release (p) from a limited number of available sites (n). During presynaptic inhibition, both n and p were reduced.

3. The binomial model provided a good description of results from non-inhibited junctions. During presynaptic inhibition, results from some junctions could be described by the binomial model, while those from other junctions could not. An interpretation of this finding is that presynaptic inhibition differentially affects the probability of release at various release sites of the neuromuscular junctional complex.

4. A morphological study of the region of transmitter release under the macro-patch electrode was made. Release-dependent uptake of horseradish peroxidase (HRP) into presynaptic terminals was restricted to the region under the recording electrode, by perfusing the preparation with calcium-free solution containing HRP. Transmitter release, and HRP uptake, occurred only at the site of the electrode, which was filled with a calcium-containing solution. Subsequently, serial sections were prepared for electron microscopy and the region of transmitter release was reconstructed.

5. Numerous axo-axonal synapses were found in the HRP-labelled region. Thus, the morphological prerequisite for presynaptic inhibition exists at the site of transmitter release, and not exclusively at a more remote region.

6. The number of morphologically identified excitatory neuromuscular synapses exceeded the 'release sites' estimated from the binomial model (n) by a wide margin. Morphological differences among synapses were observed. It is proposed that not all morphologically identified synapses participated in transmitter release under the experimental conditions employed. Thus, morphologically defined synapses are

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likely to be non-uniform in their response properties, including probability of transmitter release (p).

INTRODUCTION

Changes in quantal release of transmitter at crustacean motor nerve terminals during presynaptic inhibition were first investigated by Dudel & Kuffler (1961), who demonstrated that presynaptic inhibition reduces the probability of transmitter release, but not quantal size. This ruled out changes in sensitivity or availability of postsynaptic receptors, and firmly established a presynaptic locus of synaptic inhibition.

Dudel & Kuffler (1961) employed a Poisson statistical analysis of extracellular synaptic potentials recorded focally at individual motor axon terminals. More recent work has demonstrated that quantal release from crustacean motor axon terminals is better described by binomial, rather than Poisson, statistics (Johnson & Wernig, 1971; Zucker, 1973; Wernig, 1975). To date, there has not been a binomial analysis of quantal release during presynaptic inhibition at the crustacean neuromuscular junction.

In the binomial model from transmitter release (del Castillo & Katz, 1954) the mean number of quantal units released by a nerve impulse (m) is thought to depend upon two parameters, n and p ($m = n\,p$). Previous work in both crustacean (Zucker, 1973) and vertebrate (Korn, Mallet, Triller & Faber, 1982) synapses has led to the general proposal that n represents a fixed number of releasable quantal units or release sites, and p the independent, uniform, and stationary probability of activating each of the n releasable quantal units or responsive release sites.

Despite uncertainties about the exact interpretation of the parameters n and p in the standard binomial analysis (Brown, Perkel & Feldman, 1976; Barton & Cohen, 1977; Lustig, Parnas & Segel, 1986), the application of binomial statistics can provide useful preliminary information about the number of responding units (Korn *et al.* 1982) or about changes in this number with physiological manipulation (Zucker, 1973; Smith, 1983; Wojtowicz & Atwood, 1986). A change in the number of responding synapses during presynaptic inhibition has been predicted by Atwood, Stevens & Marin (1984) from computer simulations of changes in the membrane potential of varicose crustacean motor terminals. The small size of synapse-bearing nerve terminals and difficulties in penetrating them with a microelectrode have prevented direct measurements of presynaptic electrical events in terminal varicosities during presynaptic inhibition, although intra-axonal recordings near the terminals have been achieved (Baxter & Bittner, 1981).

It has been predicted that increased chloride conductance in the presynaptic terminal membrane of the excitatory motor axon induced by a single quantal unit of the inhibitory transmitter at an axo-axonal synapse will significantly reduce the depolarization associated with a nerve impulse in the inhibited excitatory axon. The extent of the inhibitory effect will vary with the location of the axo-axonal synapse and with the morphology of the inhibited terminal (Atwood *et al.* 1984).

It follows that in the presence of presynaptic inhibition, the probability of activating individual quantal units located at different varicosities of a crustacean

motor axon terminal may be differentially reduced, and, if the inhibition is strong, the total number of available quantal units may be reduced also.

The objective of the present study is to test this hypothesis by analysing changes in binomial parameters of quantal release from individual motor axon terminals of a crustacean neuromuscular preparation at which presynaptic inhibition is strong. We selected the stretcher muscle of the shore crab, *Pachygrapsus crassipes*, because of the strong presynaptic inhibition observed at many of the excitatory axon terminals.

Preliminary reports of the present study have been presented (Tse & Atwood, 1985, 1986).

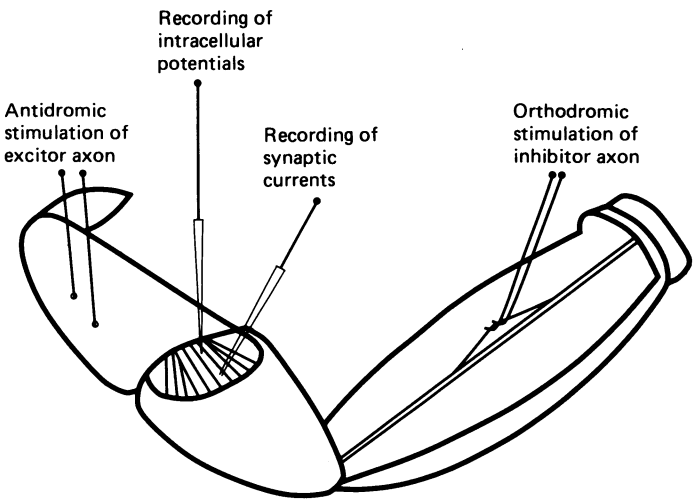


Fig. 1. 'Stretcher' muscle preparation of the crab leg: arrangement for studying presynaptic inhibition.

METHODS

Neuromuscular preparation

Experiments were performed on the leg 'stretcher' muscle (Fig. 1) of the shore crab *Pachygrapsus crassipes* (Randall). Animals were purchased from Pacific Bio-marine Supply Co. in Venice, CA, U.S.A., and had a maximum carapace width of 3–5 cm. Preparations were made from the first three walking legs; regenerated legs were not used.

The preparation of the stretcher muscle was similar to that described by Atwood, Hoyle & Smyth (1965) and Stephens & Atwood (1982, 1983). The preparation was immersed in refrigerated standard crab solution (470 mM-NaCl, 8 mM-KCl, 20 mM-$CaCl_2$, 10 mM-$MgCl_2$, 1 mM-HEPES, at pH 7·4). Superficial muscle fibres on the outer surface of the muscle (Fig. 1) were exposed by removal of a small piece of the overlying carpopodite shell. All electrophysiological data were collected from these superficial muscle fibres.

In decapod crustaceans, the stretcher muscle of each limb is innervated by three motor axons (reviewed in Govind & Atwood, 1982): the excitor (E), which also supplies the opener muscle in the propodite, the specific inhibitor (SI), which inhibits only the stretcher muscle, and the common inhibitor (CI), which inhibits all muscle groups of the limb (Wiens & Rathmayer, 1985; Rathmayer & Bevengut, 1986). The leg nerve that includes these axons was exposed in the meropodite, through removal of part of the meropodite shell and all included muscles. In crabs, the SI and CI axons inhibit the stretcher muscle fibres both pre- and postsynaptically; the presynaptic mechanism is seen when the inhibitory axon is stimulated 1–5 ms before the excitatory axon (Atwood & Bittner, 1971; Wiens & Atwood, 1975).

In grapsid crabs, presynaptic inhibition from the SI axon is usually more prominent than that from the CI axon (Atwood & Bittner, 1971; Wiens & Atwood, 1975); therefore, the present study focused on the maximal presynaptic inhibition of terminals of the E axon when the SI axon was stimulated.

A small nerve bundle which included the SI axon (but not the E and CI axons) was isolated from the leg nerve by microdissection. This nerve bundle was stimulated orthodromically with fine platinum hook electrodes. The E axon was stimulated antidromically with fine stainless-steel electrodes inserted through the shell of the propodite close to the opener muscle.

During all experiments, the preparation was superfused with the standard crab solution at a temperature between 14 and 16 °C, unless noted otherwise.

Electrophysiological recording

Excitatory postsynaptic potentials (EPSPs) were recorded from muscle fibres with intracellular glass microelectrodes filled with 3 M-KCl. These electrodes had tip resistances of 5–10 MΩ when tested in crab saline. The intracellularly recorded EPSPs were amplified by a conventional high-impedance amplifier.

Focal synaptic currents were recorded from selected terminals of the E axon via a macro-patch electrode with a tip opening of 3–10 μm (Dudel, 1981). The macro-patch electrode was filled with the standard crab saline, and had a tip resistance of a few hundred kilohms. The holder of the macro-patch electrode (E. W. Wright EH-2R) had a pressure port for application of suction, which was provided by a micrometer syringe connected to the pressure port with polyethylene tubing. When the macro-patch electrode was optimally positioned for recording synaptic currents, application of gentle suction increased the seal resistance of the electrode up to one-third the value of the tip resistance. The recorded synaptic currents were amplified by a current-to-voltage converter modified from that described in Neher, Sakmann & Steinbach (1978), which included an FET operational amplifier (Burr-Brown 3523) with a 100 MΩ resistor in the feed-back path.

All signals recorded with the macro-patch electrode were stored on an FM tape-recorder (Hewlett–Packard 3964 A) for subsequent computer analyses.

We surveyed the synaptic physiology of the superficial muscle fibres in each preparation by recording EPSPs from them while the E axon was stimulated in the absence and presence of optimally timed presynaptic inhibition from the SI axon (Fig. 2). We selected from each preparation a few muscle fibres which received strong presynaptic inhibition (i.e. in which the amplitude of the EPSP was reduced by over 70% by a preceding inhibitory impulse). In the absence of presynaptic inhibition, EPSPs of these selected muscle fibres were typically large (10–20 mV during 4 Hz stimulation), and showed little short-term facilitation.

Focal recordings of synaptic currents (Fig. 2) were obtained from individual active synaptic terminals on the surface of the selected muscle fibres. The macro-patch electrode was considered to be positioned for optimal recording of focal synaptic currents when their amplitude was maximized and their rise time minimized. Frequently, large focal currents could be recorded from sites in a cleft between muscle fibres; however, we did not use such sites because the currents could have originated from synaptic terminals associated with an unknown number of muscle fibres adjacent to the cleft.

Two sets of data, each including at least 300 synaptic currents evoked consecutively at 4 Hz, were recorded from each selected terminal of the E axon, so that amplitude–frequency histograms could be compiled. The first set of data was recorded in the absence of presynaptic inhibition; the second, in the presence of optimally timed presynaptic inhibiton. We estimated the mean and variance of the unit quantal currents by at least one of three methods. These methods were necessary for two reasons: first, in the absence of presynaptic inhibition, each evoked synaptic release from a selected terminal usually involved the simultaneous release of several quanta of transmitter; secondly, at a physiological temperature (about 15 °C), the evoked release of these quanta is too synchronous to allow unambiguous resolution of inflexions which reflect the release of individual quanta.

Method 1. This method was successfully applied in all selected experiments described in the Results section. It involved the superfusion of the preparation with a crab saline containing 30 mM-magnesium ions but no calcium ions, to reduce the overall probability of evoked quantal release (del Castillo & Katz, 1954). If the maximum number of quanta associated with each impulse was reduced by this method to less than two, the mean and variance of the unit quantal currents could

be estimated from all evoked releases that resulted in synaptic currents exceeding the maximum amplitude of instrument noise. This method had three drawbacks. First, the changes in ionic concentration in the crab saline could produce a small change in the size of the unit quantum (see Dudel, 1981), but since the glutamate-operated channel is highly selective for monovalent cations (Dekin, 1983) this effect is not a serious one. Second, in some preparations, the excitatory motor axon became inexcitable before the maximum evoked synaptic current had been reduced to include less than two quanta. Third, in some preparations, the size of the recorded quantal unit had a large variance which obscured the discrimination of single quanta from instrument noise; this led to some data being discarded.

Method 2. The mean and variance of the unit quantal currents were estimated from spontaneous synaptic currents (del Castillo & Katz, 1954, 1956; Dudel & Kuffler, 1961) that were each assumed to include only a single quantum. This method has been employed in other studies (reviewed in McLachlan, 1978); however, it had a low success rate in this preparation, because spontaneous

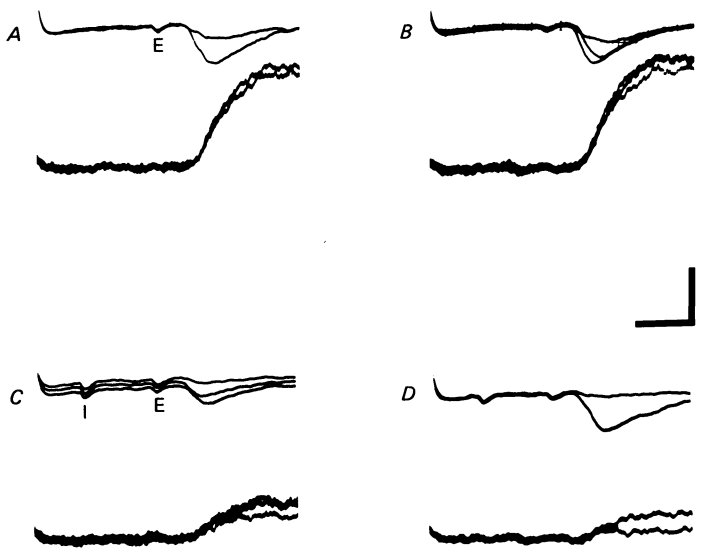


Fig. 2. Simultaneous recordings of EPSPs (bottom traces) and focal synaptic currents (top traces) from a muscle fibre receiving medium-output innervation and strong presynaptic inhibition. Only the E axon was stimulated in *A* and *B*; the E and SI axons were stimulated in *C* and *D*. The terminal current of the E axon is indicated in *A* and *C* by 'E', and that of the SI axon is indicated in *C* by 'I'. There are two sweeps of signals in *A* and *D*; three sweeps in *B* and *C*. Calibration: 2 mV, 0·5 nA (vertical); 2 ms (horizontal).

synaptic currents were rarely recorded from the selected terminals. Typically, less than ten were recorded over a period of 15 min, except when the macro-patch electrode was pressed very firmly against the surface of the muscle fibre. This procedure increased the rate of occurrence of spontaneous events, but usually damaged the axon terminal irreversibly.

Method 3. The mean and variance of unit quantal currents were estimated from asynchronous evoked releases which occurred at low temperatures (Katz & Miledi, 1965; Zucker, 1973). In this preparation, unequivocal asynchronous release was observed when the temperature was reduced to 5 °C. At this temperature, the E axon frequently became inexcitable; therefore, this method had a low success rate. Another possible drawback of this method is that the size of the quantum could be altered at unphysiologically low temperatures.

The variance of the unit quantal currents estimated by the above methods included the variance of the instrument noise; therefore, the value of the latter had to be estimated and subtracted from the former to obtain the actual variance of the quantal unit (see Wojtowicz & Atwood, 1986).

Signals recorded with the macro-patch electrode were digitized for the measurement of random

instrument noise and the amplitude of each evoked synaptic current. These measurements were made by a laboratory computer with a program described previously by Wojtowicz & Atwood (1986).

In the present study, the computer was programmed to: (1) make measurements from a specified number (usually 300) of individual evoked synaptic currents; (2) plot the fluctuations in amplitude of the synaptic current and the instrument noise as frequency histograms; and (3) calculate the mean and variance of each histogram.

Quantal analysis

All sets of focal synaptic currents selected for quantal analysis had to satisfy two criteria. First, the variance of the unit quantal currents had to be small enough so that the mean quantal size could be estimated reliably. Typically, the square root of this variance (i.e. one standard deviation) had to be less than half the mean quantal size. Secondly, the mean amplitude of subsets of fifty consecutively evoked synaptic currents from each set of data had to show no trend of non-stationarity.

The binomial statistical analysis of the present study was directly adapted from that described by Wojtowicz & Atwood (1986). The analysis involved the use of a computer program to search for a set of binomial parameters (n and p) which best describe each set of data. The computer program utilized χ^2 and Komolgorov–Smirnov statistics to compare the experimental data to simulations generated from a binomial statistical model which included the following assumptions:

(1) Evoked release of transmitter from a selected terminal occurred at a fixed number, n, of equivalent sites which could each release only one quantum when activated.

(2) The probability, p, of releasing one quantum of transmitter from each of the n sites was independent, stationary, and uniform.

(3) The release of individual quanta of transmitter gave rise to quantal synaptic currents.

(4) The size of individual quanta of transmitter released from each of the n sites was unchanged in the absence or presence of presynaptic inhibition, and had a Gaussian distribution which centred around the same mean value and had the same variance.

(5) When multiple sites were releasing simultaneously, the variance in the total size of the released quanta was equal to the product of the number of released quanta and the variance of each quantum.

(6) The random instrument noise had a Gaussian distribution and a constant variance regardless of the number of quanta released.

(7) The total variance associated with each evoked synaptic current is the sum of the variance associated with instrument noise and the variance associated with the total size of the released quanta.

The validity of these assumptions will be considered in the Discussion. The probability distribution that can be generated from the above assumptions is given in eqn (1) (after Wojtowicz & Atwood, 1986).

$$P(a,b) = \sum_{r=0}^{n} \left(\frac{n}{r}\right) p^r (1-p)^{n-r} \int_a^b 1/[\sqrt{(2\pi)}\sqrt{(r\,\mathrm{var}_M + \mathrm{var}_N)}]\,(\exp) - \left\{\frac{(x - rM)^2}{2(r\,\mathrm{var}_M + \mathrm{var}_N)}\right\} \mathrm{d}x, \quad (1)$$

where $P(a,b)$ is the probability for the amplitude of an evoked synaptic current (x) to fall between a and b (e.g. 0·0–0·1 nA, 0·1–0·2 nA, etc.); n and p are the binomial parameters; r is an integer value of n within a binomial distribution; var_N is the variance of instrument noise; var_M is the variance of the unit quantal size; and M is the mean unit quantal size.

The $(r\,\mathrm{var}_M + \mathrm{var}_N)$ term in eqn (1) gives the total variance of each Gaussian distribution that is to be multiplied for each binomial category; the term came from the initial assumptions that each released quantum contributed its variance, and that the variance of instrument noise was constant. Estimated values of the variance of instrument noise, and of the mean and variance of the unit quantal size, were required to generate the above probability density distribution; they were calculated from measurements described earlier.

In order to further confirm that the 'best-fitted' sets of binomial parameters were indeed

reasonable values, the total variance and the mean of an entire set of current amplitudes were calculated and substituted into eqns (2) and (3).

$$p = 1 - \frac{\text{variance of synaptic currents} - \text{var}_N}{\text{mean of synaptic currents} \times M} + \frac{\text{var}_M}{M^2}. \quad (2)$$

$$n = \text{mean of synaptic currents}/(Mp). \quad (3)$$

These equations, derived from the method of moments, give accurate estimates of n and p only if all of the assumptions used in the simulations are satisfied. Therefore, if the values of n and p estimated by eqns (2) and (3) deviated significantly from those of the 'best-fitted' simulation (e.g. a negative value of p), then the set of data could not be described by binomial statistics.

The simulation generated by each set of best-fitted binomial parameters reported in the Results section has a statistically significant goodness-of-fit (i.e. there was less than a 5% chance that the experimental data could not be described by the best-fitted binomial parameters).

Focal labelling of active terminals

We utilized activity-dependent uptake of horseradish peroxidase (Holtzman, Freeman & Kashner, 1971; Thompson & Atwood, 1984) to label selected synaptic terminals. Our procedure is described in detail elsewhere (Tse, Marin & Atwood, 1987). It involved the suppression of synaptic transmission in all surface muscle fibres, except at the site immediately under the macro-patch electrode (which was filled with crab saline containing 30 mM-Ca^{2+}), by superfusing the preparation with a calcium-free solution (standard crab saline with no Ca^{2+} and 30 mM-$MgCl_2$) containing 1–2% horseradish peroxidase (HRP). The E and SI axons were alternately activated by 1 min trains of 4 Hz stimuli for a total duration of $\frac{1}{2}$ to 1 h. The quantal synaptic currents and the degree of presynaptic inhibition recorded during these stimuli were similar to those recorded for quantal analysis.

Following stimulation in HRP-containing solution, the preparation was fixed for electron microscopy (2·5% glutaraldehyde and 0·2% formaldehyde in a buffer solution composed of 0·15 M-sodium cacodylate, 0·011% $CaCl_2$, 0·35% NaCl, 13·5% sucrose, and adjusted to pH 7·4 with a small amount of HCl). After 1·5 h of fixation the selected muscle fibre was processed for HRP histochemistry, dehydrated and embedded for sectioning.

Serial sectioning of the recorded region showed higher-than-background HRP uptake in E and SI axon terminals over a region with maximal radius of 10–15 μm (about twice the outer tip diameter of the macro-patch electrode). HRP-labelled vesicles were most numerous at the centre of the labelled region. Spread of Ca^{2+}-containing saline from the electrode appears to account for the fact that the region of HRP uptake is larger than the tip of the macro-patch electrode.

Reconstruction of labelled regions from serial sections (Tse *et al.* 1987) provided information on the number of neuromuscular and axo-axonal synapses under the macro-patch electrode.

RESULTS

In five out of nineteen completed experiments, focal synaptic currents recorded from individual terminals of the E axon (in the presence and absence of presynaptic inhibition) satisfied the selection criteria for quantal analysis. These criteria, and the manipulations required to obtain the data, led to a low success rate in these experiments. In three of the acceptable experiments, the mean and variance of the unit quantal synaptic currents were estimated by at least two of the three methods; the values so obtained are listed in Table 1. (Here, the variance of the unit quantal currents includes variance of unit quantal size plus variance of instrument noise.) In the two remaining experiments, the mean and variance of the unit quantal currents were estimated by the first method only.

In each of the experiments listed in Table 1, the values estimated by the second

and third methods differed by less than 20% from those estimated by the first method. From this, we inferred that the values estimated by the three methods are similar. We used the values estimated from the first method in subsequent analyses of all five selected experiments.

Information employed for quantal analysis is listed in Table 2, in which the standard deviations, instead of the variances, of the unit quantal currents, the unit quantal size, and instrument noise are given, so that they can be compared as percentages of the respective mean unit quantal size. One standard deviation of the unit quantal current ranged from 13 to 52% of the mean quantal size. For a Gaussian distribution of the unit quantal current, fewer than 10% of the unit quantal currents would have an amplitude either smaller than that of failures in evoked release, or larger than that of releases of multiple quanta. Such relatively small variances in the unit quantal currents allowed us to estimate the mean unit quantal size with the accuracy that was required for the binomial statistical analysis.

TABLE 1. Quantal size and quantal variance estimated by three different methods in successful experiments

Experiment	1st method	2nd method	3rd method
	Mean quantal size (pA)		
II	114	98	101
III	135	NA	113
V	87	NA	92
	Total variance of the unit quantal size (pA^2)		
II	593	590	503
III	250	NA	215
V	1186	NA	1230

NA, not applicable.

The estimated mean quantal sizes for the five selected experiments were of the order of 0·1 nA. This value is two to three times smaller than that estimated, with similar apparatus, from the crayfish opener muscle preparation (Dudel, 1981). The lower mean values of quantal current estimated in the present study can be completely attributed to lower seal resistance of the macro-patch electrodes (due in part to the higher salt content of crab solution compared with crayfish solution). The relatively small variation in the estimated mean unit quantal sizes among the five selected experiments suggested that the seal resistance of the macro-patch electrode was similar in all experiments.

Frequency histograms of synaptic current amplitudes from four of the five successful experiments (numbered I–V, as in Tables 1 and 2) are shown in Fig. 3*A–D*. In the absence of presynaptic inhibition, the evoked (4 Hz) quantal release from all selected experiments could be described very well by the binomial model; however, in the presence of presynaptic inhibition, only two of these experiments (I and II) could be described by the binomial model.

Results fitted by the binomial model in the presence of presynaptic inhibition

The data of experiment I (Fig. 3*B*) were obtained from a muscle fibre with 20 mV EPSPs at low frequencies of stimulation and very strong presynaptic inhibition (over

TABLE 2. Mean quantal size and dispersion for successful experiments: data used for binomial models

Experiment	Mean quantal size (pA)	S.D. of quantal currents (% of mean quantal size)	S.D. of quantal size (% of mean quantal size)	S.D. of instrument noise (% of mean quantal size)
I	82	52	45	26
II	101	22	13	18
III	113	13	12	5
IV	88	45	35	28
V	92	37	26	27

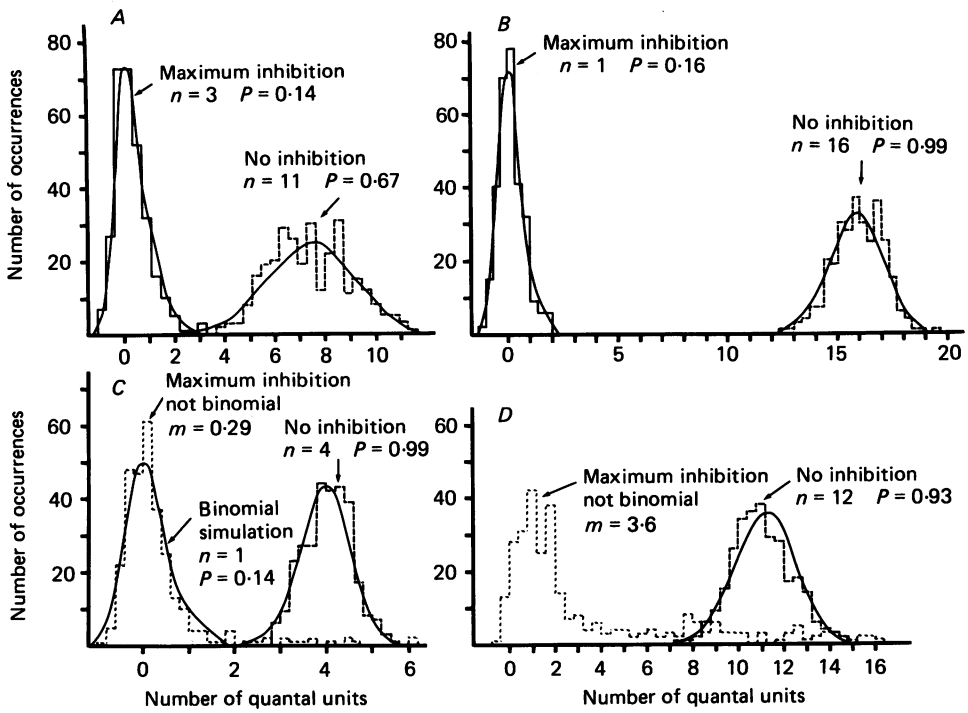


Fig. 3. Frequency histograms of synaptic current amplitudes recorded in four experiments, with and without presynaptic inhibition. Continuous lines show the results of the best-fitting binomial analysis (values for n and p indicated). Dotted lines show the actual data (labelled in the Figure). *A–D*, data and binomial analyses for experiments II, I, III and IV, respectively, as described in the text.

95% reduction in EPSP amplitude). In the absence of presynaptic inhibition, there were no failures in evoked release; the best-fitted binomial parameters were $n = 16$ and $p = 0{\cdot}99$ (the mean quantal content, $m = np$, being 15·8). In the presence of presynaptic inhibition, there were frequent failures in evoked release; the best-fitted binomial parameters were $n = 1$ and $p = 0{\cdot}16$ ($m = 0{\cdot}16$). The overall reduction in mean quantal content during presynaptic inhibition was almost a hundredfold.

A similar case is presented by experiment II (Fig. 3*A*), in which presynaptic inhibition reduced EPSP amplitude by over 90%. Both n and p were reduced, as

shown in the Figure, and mean quantal content (m) was reduced over seventeenfold (from 7·37 to 0·42).

Results not fitted by the binomial model in the presence of presynaptic inhibition

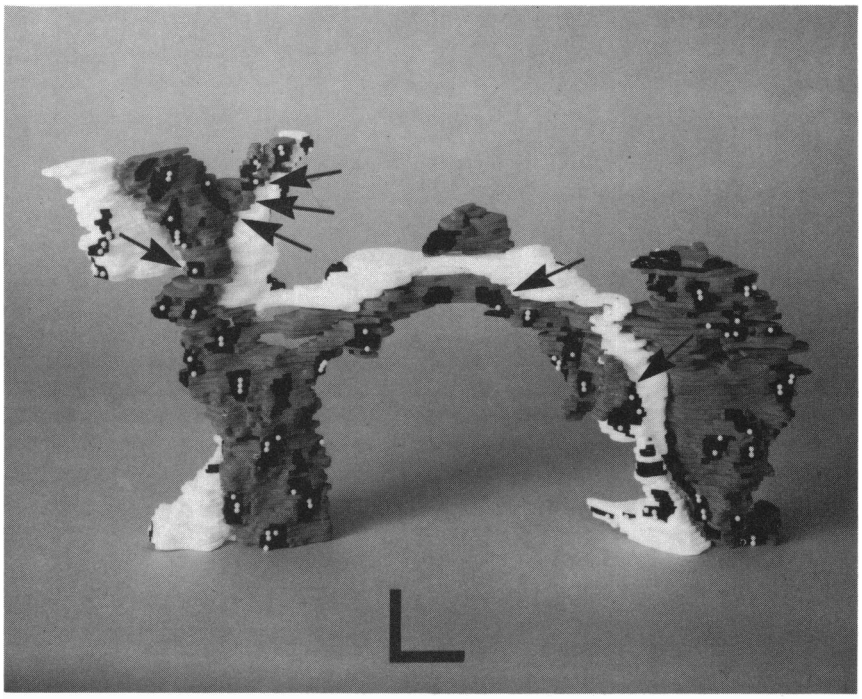
Three key features suggested that, in experiments III, IV and V, evoked quantal releases in the presence of presynaptic inhibition could not be described by the binomial model. First, the simulations generated by the set of best-fitted binomial parameters for these experiments did not produce a statistically significant goodness-of-fit to the data. Secondly, the mean quantal content (m) estimated directly from the quotient of the average amplitude of a set of evoked synaptic currents to the estimated mean unit quantal size had a value that was inconsistent with the product of the best-fitted binomial parameters ($np = m$). Thirdly, the value of p was negative when it was calculated by an equation derived from the method of moments (eqn (2)).

Two examples illustrating the problems encountered with the binomial model during presynaptic inhibition are shown in Fig. 3*C* and *D* (from experiments III and IV respectively).

The data of experiment III were obtained from a muscle fibre with 15 mV EPSPs and strong presynaptic inhibition (over 90% reduction in EPSP amplitude). In the absence of presynaptic inhibition there were no failures in evoked release; the best-fitted binomial parameters were $n = 4$ and $p = 0{\cdot}99$. Presynaptic inhibition reduced the mean quantal content (m) by over fourteenfold (from 4·0 to 0·29), and produced frequent failures in evoked release. However, there were also occasional releases of multiple quanta. When we arbitrarily excluded all synaptic currents which exceeded the average amplitude of two quanta, the quantal release in the presence of presynaptic inhibition could be described by the binomial model ($n = 1$ and $p = 0{\cdot}14$). In this experiment, the quantal releases during presynaptic inhibition deviated from the binomial model because of the 'tail' of larger events.

In experiment IV (Fig. 3*D*), the muscle fibre had 9 mV EPSPs and showed moderately strong presynaptic inhibition (70% reduction in EPSP amplitude). Presynaptic inhibition reduced the mean quantal content (m) by over threefold (from 11·2 to 3·6). In the presence of presynaptic inhibition, there were frequent releases of one or two quanta; however, there were also occasional failures in evoked release, and some releases of several quanta. This gave a pronounced 'tail' in the distribution, and a binomial fit could not be made.

Fig. 4. Reconstructed three-dimensional model of a serially sectioned 12 μm length of E and SI synaptic terminals. The two sides of the reconstructed model are shown. The synaptic terminals of the E axon are grey; synaptic terminals of the SI axon, white; individual synaptic contact areas, black areas on terminals; presynaptic dense bars, small white circles on synaptic contact areas. The region with the highest number of vesicles labelled with HRP in the E axon is indicated by a large star. The distribution of axo-axonal synapses is also shown. Each axo-axonal synapse with at least one presynaptic dense bar is indicated by an arrow-head. The polarity of all axo-axonal synapses indicates that the SI axon releases transmitter onto the E axon. Scale bar = 2 μm (vertical and horizontal).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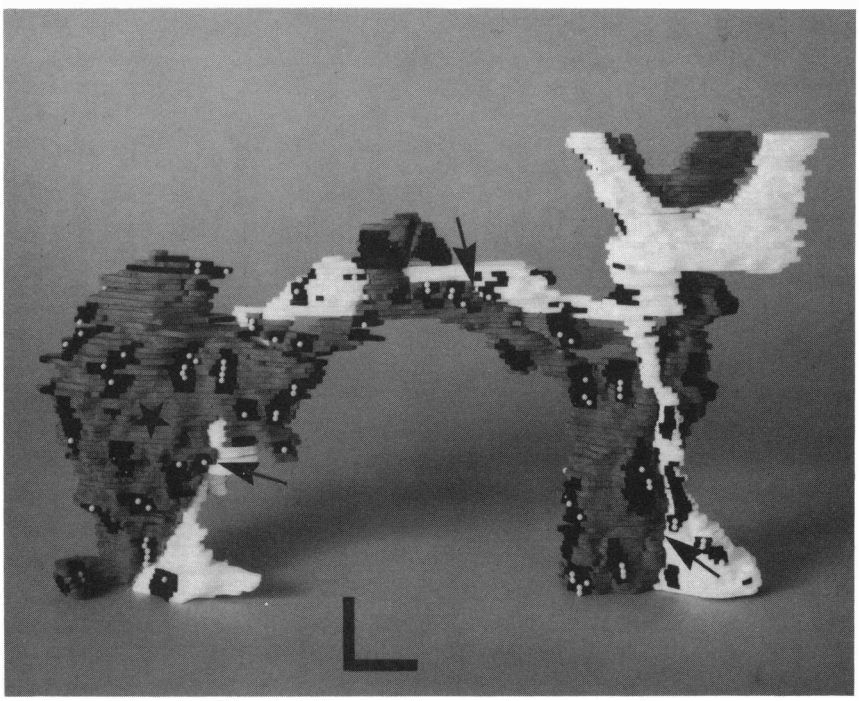
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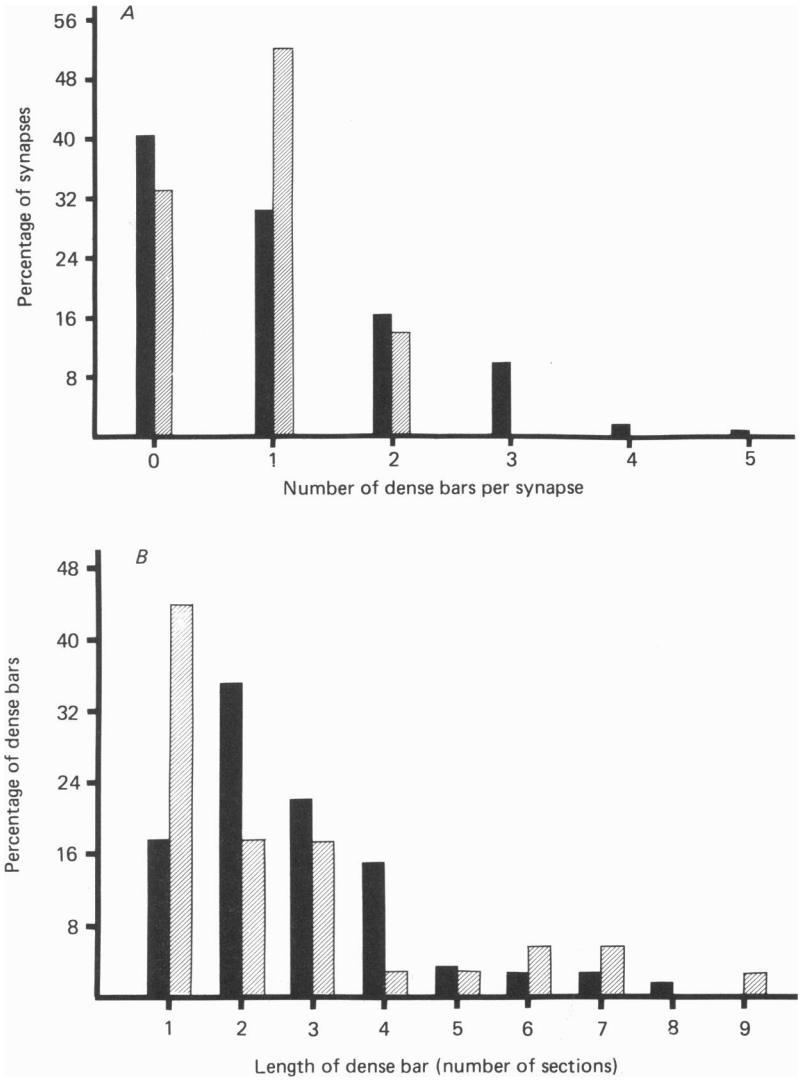

Fig. 5. Histograms of data from Fig. 4 showing: *A*, number of presynaptic dense bars in each synapse; *B*, lengths of individual dense bars (each section has a thickness of about 70 nm). In each case, percentage values are given. Filled bars show data for the E axon; hatched bars, those for the SI axon (including axo-axonal synapses).

Synapses of labelled terminals

Reconstructions from serial sections of labelled terminals were successfully made in four instances. The most complete reconstruction (Fig. 4) includes a 12 μm length of nerve terminal with two varicosities. Data obtained from this reconstruction were representative of observations made in the less complete series.

Nine axo-axonal synapses were identified in the HRP-labelled region of Fig. 4. Therefore, the synaptic requirements for presynaptic inhibition are localized in the region of excitatory transmission. This conclusion is supported by the observation

that presynaptic inhibition takes place at the macro-patch electrode in calcium-free solution.

A striking feature of the reconstructions was the large number of neuromuscular synapses identified by morphological criteria (Table 3). For the excitatory terminal, 109 individual synapses were counted. However, some were very small, and, as shown in Fig. 5*A*, 40% of the identified excitatory synaptic contact areas did not possess a presynaptic dense bar (thought to be equivalent to the active zone of vertebrate neuromuscular junctions: see Jahromi & Atwood, 1974; Pearce, Govind & Shivers, 1986). The number of neuromuscular synapses and dense bars counted for the SI axon in the same region was less than for the E axon (as in previous comparisons of excitatory and inhibitory terminals: see Jahromi & Atwood, 1974; Atwood & Kwan, 1976).

TABLE 3. Morphological data for the labelled terminals of E and SI axons reconstructed in Fig. 4

Morphometric parameter	E axon	SI axon
Total number of synapses	109	42
Total number of dense bars	113	34
Average number of dense bars per synaptic contact area	1·04	0·81
Maximum number of dense bars per synaptic contact area	5	2
Average length of individual dense bars (μm)	0·28	0·29
Maximum length of individual dense bars (μm)	0·8	0·9

Individual synapses of both E and SI axons exhibited considerable variation in number and size of presynaptic dense bars (Table 3; Figs 4 and 5). The E axon possessed more of these structures than the I axon, but the I axon had a higher percentage of dense bars greater than 0·4 μm in length (Fig. 5*B*). This observation also is in accord with previous studies (Jahromi & Atwood, 1974; Atwood & Kwan, 1976; Atwood, Govind & Kwan, 1978).

All of the axo-axonal synapses had at least one presynaptic dense bar, and the longest presynaptic dense bar of the SI axon was located at an axo-axonal synapse.

DISCUSSION

Presynaptic inhibition of crustacean motor nerve terminals (reviewed in Nicoll & Alger, 1979) is thought to involve reduced depolarization of the excitatory nerve terminal by its action potential. GABA is released from axo-axonal synapses of the inhibitory axon which impinge upon the excitatory motor nerve terminals; the released GABA increases the chloride conductance in these terminals to shunt depolarizing currents which normally trigger synaptic release. This general picture is confirmed by recent intra-axonal recording near inhibited nerve terminals of crayfish (Baxter & Bittner, 1981).

Computer simulations (Atwood *et al.* 1984) have led to several predictions for inhibitory effects in varicosities of a motor axon terminal connected in series to the

pre-terminal axon by one or more bottlenecks. First, the increased chloride (shunt) conductance associated with release of even a single quantum of GABA from an inhibitory axo-axonal synapse can significantly reduce the depolarization of all varicosities distal or adjacent to the axo-axonal synapse in question. Secondly, although each individual varicosity of a motor axon terminal is essentially isopotential, serial distribution of several inhibitory axo-axonal synapses along the terminal can produce different strengths of inhibition even at adjacent varicosities.

The morphological studies completed for the present experiments confirm that presynaptic inhibition is mediated by axo-axonal synapses close to the site of release of excitatory transmitter substance, rather than at a more remote location (Smith, 1978; Atwood *et al.* 1984). Not only are axo-axonal synapses present in substantial numbers at sites where presynaptic inhibition was focally recorded, but they continue to exert their effect in zero-calcium solution when all synaptic transmission except that under the focal electrode is blocked.

The binomial analyses of the present study indicate that strong presynaptic inhibition at crab neuromuscular junctions could involve a reduction in both the probability of transmitter release and in the number of available quantal units. This is in accord with the predictions drawn from three-dimensional ultrastructure and electrical models (Atwood *et al.* 1984). However, the finding that the binomial model does not fit the data at some of the inhibited nerve terminals suggests that certain of the underlying assumptions are not valid for such sets of data. In particular, the assumption of uniform probability is questionable.

At crustacean neuromuscular junctions, the physical identity of the releasable quantal unit n has not been firmly established. Studies by Korn, Triller, Mallet & Faber (1981) provided an almost perfect correlation of the binomial parameter n and the number of presynaptic boutons supplying each identified Mauthner cell in the brain of the goldfish. Moreover, the same group of investigators (Triller & Korn, 1982) have inferred, from ultrastructural evidence, that each bouton normally releases zero or one quantal unit of transmitter in response to a presynaptic nerve impulse.

At crustacean neuromuscular junctions, the number of morphologically identified synapses seems to be considerably larger than the estimated values for n at a particular recording site. This is borne out by the data of Fig. 4 and Table 3, in which it is shown that the number of morphologically identified synapses in a focally recorded region of the E axon terminal exceeds the estimated binomial parameter n in any of the successful experiments by a factor of 5. This would suggest that not all of the morphologically identified synapses are participating in transmitter release under the conditions of the experiment. If probability of release is not uniform among the morphological synapses at a recording site, the simple binomial model would not apply, and a more comprehensive model would have to be developed.

The morphological details of the individual synapses confirm the impression of non-uniformity (Figs 4 and 5). Some lack presynaptic dense bars, others have one or more, and the size of the individual dense bars is quite variable. The number of synapses with dense bars over 0·4 μm in length matches more closely the binomial parameter n for the E axon than does the total number of synapses (Fig. 5*A*). Furthermore, the SI axon has a higher percentage of this type of synapse, though the

total number of synapses is lower. Previous studies have shown that quantal content of transmission for crustacean specific inhibitory axons is generally higher than for excitatory axons innervating the same muscle fibre (Atwood & Bittner, 1971). Although it seems likely that the presynaptic dense bars are *potential* sites of transmitter release (Pearce *et al.* 1986), it is also becoming increasingly likely that normal physiological functions involve only a limited proportion of the total available (Atwood *et al.* 1978; Atwood & Wojtowicz, 1986).

The conclusion that synapses are non-uniform in physiological effectiveness is in agreement with those of several other studies. In the cat spinal cord, Redman & Walmsley (1983*a*, *b*) found that different boutons of an identified Group Ia afferent terminal do not appear to have the same probability of quantal release; therefore, the standard binomial analysis does not apply. In a recent study on presynaptic inhibition of synaptic potentials of cat spinal motoneurones, Clements, Forsythe & Redman (1987) showed non-uniform effects of presynaptic inhibition on different unitary components of the EPSP produced by a single Ia afferent axon. Deconvolution procedures were used to extract the changes in response probability associated with each unitary component. Thus, in the vertebrate spinal cord, presynaptic inhibition may affect particular boutons selectively. This result parallels those of the present study in many respects.

When presynaptic inhibition is occurring in crab excitatory motor terminals, the binomial model does not provide a good fit for the data in a significant number of cases. Possible reasons for this include: (a) variable release of inhibitory transmitter at different axo-axonal synapses; (b) non-uniform influence of inhibition on the different responding quantal units. The latter possibility appears likely from previous ultrastructural work and modelling (Atwood *et al.* 1984). Concerning the former possibility, it is known that quantal content of inhibitory transmission is greater than that of excitatory transmission for the same muscle fibre (Atwood & Bittner, 1971). Since the muscle fibres of the present sample were all of the high-output type, it is likely that inhibitory transmission occurs with high probability for the fibre as a whole. However, it is also known that strength of presynaptic inhibition is variable among terminals of a given muscle fibre (Dudel & Kuffler, 1961; Wiens & Atwood, 1975). Thus, at some of the sites selected for the data sets of the present study, variable inhibition could have played an important role in producing dispersion of the data.

The questions about non-uniform probability and non-stationarity of the inhibitory effect would add weight to objections raised by others to the simple version of the binomial model. Several studies (Brown *et al.* 1976; Barton & Cohen, 1977; Lustig *et al.* 1986) have indicated that conventional methods of analysing quantal fluctuations in synaptic release can fortuitously estimate a set of seemingly good-fitting (but meaningless) values of n and p from data simulated under conditions which cannot be described by binomial statistics (e.g. non-uniform probabilities of release from a population of quantal units).

Development of a more complete model for transmission at crustacean terminals would have to take into account possible non-uniformities of transmitter release probability (p) for different responding quantal units (n). Also, some of the data for presynaptic inhibition may be very difficult to model if the probability of inhibitory

transmitter release is variable or non-stationary. More advanced methods of analysis, such as those currently employed by Clements *et al.* (1987), may provide a better description of non-uniform release probabilities during presynaptic inhibition.

Dr Leo Marin collaborated in developing the method for focal labelling of active terminals with horseradish peroxidase (Tse, Marin & Atwood, 1987) and prepared the sections used for electron microscopic studies. Marianne Hegström-Wojtowicz assisted with preparation of Figures and manuscript. Dr M. Wojtowicz provided suggestions and help with the statistical programs used in the analysis. The work was supported by a grant from NSERC (Canada), with supplementary support from a grant from the MRC (Canada). F. Tse received financial support from an Ontario Graduate Scholarship.

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