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THE BINOMIAL NATURE OF TRANSMITTER RELEASE AT THE CRAYFISH NEUROMUSCULAR JUNCTION

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SUMMARY

1. Transmitter release at excitatory junctions on the opener muscle of the crayfish dactyl was studied by recording junctional potentials with extracellular micro-electrodes.

2. At low temperatures, evoked release was dispersed sufficiently in time for potentials produced by individual quanta to be counted, and the mean (m) and variance (σ^2) of the quantum content distribution for a series of trials measured directly. These values were used to calculate the average probability of quantal release (p), assuming a binomial distribution.

3. For all values of m and p, the observed release pattern (number of 0, 1, 2, 3, ... quantal releases during a series of trials) was approximated closely by the corresponding binomial distribution. However, Poisson predictions differed significantly from the observed quantal distribution for values of $p > 0{\cdot}2$.

INTRODUCTION

There now exists considerable evidence for the quantal nature of transmitter release from presynaptic nerve terminals. The model originally proposed by del Castillo & Katz (1954*b*) to describe this process assumes the presence of a number (n) of discrete packets or 'quanta' of transmitter within the terminal, having an average probability of release (p) in response to a nerve impulse. In a large series of trials, the average number of quanta released per impulse (m) is given therefore by

$$m = np. \tag{1}$$

If the average probability of release is constant, then it follows from the hypothesis that the relative frequency of 0, 1, 2, 3, ... quantal releases in a series of trials is given by the successive terms in the binomial expan-

sion $(p+q)^n$, where $q = 1-p$ is the average probability that a quantum will not be released. In a given trial, the probability that x quanta will be released (p_x) is given by the binomial term

$$p_x = \frac{n!}{(n-x)!x!} p^x q^{n-x}. \tag{2}$$

If the probability of quantal release is low (such that $p \to 0$), then the binomial expression (2) is well approximated by a Poisson distribution where

$$p_x = \frac{e^{-m}m^x}{x!}. \tag{3}$$

Under these circumstances (low p), the relative frequency of observing x quanta per stimulus in a series of trials can be predicted from m alone, in contrast to binomial statistics which require determination of both n and p.

The original experiments of del Castillo & Katz (1954*a*, *b*) were done on preparations bathed in solutions containing high Mg. The low values of m observed in these solutions were attributed to reductions in the release probability. Consequently, the observed pattern of end-plate potential amplitude fluctuations was compared with, and found to approximate closely, the Poisson distribution. This observation has since been extended to include a wide variety of vertebrate and invertebrate synapses (see Martin, 1966). However, Atwood & Parnas (1968) and Atwood & Johnston (1968) reported a deviation from Poisson statistics at some synapses in crustacean neuromuscular preparations. More recently, Bittner & Harrison (1970) confirmed these observations, which they considered to violate a 'compound Poisson hypothesis' for transmitter release. The theory proposed originally by del Castillo & Katz, however, was essentially a binomial one, and therefore it was of interest to determine if non-Poisson release in the crayfish did indeed violate this theory.

As outlined above, in order to apply binomial statistics it is necessary to measure n and p. Katz & Miledi (1965) demonstrated that both the synaptic delay and the time course of transmitter release in the frog were prolonged at low temperature, to the extent that it was possible to count individual quanta as they were released following a nerve impulse. Using this technique, m was determined directly from the relationship

$$m = \frac{\text{Total number of quanta released}}{\text{Number of trials}} = \frac{\Sigma n_x x}{N}, \tag{4}$$

where x = the number of quanta released (0, 1, 2, 3, ...) following a nerve impulse, and n_x represents the frequency of occurrence of each value of x during a series of trials (N).

As noted by Ginsborg (1970), this type of analysis can be carried further. Since, for a binomial distribution, the variance (σ^2) is given by $\sigma^2 = npq = m(1-p)$, p can be estimated from the relation

$$p = 1 - \frac{\sigma^2}{m}. \tag{5}$$

In the present experiments, the number of quanta released during each of a series of trials at low temperature was counted. The mean quantum content of the junctional potential (e.j.p.) for the series was measured directly (eqn. (4)) and an estimate of p obtained (eqn. (5)). The number of available quanta was then calculated from eqn. (1). In this fashion, the inaccuracies associated with deriving these parameters from amplitude measurements were avoided. The validity of the binomial distribution was then tested by comparing the observed occurrence of failures, single unit responses, doubles and so on, with those expected theoretically.

METHODS

The experiments were performed on neuromuscular junctions on the inner surface of the abductor muscle of the dactyl (opener muscle of the claw) in the first walking leg of the crayfish *Cambarus bartoni robustus*. The techniques used in dissecting the muscle and its excitatory nerve were essentially the same as those described by Dudel & Kuffler (1961). The preparation was mounted in a Lucite chamber and bathed in a modified van Harreveld's solution containing (mM): NaCl 195; KCl 5·4; $CaCl_2$ 15; $MgCl_2$ 3; Tris (hydroxymethyl) aminomethane 10; titrated to pH 7·5 with maleic acid. The bathing solution was cooled with a thermoelectric device to between 0 and 6·5° C and maintained within $\pm 0{\cdot}5$ C° in any given experiment.

The excitatory nerve was drawn up in a suction electrode, and stimulated at a frequency of 1/sec with 0·05 msec pulses at an intensity 2–3 times threshold. Conventional extracellular focal recording techniques were employed using low resistance glass micro-electrodes (5–10 MΩ) filled with 2 M-NaCl. The signal was fed through a unity gain high impedance preamplifier, and recorded on film from the screen of an oscilloscope.

To determine how well the theoretical predictions fit the experimental distributions, a χ^2 statistic was calculated for each comparison. The predicted numbers of observations (n_x) in each group containing x quanta (where $x = 0, 1, 2, 3, \ldots$) were rounded to the nearest integer, and whenever the observed $n_x < 10$ the Yates correction was applied. If the observed $n_x \geqslant 1$ and the predicted $n_x = 0$, then the observed quanta were added to the previous group. The calculated statistics were tested for significance in a χ^2 table at two degrees of freedom less than the number of groups for the Poisson comparison and three degrees of freedom less for the binomial comparison. If a calculated statistic was greater than the corresponding figure in a table at the 0·05 level, then the null hypothesis was rejected and a significant difference between the observed and theoretical distributions assumed (Cochran, 1954).

RESULTS

As demonstrated by Dudel & Kuffler (1961), potential changes resulting from both presynaptic and post-synaptic currents can be recorded with a single extracellular micro-electrode properly positioned in the vicinity of a crayfish neuromuscular synapse (see also del Castillo & Katz, 1956). The records in Fig. 1, obtained using this technique, were selected from

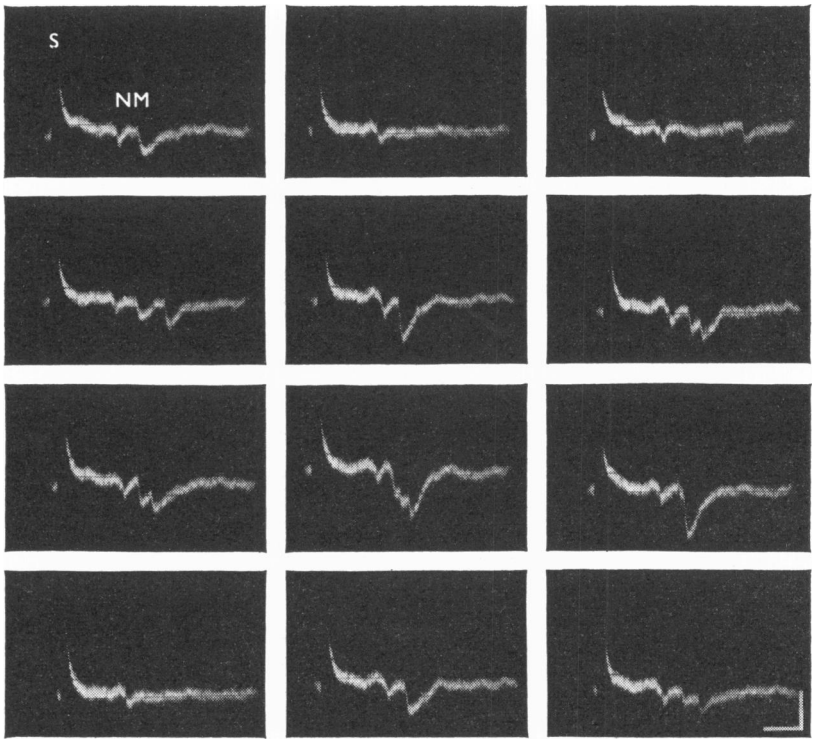

Fig. 1. Excitatory junctional potentials (e.j.p.s) recorded with an extracellular micro-electrode at 3·0° C. S, stimulus artifact. N, presynaptic nerve spike. M, post-synaptic e.j.p. Note the temporal dispersion of the e.j.p., such that potential changes produced by individual quanta may be distinguished. Calibration: 200 μV, 10 msec.

a series of 540 trials in an experiment performed at 3° C. Stimulation of the excitatory nerve (S) produced activity in the presynaptic terminal (*N*) which occasionally was followed by a post-synaptic excitatory junctional potential (M). As illustrated in Fig. 1 the quantal units making up the e.j.p. were dispersed sufficiently in time to be counted individually (cf. Katz & Miledi, 1965).

A number of experiments were performed in this fashion, from which those reported below were selected. An experiment was considered acceptable if (1) the e.j.p.s had a relatively rapid rate of rise, such that quantal multiples could be distinguished with reasonable certainty, (2) at least 150 quanta were recorded in a minimum of 450 trials, (3) during this period, the temperature remained within $\pm 0{\cdot}5°$ C of the initial value and (4) in sequential groups containing at least fifty trials, there was no apparent drift in the average value for m. Measured values of m, and calculated values for p (eqn. (6)) and n ($= m/p$) are shown in Table 1. The average values for m ranged from 0·32 to 1·4, for p from 0·044 to 0·49, and for n from 2·8 to 11.

TABLE 1. Parameters characterizing transmitter release. m is the mean and σ^2 the variance of the quantum content distribution. The average probability of release (p) was calculate dfrom eqn. (5) and the number of available quanta (n) from $n = m/p$

Expt.	Temp. (°C)	No. of trials (N)	No. of quanta	σ^2	m	p	n
I	4.0	856	278	0·31	0·32	0·044	7·3
II	3·5	1050	568	0·51	0·54	0·052	10·5
III	0·0	480	171	0·33	0·36	0·076	4·7
IV	2·0	1200	843	0·63	0·70	0·11	6·4
V	6·5	450	394	0·69	0·88	0·21	4·2
VI	3·0	540	736	0·69	1·4	0·49	2·8

As mentioned above, one of the criteria for selecting each experiment was a constant m, for a drift in m during the course of an experiment would increase the variance and thus lead to an underestimate of p and overestimate of n. Furthermore, it was of interest to determine the relative constancy of both p and n during each series of trials. As illustrated in Fig. 2, these parameters remained relatively constant throughout the duration of an experiment. At this junction, on the average, approximately 50 % of the available quanta in the terminal were released following each stimulus, and yet there was no appreciable change in n during 500 trials. In experiments characterized by a low probability of release, estimates of n and p for individual groups were less accurate. On occasion, the variance was greater than m and hence a negative value for p was obtained. Since, in such experiments, there was neither an obvious trend in the occurrence of groups with a calculated $p < 0$ nor an appreciable drift in m, it seems likely that p and n remained constant as assumed by the binomial hypothesis.

The assumption of a binomial distribution was tested by calculating the expected number of failures, singles, doubles, etc., and comparing them

with the observed quantal release pattern. Values of n_0, n_1, n_2, etc., predicted by binomial statistics, were calculated using eqn. (2), the immediately useful forms being $n_0 = Np_0 = N(1-p)^n$ and

$$n_x = n_{x-1} \frac{m-p(x-1)}{x(1-p)}.$$

Similarly, the Poisson distribution for the same value of m was calculated using corresponding relations derived from eqn. (3), namely $n_0 = Ne^{-m}$ and $n_x = n_{x-1} m/x$. The comparisons are summarized in Table 2. The

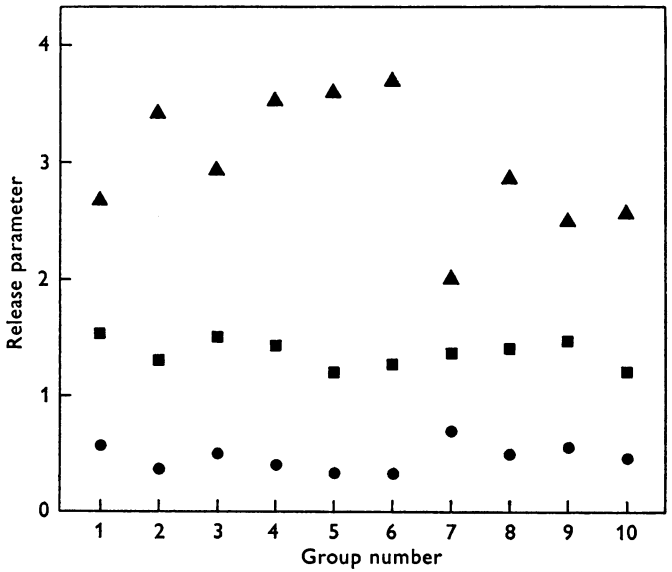

Fig. 2. Variations in mean quantal content (■), release probability (●) and number of available quanta (▲) during an experiment. Each point represents the average of fifty trials. Stimulation frequency 1/sec. Temp. = 3° C.

values calculated for n_x were rounded to the nearest integer from at least four significant figures. The rounding errors thus introduced account for occasional discrepancies in the total number of trials and total number of quanta released.

The results of Expts. I and VI are presented graphically in Fig. 3. In both cases, the observed quantal distributions were approximated closely by the binomial predictions. The observed values of n_x were also well represented by a Poisson distribution in Expt. I, but not in Expt. VI, where the Poisson calculations were appreciably different from the observed values.

Visual inspection of Table 2 and Fig. 3 suggests that the observed values of n_x are well represented by a Poisson distribution only when $p < 0{\cdot}10$. This was confirmed by χ^2 analysis, which gives a numerical measure of the 'goodness of fit' between the theoretical and observed distributions. There was no significant difference between the binomial predictions and the experimental observations in any of the experiments. In contrast, the Poisson model provided a good approximation only for those synapses characterized by low values of p ($< 0{\cdot}1$), and the predicted values differed significantly from those observed when $p > 0{\cdot}2$.

TABLE 2. Observed and predicted distribution of quantal responses. $n_{0,1,2,\ldots,x}$. Numbers of trials showing 0, 1, 2, ..., x post-synaptic unit responses

	Expt.	n_0	n_1	n_2	n_3	n_4	$n_{\geqslant 5}$	No. of trials (N)	No. of quanta
I.	Observed	615	206	33	2	0	0	856	278
	Binomial prediction ($p = 0{\cdot}044$)	615	209	31	3	0	0	858	280
	Poisson prediction	619	201	33	4	0	0	857	279
II.	Observed	604	339	94	11	2	0	1050	568
	Binomial prediction ($p = 0{\cdot}052$)	603	344	89	14	1	0	1051	568
	Poisson prediction	611	331	89	16	2	0	1049	565
III.	Observed	332	126	21	1	0	0	480	171
	Binomial prediction ($p = 0{\cdot}076$)	331	128	19	1	0	0	479	169
	Poisson prediction	336	120	21	3	0	0	480	171
IV.	Observed	573	443	154	28	2	0	1200	843
	Binomial prediction ($p = 0{\cdot}11$)	571	451	150	27	3	0	1202	844
	Poisson prediction	594	418	147	34	6	1	1200	843
V.	Observed	172	176	89	12	1	0	450	394
	Binomial prediction ($p = 0{\cdot}21$)	168	186	79	15	1	0	449	393
	Poisson prediction	188	164	72	21	5	1	451	396
VI.	Observed	80	224	200	32	4	0	540	736
	Binomial prediction ($p = 0{\cdot}49$)	83	222	191	47	0	0	543	745
	Poisson prediction	138	188	128	58	20	6	538	729

DISCUSSION

The quantum hypothesis for transmitter release (del Castillo & Katz, 1954*b*) was formulated primarily from experiments performed on amphibian neuromuscular preparations, where the quantal content of end-plate potentials was reduced by increasing the concentration of Mg in the bathing solution. This presumably decreased the mean quantal content by decreasing the probability of release, and hence reducing the binomial release process towards its Poisson limit (as $p \to 0$). Under these circumstances, the distribution of released quanta was characterized by the single parameter m. Although the quantum hypothesis assumed reality for the parameters n and p, direct determination of these quantities from

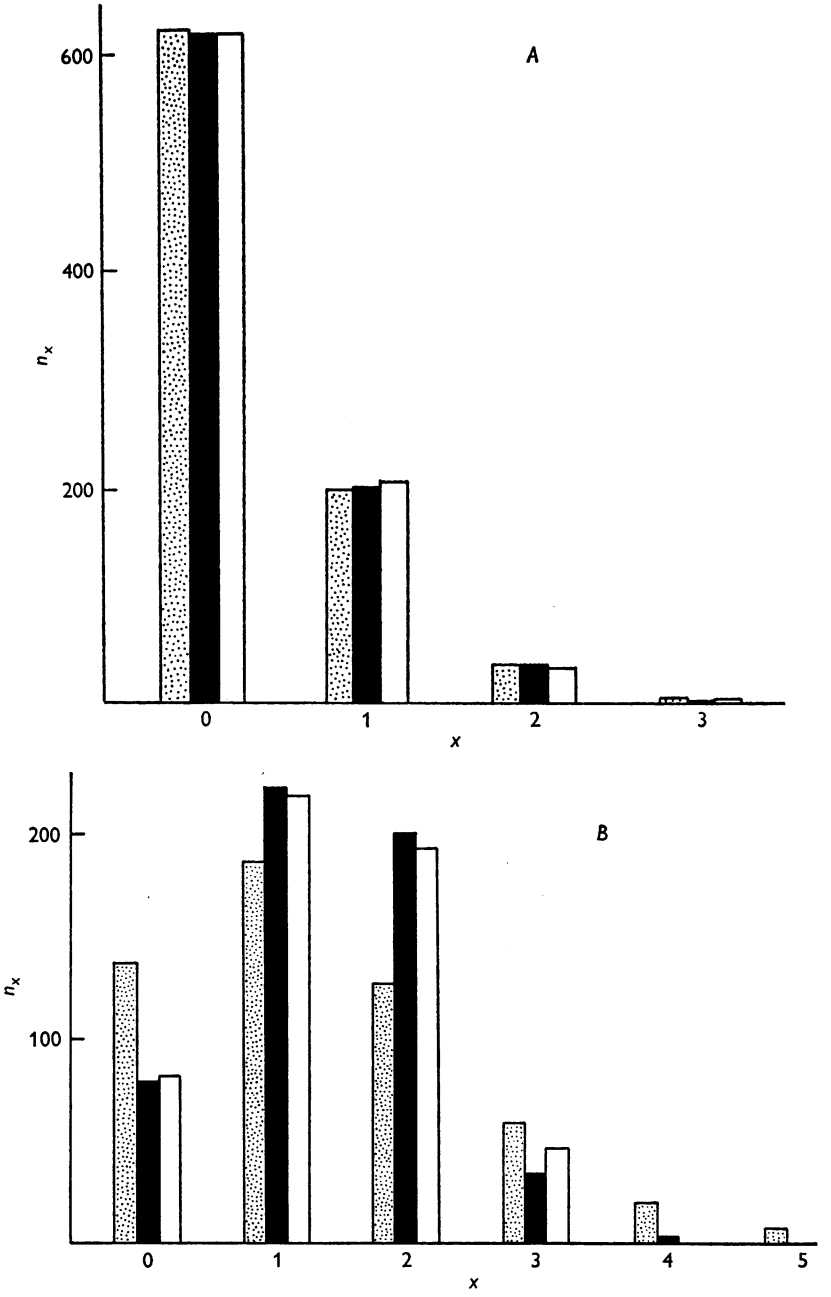

Fig. 3. Histograms of quantal distributions in Expt. I (*A*) and Expt. VI (*B*). n_x = the number of e.j.p.s containing x quanta. Black bar, observed distribution. Stippled, theoretical Poisson distribution with same m. White, theoretical binomial distribution with same m and $p = 0{\cdot}044$ (*A*) and $0{\cdot}49$ (*B*).

end-plate potential amplitude data was not possible, and the fundamental applicability of the binomial model could not be subjected to experimental verification.

In the absence of evidence to the contrary, Ginsborg (1970) proposed that quantal release might be a Poisson process *per se*, in which case n and p would be undefined. However, recent experiments by Christensen & Martin (1970) suggested that n and p were identifiable as separate parameters. These authors obtained indirect estimates of p from end-plate potential amplitude measurements at different Ca concentrations and observed that these estimates were scattered around a finite mean, rather than around a value of zero expected for a Poisson process. It should be noted that this method for estimating p requires the assumption that n is independent of external Ca, and in addition is subject to the errors associated with derivations from amplitude measurements, particularly inadequate correction for non-linear summation of unit potentials (Martin, 1955) and the Gaussian variation in quantal size.

The method presented here for determining n and p, based upon counting quanta released following nerve stimulation, avoids the inaccuracies in parameters derived from amplitude measurements, and permits a direct test for the applicability of the binomial model in transmitter release. The results provide strong support for the binomial nature of the quantum hypothesis proposed by del Castillo & Katz (1954*b*). Over the entire range of observed release probabilities (0·044–0·49), the quantal unit distributions are predicted accurately by binomial statistics. At synapses characterized by a low probability of release ($p < 0{\cdot}1$), the observed data are, of course, also well represented by a Poisson distribution. However, for higher values of p ($> 0{\cdot}2$), the theoretical quantal distribution predicted by the Poisson approximation deviates significantly from the observed values. That 'non-Poisson release' occurs at some neuromuscular junctions in crustaceans has been reported by Atwood & Parnas (1968), Atwood & Johnston (1968) and Bittner & Harrison (1970). The present results indicate that such deviations occur only at synapses where the release probability is relatively high and when the assumption of Poisson statistics is not justified.

Unless values of p are small, indirect estimates of m obtained by assuming a Poisson relationship may be considerably in error. One such statistical estimate is based on the frequency of failures, i.e. $m = \ln N/n_0$. The corresponding binomial expression is

$$m = \frac{-p}{\ln (1-p)} \ln \frac{N}{n_0}.$$

The Poisson calculation overestimates m by 4 to 39% for values

of p ranging from 0·1 to 0·5. A second statistical estimate of m frequently employed is based upon the coefficient of variation (CV), namely $m = 1/(CV)^2$. The binomial relation is $m = (1-p)/(CV)^2$. In this case, the Poisson calculation overestimates m by 11 to 100% as p increases from 0·1 to 0·5. These two binomial expressions may be combined to obtain an independent estimate of p (Kuno, 1964).

There are a number of points regarding our analysis which deserve further consideration. It is evident that low temperature prolongs the time course of quantal release (Fig. 1), and one might expect that this change would be reflected in the quantal content of the e.j.p. Experiments performed at room temperature on the synapse of the crayfish cheliped opener muscle suggest an average value for m (based on the ratio of evoked e.j.p. to spontaneous miniature e.j.p. amplitudes) of 0·78 (Bittner & Harrison, 1970, Figs. 2*a*, 3*a*, 5*a*). This compares favourably with the average measurement in the present study (at 0–6·5° C) of $m = 0{\cdot}70$ (Table 1) and suggests that cooling the preparation did not appreciably alter the number of quanta released.

Another consideration concerns the anatomical extent of the synaptic activity recorded by a 1–2 μ focal electrode. Early histological work by van Harreveld (1939) suggested that the excitatory axon branched to form approximately forty nerve terminals on each muscle fibre. More recently, Dudel & Kuffler (1961) examined the relative frequency of spontaneous junctional potentials recorded simultaneously with intracellular and extracellular micro-electrodes, and obtained an estimate of sixty synaptic contacts per muscle cell. These data suggest that the extracellular pipette monitors the activity of an entire terminal branch. It seems likely, then, that the values for m, n and p reported here may represent the release parameters for such a branch (cf. del Castillo & Katz, 1956).

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