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CHANGES IN STATISTICAL PARAMETERS DURING FACILITATION AT THE CRAYFISH NEUROMUSCULAR JUNCTION

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SUMMARY

1. Transmitter release at excitatory neuromuscular junctions of the crayfish was studied at different frequencies of stimulation ranging from 1/sec to 20/sec.

2. Over this frequency range the average number of quanta released per stimulus (m) increased with frequency by a factor of 6–7.

3. Analysis of the fluctuations in quantal release using binomial statistics indicated that the increase in m was associated with increase in the average quantal release probability (p) at stimulation frequencies between 5/sec and 20/sec. Between 1/sec and 5/sec there was an apparent increase in the number of quanta available for release (n).

INTRODUCTION

The quantum hypothesis of transmitter release proposed by del Castillo & Katz (1954*a*) states that there is a pool of n quanta in the nerve terminal, each with a small probability of being released in response to a nerve action potential. If the average of such probabilities is p, then the average number of quanta released during a series of trials will be given by $m = np$, and the number released should fluctuate from trial to trial according to binomial statistics. In a previous investigation of transmitter release at the crayfish neuromuscular junction (Johnson & Wernig, 1971), it was shown that the fluctuations in release were, in fact, consistent with the binomial hypothesis. The experiments were done with extracellular focal recording at low temperature. Because of the low quantal content at the discrete recording focus and the time dispersion of release at low temperature, the number of quanta released during each trial could be counted directly rather than calculated from amplitude measure-

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ments (cf. Katz & Miledi, 1965). Thus, m was measured directly and p calculated from m and the variance (v) of the quantal fluctuations, using the binomial relation $v = m(1-p)$. n was then calculated from $n = m/p$.

In the present investigation the methods outlined above were used to evaluate changes in the release parameters m, n and p with different frequencies of stimulation. It is known that in crayfish the average quantal release in the steady state is dependent on the rate of stimulation and is facilitated at higher frequencies (Dudel & Kuffler, 1961*a*; Dudel, 1965; Bittner, 1968; Bittner & Harrison, 1970; Bittner & Kennedy, 1970; Atwood & Bittner, 1971). Similar results have been obtained from Mg blocked vertebrate neuromuscular junctions (Hubbard, 1963; Braun, Schmidt & Zimmermann, 1966; Maeno & Edwards, 1969) and this occurrence of facilitation has been called 'frequency facilitation' (Maeno & Edwards, 1969). The results of the present study indicate that such facilitation is due to increases in p and possibly in n, the relative contributions of these parameters being dependent on the frequency ranges involved.

METHODS

Experiments were performed on excitatory neuromuscular junctions of the opener muscle of the claw in the first walking leg of crayfish. The animals were caught locally and were kept in a container with constant fresh water supply for a few weeks. Usual procedures were applied in dissecting the muscle and its excitatory nerve (Dudel & Kuffler, 1961*b*). The preparation was mounted in a Lucite chamber and bathed in a modified van Harreveld's solution containing (in mM): NaCl, 195; KCl, 5·4; $CaCl_2$, 15; $MgCl_2$, 3; Tris (hydroxymethyl) aminomethane, 10; titrated to pH 7·5 (room temperature) with maleic acid. The temperature of the bathing solution was brought to 5–6·5° C and was kept constant during the experiment ($\pm$ 0·5°).

The excitatory nerve was put on a pair of Pt electrodes and stimulated with 0·1 msec pulses at varying frequencies. The time interval between two series of stimulus trains on the same active spot was usually 1–2 min. Synaptic activity was recorded with extracellular glass electrodes (about 10 MΩ resistance) filled with 2 M-NaCl. The signals were displayed on an oscilloscope screen and photographed.

Processing of the data and statistical methods

The excitatory nerve was stimulated continuously with the same frequency for several hundred trials. The total number of quanta released in N trials was counted and the average number of quanta released per stimulus (m) was then obtained from

$$m = \frac{\text{total number of quanta released}}{\text{total number of trials}}.$$

After finishing a run with a certain stimulation rate another series with a different frequency was done with the recording electrode still in the same position. The stimulation frequencies, which were applied in no fixed order, were 1, 5, 10 and 20 stimuli/sec. In determining the steady-state level of transmitter release each

series of responses was broken up into groups of 50 or 100 and m for each group was calculated. Whenever the average release was lower in the first groups (which was especially the case with high frequency stimulation) these were discarded so that the release was essentially constant during the series.

χ^2-statistics at the 5% level were applied in comparing the observed pattern of fluctuation in release with the calculated binomial predictions. The Yates correction was applied whenever the last binomial group was < 10. All calculations were carried to four significant figures.

RESULTS

Table 1 summarizes steady state values of m at different frequencies which were obtained from six synaptic spots in the central regions of six opener muscles. At 20/sec each nerve action potential released, on the average, about 6 times more quanta than at 1/sec. At 10/sec the average release was 3 times higher, and at 5/sec less than twice as high as at 1/sec. These results demonstrate and support similar findings

TABLE 1. Observed mean quantal content (m) ± S.D. at different frequencies of stimulation. m was obtained from

$$m = \frac{\text{total number of quanta released}}{\text{total number of trials } (N)}$$

Frequency	Number of series	$m \pm$ S.D.
1/sec	5	0·36 ± 0·10
5/sec	7	0·50 ± 0·14
10/sec	7	0·94 ± 0·22
20/sec	4	2·10 ± 0·18

derived from amplitude measurements, namely that the average number of quanta released per nerve stimulus in steady state increases with frequency (Dudel & Kuffler, 1961*a*; Dudel, 1965; Bittner, 1968; Bittner & Harrison, 1970; Bittner & Kennedy, 1970; Atwood & Bittner, 1971). Employing focal recording limited the present investigation to this frequency range, since higher frequency stimulation produced small movements of the muscle and displacement of the recording electrode.

The increase in the average release per stimulus in the steady state with increasing stimulation rate indicates an even greater increase in transmitter release rate. At 20/sec an average of 41 quanta were released per second. This was about 120 times greater than at 1/sec.

Statistical evaluation of p and n

An analysis based on the assumption of binomial statistics was used to relate the increased transmitter output at higher frequencies to changes in the release parameters p and n. Measuring the mean (m) and the variance (v) of the quantal fluctuations, the average release probability

can be estimated from $p = 1 - v/m$ and n from $n = m/p$. These calculations from six experiments are listed in Table 2. The values for m, p and n for each experiment in the table were obtained by averaging the results from all series of trials at the same frequency in that experiment. N gives the total number of trials for each frequency. In most cases the increased transmitter release was found to be associated with increases in both p and n. The calculated values for these release parameters ranged from 0·05 to 0·51 for p and from 2·01 to 12·56 for n.

Before going into further evaluation of the relative changes in p and n with frequency, some consideration ought to be given to the reliability of the method and the degree of accuracy that can be expected for the calculated numbers.

Using the calculated numbers for p and n, a rough test for the accuracy of the procedures can be made for each series of trials by comparing the predicted release pattern, i.e. the number (n_x) of 0, 1, 2, 3, ..., x quanta released in N number of trials, with the actual observed numbers. According to binomial hypothesis the predicted numbers are given by

$$n_x = N \frac{n!}{(n-x)!\,x!} p^x (1-p)^{n-x}.$$

The calculated numbers of failures, single unit responses, double unit responses and so on ought to be in reasonable agreement with the observed numbers if the calculated values for p and n are accurate. Such comparisons were performed for all present experiments. Examples from Expts. II and III (Table 2) are shown in Fig. 1.

For Expt. II the more than twofold increase in transmitter release in increasing the stimulation frequency from 1/sec to 10/sec was found to be due to an increase in p from 0·14 to 0·23 and an increase in n from 2·24 to 3·50. Using these values to predict the release pattern, the expected numbers of failures, single unit responses, double unit responses, and so on, were calculated and are represented by the white columns. The black columns display the observed numbers. In Fig. 1*A* there is a satisfactory agreement at both frequencies. Similarly, there is good agreement in Fig. 1*B* (Expt. III), where p increased from 0·11 (5/sec) to 0·34 (20/sec) and n changed from 4·99 (5/sec) to 5·62 (20/sec). These findings suggest that the calculated differences in p and n with changing frequency reflect actual changes in the release parameters. The lack of total agreement in the comparisons stresses, of course, that the method does not provide exact numbers for p and n. Intrinsic statistical uncertainties and errors in the evaluation of the records easily account for the disparities.

The results from Table 2 were used to determine the average changes of the synaptic parameters as the frequency of stimulation was increased

over the range from 1/sec to 20/sec. These changes are shown in Fig. 2. The ordinate is the average ratio of the value of m, n and p at the indicated frequencies to their value at 1/sec. In experiments in which 1/sec stimulation was not used, calculated values were obtained from the average ratios at 5/sec or 10/sec. For example, m_{20}/m_1 was calculated as being

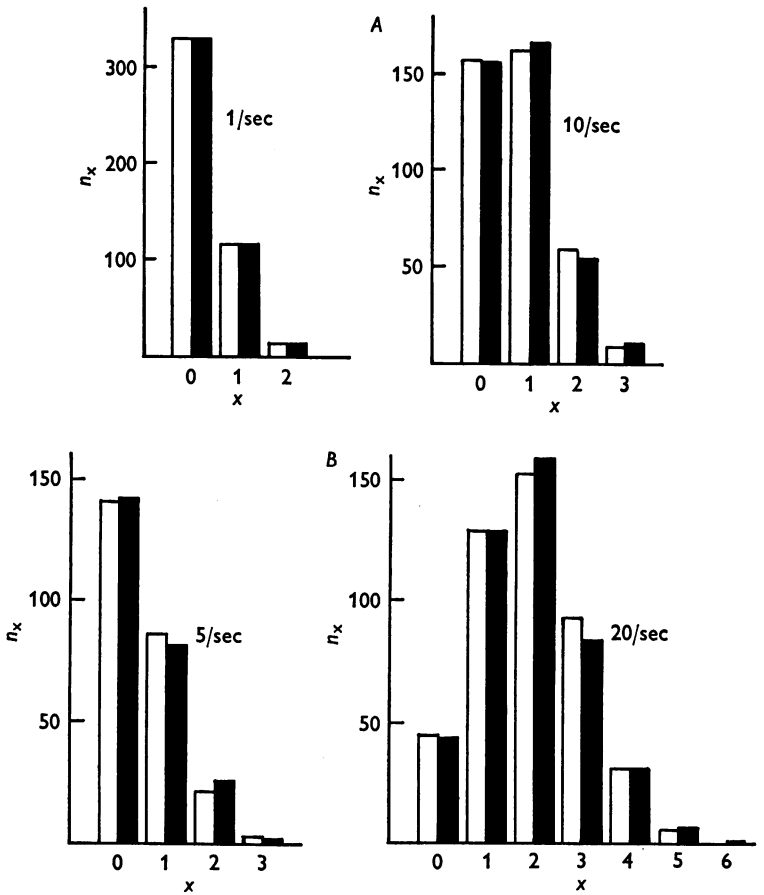

Fig. 1. Histograms of observed (black bars) and predicted (white bars) quantal distributions. n_x is the number of trials which released x quanta. Results are from Expt. II(*A*) and Expt. III(*B*).

$(m_{20}/m_5) \times (m_5/m_1)$ or $(m_{20}/m_{10}) \times (m_{10}/m_1)$, where the subscripts refer to frequency and the ratios (m_5/m_1) and (m_{10}/m_1) were obtained from other observations. All such calculations were averaged to obtain the points in Fig. 2.

Fig. 2 suggests that the facilitated output of transmitter is due to an increase in both the average release probability and the number of

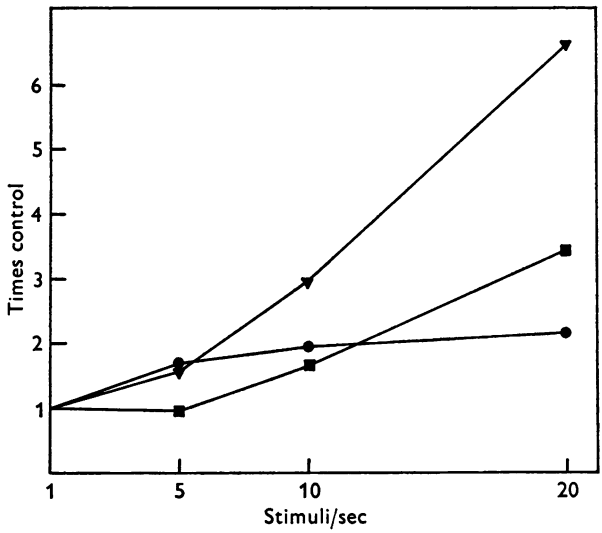

Fig. 2. Changes in m (▼), p (■) and n (●) with frequency. Grouped results from six experiments. Ordinate represents relative values referred to average values at 1/sec (see text).

TABLE 2. Release parameters at different stimulation frequencies. m is the observed mean quantal content. The average probability of release (p) was calculated from $p = 1 - v/m$, where v is the variance, and the number of available quanta (n) from $n = m/p$

Expts.	Frequency	Number of trials (N)	m	p	n
I	1/sec	774	0·28	0·14	2·01
	10/sec	236	0·74	0·18	4·09
II	1/sec	459	0·31	0·14	2·24
	5/sec	863	0·49	0·11	4·53
	10/sec	387	0·79	0·23	3·50
III	5/sec	255	0·53	0·11	4·99
	10/sec	175	1·16	0·25	4·57
	20/sec	457	1·89	0·34	5·62
IV	5/sec	647	0·40	0·09	4·33
	10/sec	448	0·78	0·11	7·12
	20/sec	330	2·02	0·39	5·13
V	1/sec	975	0·45	0·05	8·55
	5/sec	250	0·74	0·06	12·56
VI	5/sec	920	0·46	0·16	3·04
	10/sec	729	1·16	0·33	3·59
	20/sec	300	2·25	0·51	4·71

quanta available for release. However, the relative contributions of the two parameters depend on frequency. Frequency facilitation at 5/sec appears to be caused mainly by an increase in n, whereas at higher frequencies the additional increase in m is due to an increase in release probability (see Discussion).

Further observations

It was suggested by Johnson & Wernig (1971) that the release parameters p and n remain relatively constant on the average during each series of trials as long as the average release stays the same. However, fluctuations in the parameters from trial to trial might occur. In the present experiments an attempt was made to detect such fluctuations. During fluctuation of the release following each stimulus, individual responses may contain as many, or sometimes even more, quanta than the average value calculated to be available for release. One could imagine that such a high release 'empties' the pool, so that the next response would be depressed. To determine whether this was the case, all responses in a given series obtained after high releases ($x \geqslant 3$) were grouped and their mean quantum content (m_a) was compared with the over-all m. The average ratio m/m_a from nine series of trials was $1{\cdot}0048 \pm 0{\cdot}1766$ (S.D.). It appears therefore that the pool is 'restored' rapidly to its average content after the release, regardless of the previous history. In an equivalent analysis the first trials following failures (0 release) were examined to see whether or not the average quantal release (m_b) was increased. The average ratio of m/m_b from eleven series of trials was $1{\cdot}0187 \pm 0{\cdot}0895$, indicating no effect. Both tests, then, supported a requirement of the binomial theorem, namely that the release parameters remain constant during a series of trials.

DISCUSSION

The present analysis of facilitation relies on the applicability of binomial statistics to the release process as implied by the quantal hypothesis (del Castillo & Katz, 1954*a*). The results obtained previously from the crayfish neuromuscular junction (Johnson & Wernig, 1971) and the results obtained in the present series of experiments (e.g. Fig. 1) are consistent with this idea. It should be remembered, however, that the binomial calculations become increasingly less accurate as p decreases below 0·1–0·2.

The results confirm previous observations that, in the cray fish, the rate of transmitter release increases markedly with stimulus frequency (Dudel & Kuffler, 1961*a*; Dudel, 1965; Bittner, 1968; Bittner & Harrison, 1970; Bittner & Kennedy, 1970; Atwood & Bittner, 1971) and suggest that this

increase is due to an increase in both the release probability, p, and the size of the available pool of quanta, n. The increase in n appears to occur at low frequencies (1/sec to 5–10/sec) where, because of the low values of p, the analysis is least accurate. At higher frequencies (up to 20/sec) the further increase in quantal output can be attributed with much more reliability to a continuing increase in p. However, it is clear that at higher frequencies maintenance of the transmitter pool continues to play an important role in the facilitation process. Without continuing mobilization of transmitter into the pool the combined increase in release probability and stimulus frequency would be expected to lead to serious depletion. Thus if we refer to Fig. 2, p increases by a factor of about 2 when the stimulus frequency is increased from 10/sec to 20/sec. In order for n to remain constant over this range the rate of replenishment of the pool must be 4 times greater at the higher frequency than at the lower. It may be noted that, in the sense used here, 'mobilization' refers to maintenance of the transmitter pool and not to an increase in n, which remains relatively constant over the higher range of frequencies (cf. Hubbard, 1963; Braun *et al.* 1966; Maeno & Edwards, 1969).

The differences in the rate of increase with frequency of n and p may possibly be related to the two components of facilitation reported by Mallart & Martin (1967, 1968) at the frog neuromuscular junction. Using single conditioning shocks or short trains, these investigators found that there was an early phase of facilitation which decayed with a time constant of about 35 msec and a later phase lasting several hundred msec. It is possible that the early phase represents the increase in p observed here at the higher frequencies and that the late phase corresponds to the increase in n observed at the lower frequencies. Direct comparison cannot be made, however, because of the different preparations used and the different temperatures at which the two series of experiments were done. In any case, it is clear from the present results that facilitation at short intervals is associated with a marked increase in release probability. This facilitation has been attributed to left over 'active Ca' inside the nerve terminal which combines with the newly entering Ca during a second response to create an increased number of active release sites (del Castillo & Katz, 1954*b*; Jenkinson, 1957; Dodge & Rahamimoff, 1967; Rahamimoff, 1968; Katz & Miledi, 1968; Katz & Miledi, 1970). It is more difficult, however, to account for the apparent increase in n, which appears to have a much slower time course.

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