The Quantal Nature of Synaptic

Transmission at the Neuromuscular

Junction of a Spider

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ABSTRACT Spontaneous and evoked release of transmitter at neuromuscular junctions in three different leg muscles of a tarantula (*Dugesiella hentzi*) was investigated. In most cases the spontaneous miniature potentials were released independently, although bursts from single synaptic junctions occasionally occurred. In contrast to recent findings in other arthropod muscles, focal extracellular recording from junctional areas revealed that the evoked release of transmitter quanta followed Poisson's theorem at low quantal content synaptic junctions in arachnid muscles.

INTRODUCTION

The quantum hypothesis (del Castillo and Katz, 1954 a) for the release of neurotransmitter by chemically mediating synapses has gained wide acceptance. This hypothesis states that neurotransmitters are released from the presynaptic nerve terminals in discrete packets, called quanta. Some of the quanta are released spontaneously causing small discrete postsynaptic depolarizations (miniature potentials). During the depolarization of the presynaptic terminal by an action potential, the probability of release of quanta transiently increases. The resulting postsynaptic potentials are multiples of unitary potentials which themselves are identical in amplitude to the miniature potentials.

The hypothesis of quantal transmitter release was first worked out for the transmission at the motor end plate in frog skeletal muscle. It has since been confirmed in a number of vertebrates and in crustaceans (Dudel and Kuffler, 1961 a; Johnson and Wernig, 1971; for review, see Martin, 1966). There are, however, only few data for other arthropod muscles. The occurrence of miniature potentials was demonstrated in insects (Usherwood, 1963) and chelicerates (scorpion, Gilai and Parnas, 1970; *Limulus*, Parnas et al., 1968). These studies did not investigate, however, if the transmitter released in response to nerve stimulation is composed of quantal units. Recently, Bittner

and Harrison (1970) found transmitter release patterns at crayfish neuro-muscular junctions which did not fit the predictions by the compound Poisson hypothesis, and Usherwood (1972) reported similar findings for an insect muscle. Therefore it seemed worthwhile to test the quantal release hypothesis on nerve muscle preparations of arthropods other than crustaceans and insects.

METHODS

The experiments were done on superficial fibers of the musculus promotor tibiae, the musculus flexor metatarsi maior, and the musculus flexor metatarsi bilobatus in the legs of the tarantula *Dugesiella hentzi*. These muscles are innervated by at least three excitatory motor axons (Rathmayer, 1965 a) and the promotor tibiae, in addition, receives inhibitory innervation (Brenner, 1972). Singly innervated muscles which would be more convenient for the following experiments (for reasons described below) have not been found in the tarantula leg (Rathmayer, unpublished data). The muscles were exposed by removing the cuticle on the anterior side of the leg. The motor nerve was dissected in the femur and stimulated via a suction electrode. Since the axons could not be separated anatomically, the ones with the distinctly lowest stimulus threshold were always used. Because they produce junction potentials below 2 mV the correction for nonlinear summation is negligible (Martin, 1955). In the promotor tibiae muscle the possibility of simultaneous stimulation of excitatory and inhibitory axons cannot be excluded. The inhibitory axon of this muscle, however, has nearly always a higher stimulus threshold than the excitatory axons that were stimulated with a strength just above threshold. Also, the inhibitory axon rarely innervates the surface fibers used for this investigation.

The electrophysiological techniques were conventional. Glass micropipettes were filled with 3 M KCl (resistance 10–20 MΩ) for intracellular measurements. For extracellular recordings the micropipettes were filled with 3 M NaCl and had a resistance of 1–5 MΩ. Experiments were carried out at room temperature unless otherwise specified. The saline used has been described by Rathmayer (1965 b); it was buffered at pH 7.2 with Tris chloride. Nervous conduction was blocked with tetrodotoxin (Sankyo Ltd., Tokyo) in experiments testing the spontaneous release of transmitter. The abolition of junction potentials upon stimulation indicated the blockage of impulse conduction in the axons.

RESULTS

Spontaneous Miniature Potentials

While recording intracellularly from single muscle fibers at any point along their length, small depolarizing potentials were observed (Fig. 1 a). They averaged about 100 μV with the largest reaching approximately 500 μV. Since they did not disappear after blocking nerve conduction by tetrodotoxin (10^{-5} g/ml for 1 h), it is unlikely that these potential changes were due to spontaneous electrical activity in the terminals of the motor axons.
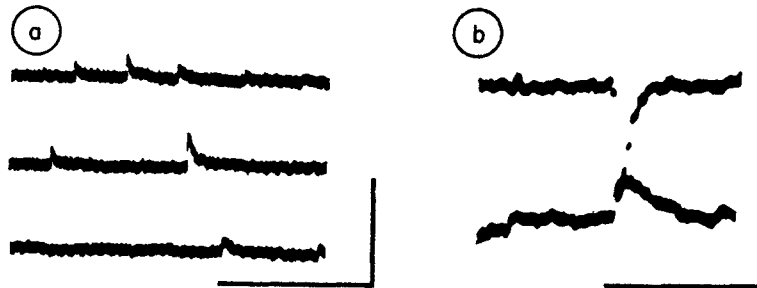
FIGURE 1. (a) Miniature excitatory junction potentials (mejp's). Note difference in time courses of rising phases. Calibration: 1 mV; 300 ms. (b) Simultaneous extracellular (upper trace) and intracellular (lower trace) recording of a mejp. Calibration: extracellular, 0.5 mV; intracellular, 1 mV; 20 ms.

The time intervals between two successive potentials usually ranged between 200 ms and several seconds. Higher frequencies of up to 10/s were occasionally observed. The plot of the interval frequency distribution of these spontaneous potentials is well described (dashed line in Fig. 2 a) by the equation

$$n_t = N \frac{\Delta t}{T} \exp \left(-\frac{t}{T} \right), \qquad (1)$$

where N = number of time intervals measured, n_t = frequency of observed time intervals ranging between t and $t + \Delta t$, T = mean time interval. This equation was first applied by Fatt and Katz (1952) to miniature potentials at the frog motor end plate. The good fit (Fig. 2 a) of the experimentally observed data by Eq. 1 suggests the absence of interaction between successive potentials and is consistent with a random process producing these potentials. Because of their similarity to spontaneous miniature potentials in vertebrates (Fatt and Katz, 1952) and invertebrates (Dudel and Orkand, 1960; Usherwood, 1963) these potentials will be referred to as miniature excitatory junction potentials (mejp's).

Fig. 2 b gives a typical amplitude histogram of mejp's recorded from a fiber in the musculus flexor metatarsi maior. The skewed shape of the distribution is to be expected since these muscle fibers are innervated multiterminally along their entire length (Rathmayer, 1965 b). The potentials are generated by numerous synapses at various distances from the recording electrode and thus are attenuated by the cable properties of the fiber before reaching the electrode.

The following analysis was made to test whether the shape of the amplitude histogram is in quantitative agreement with the known morphological features of these spider muscle fibers. It will be assumed that the junctional areas are approximately
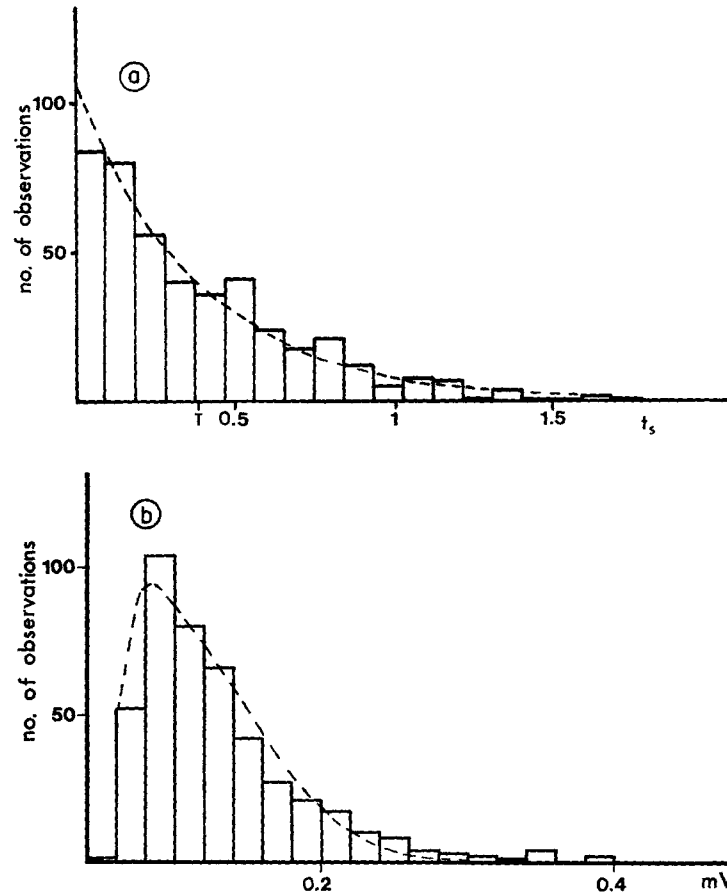
FIGURE 2. (a) Frequency distribution of the intervals between 443 successive miniature potentials. Dashed line drawn according to Eq. 1. T = mean time interval. (b) Amplitude distribution of mejp's. Same experiment as in Fig. 2 a. Dashed line drawn according to Eq. 2. Computed values for x = 140 μm; y = 0.14 mV; σ = 0.055 mV. Mejp's recorded from the center of a fiber (total length = 4.3 mm, space constant = 2.7 mm). For further explanation see text.

evenly distributed over a given muscle fiber, and that the amplitude distributions of the mejp's at each ending are normally distributed, and that the frequency of discharge is approximately equal at all junctions. Thus, the recorded mejp amplitude distribution should be the sum of a set of distributions. The shape and mean of the distribution recorded from a given junctional area are determined by the distance from the recording electrode and the cable properties of the muscle fiber. The following expression is the sum of probabilities of recording a mejp with the amplitude z, assuming normally distributed amplitudes at n junctions:

$$F(z) = \sum_{i=1}^{n} \exp\left(-(z \exp(ix/\lambda) - y)^2/2\sigma^2\right), \qquad (2)$$

where x, y, and σ are, respectively, the mean values of the distance between two neighbouring junctions, the mean amplitude of the mejp's at a single release site if there is no spatial decrement, and the corresponding standard deviation. The value for n is obtained from $n = L/x$ where L is the length of the fiber, and λ is the space constant for the decrement of mejp's travelling along the fiber (Gage and McBurney, 1972). The exponential expression inside the parentheses corrects each mejp amplitude for its attenuation by the cable properties of the muscle fiber. The relative values $F(z)$ have to be normalized to the number of observed spontaneous discharges. Using Eq. 2 and taking into account the relative position of the recording electrode along the fiber, the dashed curve in Fig. 2 b was fitted to the experimentally observed data, using a curve-fitting computer program (Chandler, 1965). The computed value for x (140 μm in Fig. 2 b) lies in the range of the histologically observed distances (Rathmayer, 1965 b). In addition, both y and σ have reasonable values. To exclude the possibility of a fit by chance through the curve derived from Eq. 2, mejp's were recorded simultaneously at the end and the center of a fiber in the locust extensor tibiae muscle, and curves based on Eq. 2 were calculated from both of the amplitude histograms taking into account the different positions of the electrodes. The agreement between theoretical prediction and experimental measurements was reasonably good and the computed parameters x, y, and σ for the curves were approximately equal (Brenner, unpublished data).

Quantal Composition of the Excitatory Junction Potentials

To test whether the excitatory junction potentials (ejp's) are multiples of the mejp's, a different recording method has to be used, because intracellularly recorded ejp's are the summated response of many activated junctional areas of the same fiber (Dudel and Kuffler, 1961 a).

In the extracellular vicinity of a junctional area the density of the synaptic current is sufficiently high to produce a voltage drop across the resistance of the bathing fluid (Fatt and Katz, 1952), making possible the focal extracellular recording of the ejp's. By moving an extracellular electrode along the clefts between two muscle fibers, one finds spots from which sharp negative-going potential changes can be recorded upon stimulation of a motor axon. Occasionally, such a deflection occurs in the absence of preceding nerve stimulation. This event coincides then with an intracellularly recorded mejp (Fig. 1 b) indicating that they are, in fact, identical.

Fig. 3 shows superimposed records of successive extracellularly recorded ejp's. The amplitudes of these potentials vary in discrete steps which are approximately equal and have the same size as an extracellular mejp. The extracellular ejp's are preceded by a small diphasic voltage deflection, the excitatory nerve terminal potential (entp). The time between the entp and the onset of the ejp's is about 1 ms. This synaptic delay is similar to that reported for the crayfish neuromuscular junction (Dudel and Kuffler, 1961 a). The extracellularly recorded miniature potentials occasionally occurred in

Figure 3. Superimposed records of three extracellular recorded ejp's and one mejp from the same recording site (different time scale for mejp recording). Note ejp fluctuation in steps which correspond to mejp amplitude. Time calibration: 2 ms (for ejp's); 10 ms (for mejp).

bursts, as has been reported for crab and locust neuromuscular preparations (Atwood and Parnas, 1968; Usherwood, 1972). It could not be decided, however, whether these bursts were artifactual, e.g., due to mechanical deformation of the nerve endings by the microelectrode, or whether they were an intrinsic feature of these endings. In any case, the junctions producing mejp bursts showed the same release pattern of evoked extracellular ejp's as did junctions not exhibiting mejp bursts.

Often, we observed at a single recording site two or three populations of extracellular miniature potentials which differed in amplitude. This may have been caused either by the presence of junctions of two different axons, with one at a greater distance from the recording electrode, or by the presence of different release sites of the same nerve branch. There is electron microscope and electrophysiological evidence for both of these possibilities (Brenner and Rathmayer, unpublished observations) in spider muscles. To avoid this complication records from single release sites were preferred for analysis. Furthermore, the junctions formed by these axons release fewer quanta per impulse, a property which reduces possible disturbing influences from neighboring release sites.

Statistical Analysis of the Extracellular ejp Amplitudes

According to the model of del Castillo and Katz (1954 a) a presynaptic terminal contains a pool of n transmitter quanta each of which has the same probability, p, of being released when an action potential invades the terminal. This means that the observed numbers of failures of responses, of unit potentials and of their multiples follow binomial statistics. If p is very small, the binomial expression is approximated by the Poisson distribution:

$$n_x = N \frac{e^{-m} m^x}{x!}, \tag{3}$$

where, in a series of N trials, n_x is the number of responses containing x

quanta, and m is the average number of quanta released per nerve impulse. The parameter m can be calculated by dividing the average amplitude of extracellular ejp's by the average amplitude of the spontaneous potentials. Fig. 4 shows the amplitude histogram of the extracellular ejp's recorded from the promotor tibiae muscle in a series of 356 stimulations. The dashed line represents the prediction if the release of the transmitter quanta follows Poissons theorem (for procedure, see Martin, 1966). The variance of the unit potentials had to be determined by trial and error since there were only 10 miniature potentials recorded in this experiment (successive values for the variance were tried until the best fit for the first peak of the histogram was found). The good agreement in Fig. 4 between the theoretically expected and the observed frequencies of extracellular ejp's containing failures (1, 2, or 3 quanta) indicates the validity of the quantum hypothesis at the spider neuromuscular junction. In some experiments, a different method of measuring the quantum content of the extracellular ejp's was used. Katz and Miledi (1965) showed that the process of transmitter release is prolonged at low temperature. In this circumstance, one can count quanta within an extracellular ejp. The method has successfully been used by Johnson and Wernig (1971) and Wernig (1972 a and b) for analyzing the transmitter release at the crayfish neuromuscular junction. Neither the average amplitude nor the variance of the miniature potentials are needed. The observed frequencies of failures and of responses containing 1, 2, 3, 4, . . . quanta can
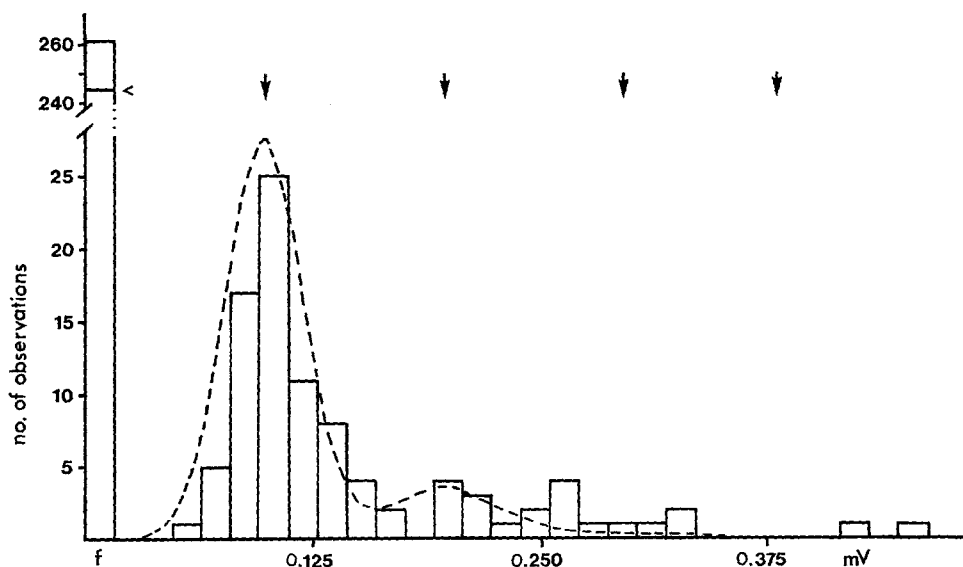

FIGURE 4. Amplitude histogram of extracellular recorded ejp's. Dashed line represents prediction by Poisson's theorem. Arrows indicate average mejp amplitude and its multiples. Arrowhead shows expected number of failures (f). 356 trials.

directly be compared with the expected values from equation (3). Table I shows the results of two experiments, performed at 9°C at the promotor tibiae muscle of the tarantula.

This method could not be used to analyze transmitter release from junctions with quantal contents higher than approximately one, because at 9°C the process of transmitter release was not sufficiently prolonged to make more than three quanta within an extracellular ejp clearly distinguishable. Cooling the preparation below 8–9°C, however, blocked nerve conduction in this preparation.

The mean quantal content can also be calculated from the percentage of failures of responses: $m_o = \log_e (N/n_o)$. Agreement between m_o and the m determined from the average amplitudes of extracellular ejp's, $\bar{v}$, and mejp's, $\bar{q}$, indicates transmitter release according to Poisson's theorem. Because the sum of a set of Poisson distributions is also Poisson (Hubbard et al., 1969) this method was used to analyze data obtained from double and triple release sites. Fig. 5 shows the results of eight experiments on the promotor

TABLE I

PREDICTED AND OBSERVED NUMBERS OF FAILURES AND 1, 2, OR 3 QUANTA RELEASED IN RESPONSE TO STIMULATING THE NERVE AT 1/S

Two experiments at 9°C. Prediction based on Eq. 3. p = predicted, o = observed.

Exp.	N	n_0		n_1		n_2		n_3	
		p	o	p	o	p	o	p	o
I	330	220	215	90	97	18	16	4	2
II	200	182	182	17	17	1	1	0	0

tibiae and the flexor metatarsi maior muscles. The good agreement between m_o and m further supports the validity of the quantum hypothesis for transmitter release at the investigated spider neuromuscular junctions. Since a junctional area in these muscles may contain endings from several axons close to each other, it is likely that some of the extracellular mejp's originated from synapses of axons other than the one stimulated during measurement of the extracellular ejp's. However, two results argue that this probably did not lead to a serious error in estimating the average mejp amplitude. First, all the values for m_o which are not based on the mejp distributions, agree with the values for m. Second, there is good agreement between m_o and another estimate of the mean quantal content which in some experiments again is little influenced by the mejp's. This estimate is given by Eq. 4 (del Castillo and Katz, 1954 a; Martin, 1966):

$$m_{CV} = (1 + [cv]^2)/(CV)^2, \qquad (4)$$

where m_{CV} = mean quantal content, cv = coefficient of variation of quantal amplitude, and CV = coefficient of variation of the extracellular ejp's.

In Table II, m_{CV} and m_o are compared for the same experiments shown in Fig. 5. The third column of the table gives the values for $1 + (cv)^2$ which are the corrections for the mejp variability.

There is good agreement between m_{CV} and m_o, both in the experiments where this correction is small and in the experiments where it is relatively
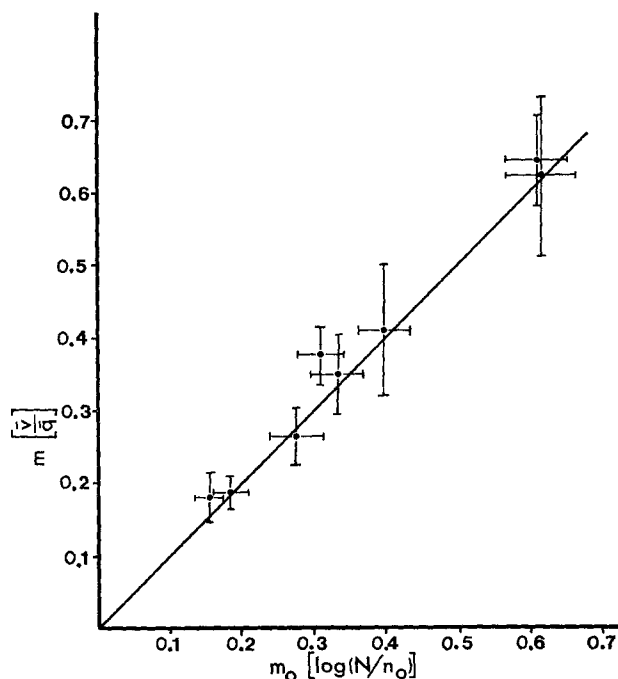

FIGURE 5. Plot of m_o ($\pm$SE) versus m ($\pm$SE). Equality of m_o and m (solid line) indicates quantal transmitter release according to Poisson's theorem.

TABLE II

COMPARISON OF $m_{cv} = (1 + [cv]^2)/(CV)^2$ AND $m_o = \log_e(N/n_o)$
SE CALCULATED ACCORDING TO MARTIN (1966).

Same set of experiments as in Fig. 5.

Exp.	$m_o \pm$ SE	$m_{CV} \pm$ SE	$1 + (cv)^2$
I	0.276±0.036	0.283±0.076	1.12
II	0.617±0.048	0.716±0.168	1.55
III	0.310±0.032	0.268±0.061	1.04
IV	0.397±0.034	0.510±0.090	1.55
V	0.156±0.020	0.174±0.045	1.29
VI	0.183±0.024	0.175±0.043	1.10
VII	0.613±0.043	0.732±0.103	1.28
VIII	0.334±0.033	0.335±0.067	1.13

large. This indicates then that a serious error in estimating the mejp distribution has not been made.

Facilitation

The experiment in Fig. 6 shows that the release of transmitter remains Poisson when the ejp is facilitated by a conditioning stimulus arriving 43 ms before the test stimulus. The intracellularly recorded response to the second stimulus is greater than that of the first, and the extracellular records show a smaller percentage of failures among the second responses. The mean quantal content of the second responses is greater with the quantal amplitude remaining constant. Fig. 6 a is an amplitude histogram of the extracellular
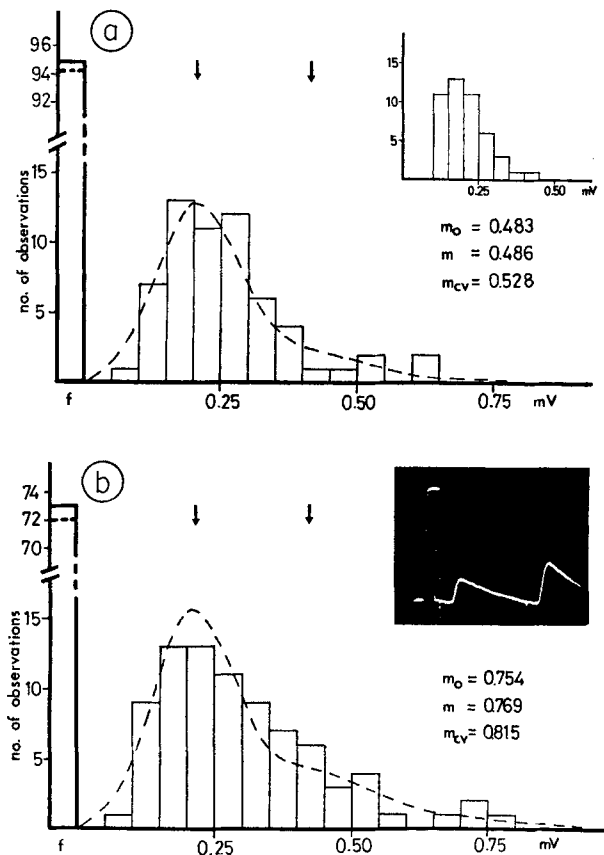

FIGURE 6. Amplitude histograms with Poisson predictions of the conditioning (a) and facilitated (b) ejp's using paired stimuli. Time interval between pairs, 4 s. Inset (a): spontaneous mejp's; inset (b): intracellular recordings of 32 pairs, averaged by means of a Biomac 1000 averager (Data Laboratories Ltd., London, England). Calibration pulse 10 mV/5 ms. Facilitation measured intracellularly is $V_{cond}/V_{test} = 1.69$; extracellular facilitation: $m_{cond}/m_{test} = 1.59$.

ejp's with the theoretical Poisson prediction for the conditioning stimulus, and Fig. 6 b is the histogram of the facilitated ejp's. The facilitation of the intracellular ejp's is accounted for by the increase in m only. Also, the level of facilitation of the second ejp of the pair is independent of whether the conditioning ejp was a failure or a response. In Fig. 6 b the mean amplitude of the test ejp's is 144 μV and the mean amplitude of only those pairs in which the first ejp was a failure is 140 μV.

DISCUSSION

The data presented in this paper confirm the validity of the quantum hypothesis for transmitter release at arachnid neuromuscular junctions. Recently, Atwood and Parnas (1968), Bittner and Harrison (1970), and Usherwood (1972) reported release patterns at several arthropod neuromuscular preparations which did not fit the predictions based on a Poisson process. Usherwood (1972) made reservations about his conclusion because in the locust retractor unguis muscle the synaptic contacts of the terminals are located at the inner faces of the closely packed muscle fibers and they are widely dispersed. Therefore, the release sites were not directly accessible for the extracellular electrodes.

The morphological situation of the terminals in spider muscle is similar. Because the synaptic junctions are located in the clefts between two adjacent fibers the space constant for the extracellular synaptic current density is probably even longer than that assumed by del Castillo and Katz (1956) for the frog motor end plate (5–10 μm). This emphasizes the necessity for caution in interpreting results of extracellular recordings from arthropod muscles.

In the present investigation only the relatively rare records showing extracellular ejp's with fast rise times were analyzed. Such records indicated that the recording was restricted to only a few localized release sites. Under this condition failures could easily be distinguished from responses thereby allowing accurate estimates of m and m_o. All data obtained in this way revealed approximate equality of m and m_o (Fig. 5), although only in a few cases were single release sites found.

The concept of the Poisson nature of synaptic transmission at arachnid neuromuscular junctions is further supported by the fact that facilitation can be interpreted purely as an increase of the probability of the release of transmitter packets. These results agree well with the findings in vertebrate (del Castillo and Katz, 1954 b; Boyd and Martin, 1956) and invertebrate neuromuscular junctions (Dudel and Kuffler, 1961 b).

Release patterns according to a binomial distribution (Johnson and Wernig, 1971; Wernig, 1972 a and b) could not be established in this investigation. The junctions of the axons causing large ejp's (up to 7 mV), however,

might release transmitter substance with a higher probability than those investigated in the present study. The extracellular ejp amplitude histogram might then be described by the binomial rather than the Poisson distribution.

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