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THE EFFECT OF CALCIUM IONS ON THE BINOMIAL STATISTIC PARAMETERS WHICH CONTROL ACETYLCHOLINE RELEASE AT SYNAPSES IN STRIATED MUSCLE

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SUMMARY

1. A study has been made of the effects of changing $[Ca]_o$ and $[Mg]_o$ on the binomial statistic parameters p and n which control the average quantal content ($\overline{m}$) of the synaptic potential due to acetylcholine release.

2. When $[Ca]_o$ was varied in the range 0·1 to 1·0 mM, p increased as the first power of $[Ca]_o$ whereas n increased as the third power of $[Ca]_o$.

3. Increasing $[Mg]_o$ depressed both p and n, however variations of $[Ca]_o$ in the presence of high $[Mg]_o$ did not significantly change the power relationship between either p and $[Ca]_o$ or between n and $[Ca]_o$.

4. The facilitated increase in $\overline{m}$ during a short train was due to an increase in n, whereas the post-tetanic increase in $\overline{m}$ during a tetanus was due to an increase in p. These results are considered in terms of the role of Ca ions in facilitation and post-tetanic potentiation.

INTRODUCTION

The evoked release of transmitter at synapses in both vertebrate (Bennett & Florin, 1974) and invertebrate (Johnson & Wernig, 1971) striated muscles obeys binomial statistics, in which the average quantal content of the end-plate potential (e.p.p.) evoked by a nerve impulse is dependent on a probability parameter p acting on a quantal release parameter n. This evoked transmitter release increases as the fourth power of the external Ca concentration $[Ca]_o$ (Dodge & Rahamimoff, 1967) and this action is depressed by Mg in such a way (Jenkinson, 1957) as to suggest that the release of transmitter is mediated by the formation of a Ca-receptor complex (CaX). We have examined the dependence of the binomial release parameters p and n on $[Ca]_o$, and found that the n parameter is dependent on a third power of $[Ca]_o$, indicating that n is dependent on the formation of CaX.

It has been suggested that facilitation is due to a conditioning impulse

leaving some residual CaX at the nerve terminal, so that the amount of CaX present following a subsequent test impulse is increased leading to an enhanced transmitter release (Katz & Miledi, 1968; Rahamimoff, 1968; Miledi & Thies, 1971; Barrett & Stevens, 1972; Rahamimoff & Yaari, 1973; Younkin, 1974); thus if n is dependent on the formation of CaX, facilitation should be accompanied by an increase in n. On the other hand post-tetanic potentiation has a multiplicative effect on facilitation (Magleby, 1973*a*, *b*) and therefore would be attributed to an increase in p. The changes in p and n accompanying facilitation and potentiation have therefore been determined to see if they accord with these predictions.

METHODS

The statistics of transmitter release at mature synapses was studied in the rat (100–300 g) hemidiaphragm, extensor digitorum longus and soleus muscles. For studies of newly formed synapses, reinnervated rat soleus and extensor digitorum longus muscles were prepared in the manner previously described (Bennett, McLachlan & Taylor, 1973; Bennett, Florin & Woog, 1974).

The normal or reinnervated muscle and its nerve supply were dissected free from any surrounding connective tissue and tendinous insertions, and mounted in a Perspex organ bath of about 10 ml. capacity. This was perfused with a modified Krebs solution of the following composition (mM) Na 151, K 4·7, Ca 1·8, Mg 1·2, Cl 142, H_2PO_4 1·3, SO_4 1·2, HCO_3 16·3, glucose 7·8 and gassed continuously with 95 % O_2 and 5 % CO_2. Solutions were maintained at 28–30° C and flowed continuously through the bath at a high rate (about 15 ml. min^{-1}). Stimulation of the nerve was by means of a suction glass capillary electrode, with current pulses of 0·01–0·1 ms duration and 1–10 V amplitude. Intracellular recordings were made with glass microcapillary electrodes filled with 2M-KCl of 10–50 MΩ resistance. The signals were led through a high impedance unity gain preamplifier, displayed on an oscilloscope and photographed on moving film.

Changes in the external Ca concentration $[Ca]_o$ or external Mg, $[Mg]_o$ were made by changing the level of $CaCl_2$ or $MgCl_2$ present in the reservoir of Krebs supplying the organ bath. At no stage was the total divalent cation concentration allowed to fall below 0·7 mM so as to avoid possible changes in the conduction of the nerve impulse (Frankenhaeuser & Hodgkin, 1957). In general when changing $[Ca]_o$ or $[Mg]_o$, $CaCl_2$ or $MgCl_2$ were progressively increased so as to keep the time taken to reach a new steady state in the average quantal content of the e.p.p.s to a minimum. If the ion changes were such as to decrease $[Ca]_o$ or $[Mg]_o$, at least 20 min was required before the average quantal content of the e.p.p.s reached a steady state.

In experiments for which changes in $\bar{m}$, p and n were to be determined at different $[Ca]_o$ or $[Mg]_o$, the nerves were stimulated at 0·5 Hz and the e.p.p.s recorded from surface fibres were photographed on continuously moving film. At this rate of stimulation there is neither facilitation, depression nor post-tetanic potentiation of responses (Hubbard, 1963), and the combinations of $[Ca]_o$ and $[Mg]_o$ chosen were such that the e.p.p. was always less than 6 mV, thus ensuring that no serious corrections for non-linear summation (Martin, 1955) were required. In most experiments the microelectrode was maintained at the same impalement for at least the time required for the average quantal content of the e.p.p. to reach the steady state in two different $[Ca]_o/[Mg]_o$ solutions but it was rare for the quality of the impalement to be sustained

over a period sufficient for four or more solution changes. The criteria used for determining the quality of an impalement after the steady state had been reached in a given solution were: a shift in the resting potential; a shift in the mean amplitude of the m.e.p.p.s collected at the end of exposure to the solution compared with the mean amplitude at the time of reaching the steady state in average e.p.p. quantal content in that solution; any trend in the average e.p.p. quantal content once a steady state had been reached. At those synapses where these criteria were fulfilled an amplitude-frequency histogram of 100–300 e.p.p.s in any particular solution was constructed and the average m.e.p.p. amplitude recorded in that solution was taken as a measure of quantal size; the histograms were compared with the predictions of binomial statistics, given by eqn. (1) in Bennett & Florin (1974). In the single case where a comparison was made between amplitude-frequency histograms from different cells (Fig. 1), the binning size for the e.p.p.s was made equal to the mean amplitude of the m.e.p.p.s; the abscissa in Fig. 1 is therefore labelled in quantal units, instead of mV.

At those junctions where the changes in p and n during facilitation were studied, the nerves were stimulated with short trains of up to 10 impulses at high frequencies (10–100 Hz), and these trains repeated 50–100 times, with an interval of 40–60 s between trains in order to avoid any effects due to post-tetanic potentiation (Hubbard, 1963). Amplitude-frequency histograms were constructed for the e.p.p.s of each impulse (up to 10) over the 50–100 trains and these compared with the predictions of binomial statistics.

When the changes in p and n during post-tetanic potentiation were studied, the nerves were stimulated with long trains at high frequencies (10–100 Hz) lasting up to 60s; this procedure was seldom repeated twice for the same muscle but when it was, an interval of at least 20 min was left between trains. The average quantal content of groups of between 100 and 200 e.p.p.s, collected after the first 500 ms of stimulation, were determined for different intervals during the long train. Amplitude-frequency histograms were constructed for the e.p.p.s in each group and these also compared with the predictions of binomial statistics. For all amplitude-frequency histograms the 'goodness of fit' of the binomial distribution to the observed distribution was determined by a χ^2 test.

The binomial probability distribution for e.p.p. amplitudes (and therefore quantal contents), together with the statistical methods employed in determining $\overline{m}$, p and n and their respective standard errors of the mean, are given in Bennett & Florin (1974) and Robinson (1975). The derivation of the mass action equation describing the competitive actions of Ca and Mg at a point or points in the release process is given by Jenkinson (1957) and Dodge & Rahamimoff (1967), as is the general method for determining the dissociation constants in the equation.

RESULTS

Binomial nature of transmitter release

Although it has been shown that transmitter release at growing vertebrate synapses conforms to binomial statistics (Bennett & Florin, 1974), it is necessary to show that such statistics are adequate to describe transmitter release at mature vertebrate synapses. Acetylcholine release at synapses for which the e.p.p. was made subthreshold by altering the $[Mg]_o$ or the $[Ca]_o$ were studied in the present work, and in all cases which fulfilled the criteria for a successful impalement (see Methods), χ^2 tests

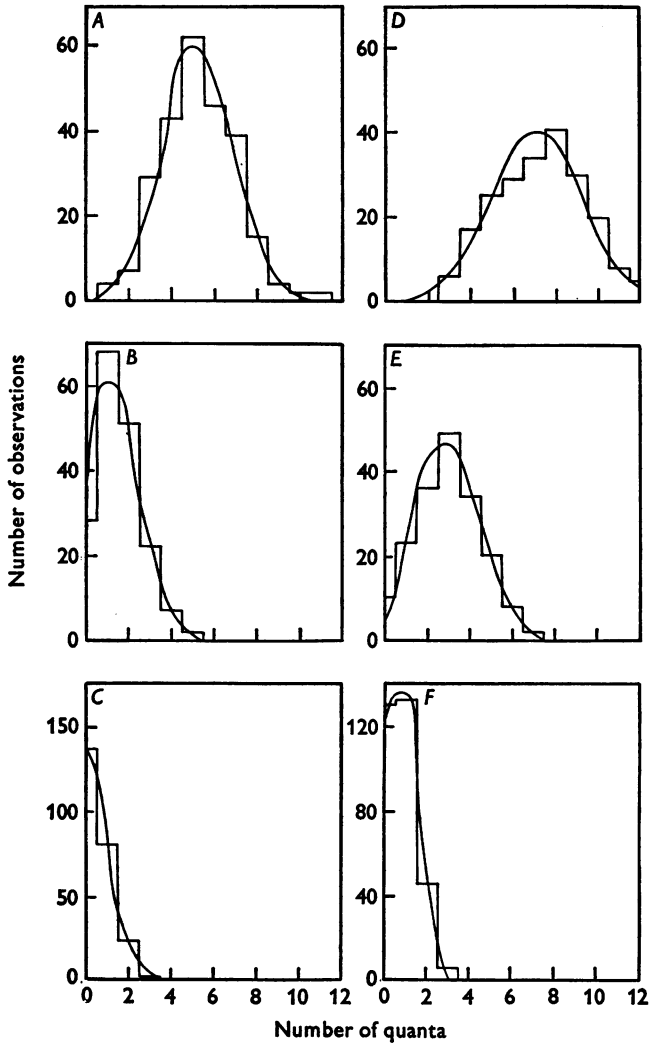

Fig. 1. Amplitude-frequency histograms of e.p.p. quantal content in different $[Ca]_o$ and different $[Mg]_o$. *A*, *B* and *C* give the effect of increasing $[Mg]_o$ in a constant $[Ca]_o$ of 1·8 mM: $[Mg]_o$ in *A* 6·35 mM, *B* 11·2 mM, *C* 16·2 mM. *D*, *E* and *F* give the effect of decreasing $[Ca]_o$ in a constant $[Mg]_o$ of 1·2 mM: $[Ca]_o$ in *A* 0·5 mM, *B* 0·4 mM, *C* 0·25 mM. The binomial prediction for the quantal content of more than 150 e.p.p.s collected during continual stimulation at 0·5 Hz is given by the continuous line for each set of ionic conditions. The values of $\bar{m}$, p and n for increasing $[Mg]_o$ were $5{\cdot}28 \pm 0{\cdot}15$, $1{\cdot}49 \pm 0{\cdot}08$, $0{\cdot}57 \pm 0{\cdot}05$ and $0{\cdot}467 \pm 0{\cdot}050$, $0{\cdot}214 \pm 0{\cdot}64$, $0{\cdot}136 \pm 0{\cdot}064$ and $11{\cdot}31 \pm 1{\cdot}26$, $3{\cdot}19 \pm 0{\cdot}32$, $4{\cdot}18 \pm 0{\cdot}26$ respectively. The values of $\bar{m}$, p and n for decreasing $[Ca]_o$ were $7{\cdot}36 \pm 0{\cdot}22$, $3{\cdot}03 \pm 0{\cdot}07$, $0{\cdot}90 \pm 0{\cdot}03$ and $0{\cdot}403 \pm 0{\cdot}065$, $0{\cdot}244 \pm 0{\cdot}061$, $0{\cdot}278 \pm 0{\cdot}061$ and $18{\cdot}25 \pm 2{\cdot}61$, $12{\cdot}40 \pm 1{\cdot}18$, $3{\cdot}08 \pm 0{\cdot}21$ respectively.

of the 'goodness of fit' of the binomial predictions to the observed distributions were such that P exceeded 0·50 in twenty-five out of thirty-three cases, the mean value of P being 0·61. Values of $\overline{m}$, p and n were only accepted if their individual S.E. of the mean were less than 20% of the mean. When the S.E. of n becomes comparable to the value of n, which it frequently does at junctions with low p if insufficient numbers of e.p.p.s are sampled for analysis, then it is not possible to distinguish the predictions of binomial statistics from those of Poisson statistics.

The effect of changes in $[Ca]_0$ on the statistical parameters governing transmitter release by a single impulse

$[Ca]_0$ was varied in the range from 0·1 to 1·0 mM, and the values $\overline{m}$, p and n determined for synapses by an analysis of 100–300 e.p.p.s collected during continual stimulation at 0·5 Hz at each $[Ca]_0$. The quantal content of the e.p.p. increased with the fourth power of $[Ca]_0$ over this concentration range (Fig. 2*A*), and this was primarily due to the third power dependence of n on $[Ca]_0$ (Fig. 2*A*), as p only changed linearly with $[Ca]_0$ (Fig. 2*A*).

It is known that an increase in $[Mg]_0$ depresses the quantal content of the e.p.p. in a manner consistent with a competitive inhibition between Ca and Mg at some point in the release process (Jenkinson, 1957; Dodge & Rahamimoff, 1967; Hubbard *et al.* 1968). High $[Mg]_0$ depressed both p and n (Fig. 2*B*) by similar percentage amounts, and variation of $[Ca]_0$ in the presence of a fixed high $[Mg]_0$ produced an approximately parallel change in the values of p and n to that in normal low $[Mg]_0$ (Fig. 2*A*). These results suggest that high $[Mg]_0$ competitively blocks the action of Ca in increasing both p and n at some point or points in the release process.

Dissociation constants governing transmitter release

If it is supposed that the increase in $[Mg]_0$ independently inhibits the action of Ca in increasing p and n, then it is possible to derive the dissociation constants (K_1 and K_2) in the expressions describing this competitive inhibition for both p and n, which are of the form

$$n = L\left\{\frac{[Ca]_0}{1 + \frac{[Ca]_0}{K_1} + \frac{[Mg]_0}{K_2}}\right\}^R, \qquad (1)$$

where R is the slope of the relationship between log $[Ca]_0$ and log n and L is a proportionality constant which includes the total number of X receptors which the synapses possess (for derivation see Jenkinson, 1957). The R values for $\overline{m}$, p and n (from Fig. 2*A*) were 4·3, 1·1, and 3·3 respectively.

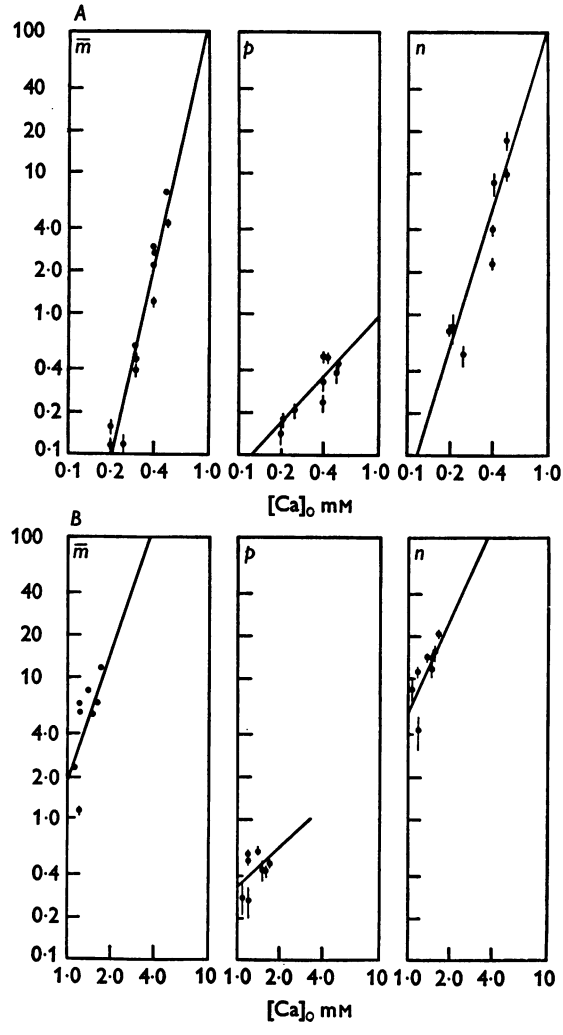

Fig. 2. Dependence of the statistical parameters describing transmitter release on $[Ca]_o$. The effect of changing $[Ca]_o$ at two different $[Mg]_o$ (*A*, 1·2 mM; *B*, 6·35 mM) on the quantal content ($\bar{m}$) of the e.p.p. and on p and n are shown on log–log co-ordinates. Each point was determined from a binomial statistic analysis of at least 200 e.p.p.s; ± one S.E. of mean is indicated for each point; if the S.E. of mean was greater than 20 % of the mean the value was not included in the graphs. In *A*, log $\bar{m}$, log p and log n increase along a gradient of 4·3, 1·1 and 3·3 respectively with an increase in log $[Ca]_o$. Least-square regression lines to the data in each of the graphs are shown.

K_2 can be determined (see Dodge & Rahamimoff, 1967) by considering two different $[Ca]_o/[Mg]_o$ mixtures (subscripts 1 and 2) for which n is constant and equating the right hand side of eqn. (1) for each of the mixtures, obtaining

$$K_2 = \frac{[Mg]_1 [Ca]_2 - [Mg]_2 [Ca]_1}{[Ca]_1 - [Ca]_2}. \quad (2)$$

The data in Fig. 2 gave the following values of the K_2 constant for $\bar{m}$, p and n of 2·1, 1·6 and 2·0 mM respectively.

Rearrangement of eqn. (1) to the form

$$n^{1/R} = \frac{L^{1/R}[Ca]_o}{\left(1 + \frac{[Ca]_o}{K_1} + \frac{[Mg]_o}{K_2}\right)} \quad (3)$$

allows a double reciprocal plot(Lineweaver & Burk, 1934) of $n^{-1/R}$ against $[Ca]_o^{-1}$ which gives a straight line, which on extrapolation to $n^{-1/R} = 0$ gives $-([Ca^*]_o)^{-1}$, where $K_1 = \frac{[Ca^*]_o}{1 + \frac{[Mg]_o}{K_2}}$. These double reciprocal plots for $\bar{m}$, p and n, obtained from the data in Fig. 2, are given in Fig. 3; the experimental points lie approximately on a straight line giving K_1 constants for $\bar{m}$, p and n of 1·1, 1·5 and 0·7 mM respectively. The K_1 and K_2 constants for the average quantal content $\bar{m}$ of 1·1 and 2·1 mM are comparable to those of 1·1 and 3·0 mM determined by Dodge & Rahamimoff (1967).

The effect of changes in $[Mg]_o$ on the statistical parameters governing transmitter release by a single impulse

A test for the existence of a competitive inhibition of the action of Ca by Mg at some site in the release process, and therefore of the applicability of eqn. (1), is to vary $[Mg]_o$ over a wide range while maintaining $[Ca]_o$ constant and to observe if the resultant changes in p and n are successfully predicted by this equation. At mature junctions in striated muscle the average quantal content must be lowered sufficiently to allow the e.p.p. to become subthreshold by changing the ratio of $[Ca]_o$ to $[Mg]_o$, and under these conditions p is always less than 0·6; it is therefore not possible to study changes in p with changes in $[Mg]_o$ over a sufficiently extensive range to check the applicability of eqn. (1) to the case of p. However synapses occur in reinnervated muscles which possess both subthreshold e.p.p.s and high values of p (Bennett & Florin, 1974) and so these were studied in addition to the mature synapses.

Increases in $[Mg]_o$ over the range from 6 to 16 mM at mature synapses led to a decrease in $\bar{m}$, p and n as shown in Fig. 4*A*. Increases in $[Mg]_o$

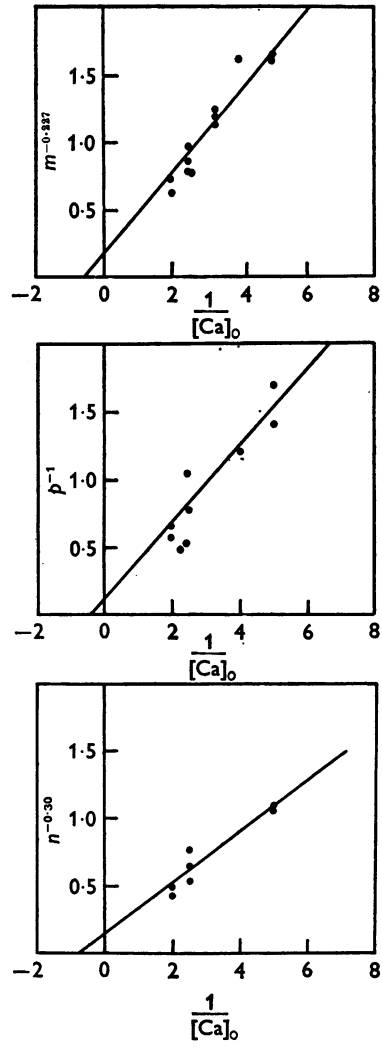

Fig. 3. Double reciprocal plots for the relationship between $(1/\overline{m})^{1/4\cdot 4}$ and $1/[Ca]_o$; $(1/p)$ and $1/[Ca]_o$; $(1/n)^{1/3\cdot 3}$ and $1/[Ca]_o$.

Linear co-ordinates. $[Mg]_o$ in all cases is 1·2 mM. The data shown in Fig. 2*A* have been replotted on these co-ordinates and lines of best fit drawn. The lines intercept the abscissa at $[Ca]_o$ of −1·67, −2·5 and −1·2 mM for the $\overline{m}$, p and n plots respectively.

over the lower range from 1·2 to 6 mM at newly formed synapses also produced a decline in $\overline{m}$, p and n (Fig. 4*B*). However the values of $\overline{m}$ and n for newly formed synapses were smaller than those for mature synapses at the same $[Mg]_o$ (Fig. 4), whereas the values of p were about the same.

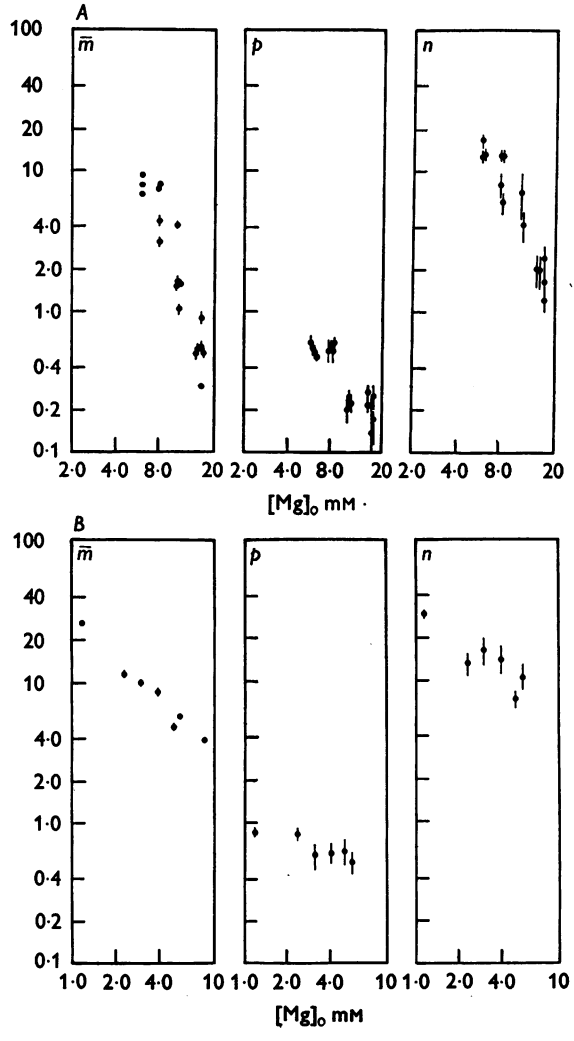

Fig. 4. Dependence of the statistical parameters describing transmitter release on $[Mg]_o$. The effect of changes in $[Mg]_o$ at a constant $[Ca]_o$ of 1·8 mM on the quantal content of the e.p.p. ($\bar{m}$) and on p and n are shown for both mature synapses (A) and newly formed synapses (B). Log–log co-ordinates. Each point was determined from a binomial statistic analysis of at least 100 e.p.p.s; ± 1 S.E. of mean is indicated for each point; if the S.E. of mean was greater than 20 % of the mean the value was not included in the graphs.

This is likely to be due to the presence of fewer X receptors at the newly formed synapses which are small compared with mature synapses (Bennett *et al.* 1973), thus giving a smaller value of L in eqn. (1) for the former with a consequent lowering of the curve relating $\bar{m}$ and n to $[Mg]_o$. The similarity

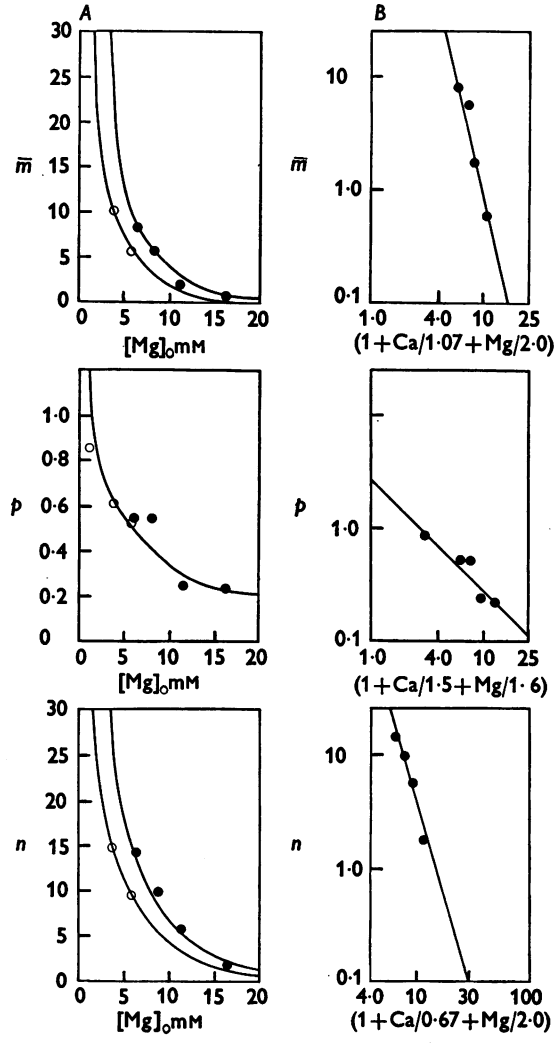

Fig. 5. Comparison between the predicted and observed results for the dependence of the statistical parameters describing transmitter release on $[Mg]_o$. *A*: $\bar{m}$, p and n plotted against $[Mg]_o$ on linear co-ordinates; the values given in Fig. 4 have been averaged into 2·5 mM $[Mg]_o$ bins; filled circles mature synapses, open circles newly formed synapses; the theoretical curves have been drawn according to eqn. (1) with the appropriate dissociation constants given in the text, with a $[Ca]_o$ of 1·8 mM and with L values (see text) which gave a best fit to the points; for $\bar{m}$, $L1 = 391$ and $L2 = 833$; for p, $L = 1{\cdot}56$; for n, $L1 = 579$, and $L2 = 1239$. *B*: $\bar{m}$, p and n plotted against $(1+[Ca]_o/K_1+[Mg]_o/K_2)$ on log–log co-ordinates; the values given in Fig. 4 have been replotted on these co-ordinates; the lines have been drawn through the points with the same slopes as those in Fig. 2*A*, namely 4·3, 1·1 and 3·3 respectively; the appropriate dissociation constants given in the text have been used with a $[Ca]_o$ of 1·8 mM.

of the p values at both synapse types is to be expected as this statistical parameter is independent of the size of the nerve terminals (Bennett & Florin, 1974).

A comparison between the changes in $\overline{m}$, p and n in different $[Mg]_0$ and the theoretical predictions of eqn. (1), for both mature and newly formed synapses is shown in Fig. 5. There is reasonable agreement between the theoretical curves and the experimental values for different $[Mg]_0$ (Fig. 5*A*) for both mature (open circles) and newly formed (filled circles) synapses;

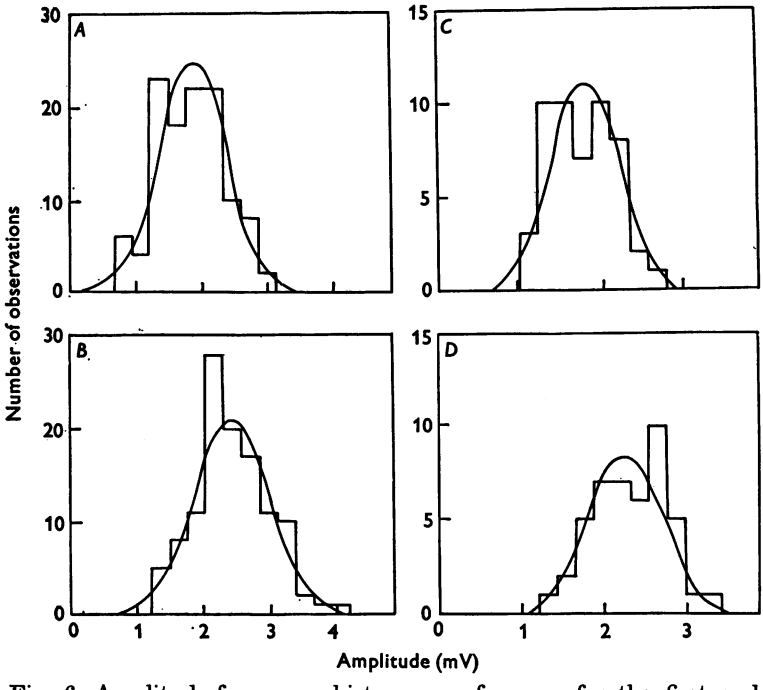

Fig. 6. Amplitude-frequency histograms of e.p.p.s for the first and xth ($2 \leqslant x \leqslant 10$) impulse of a short train (50 Hz) during facilitation at two different junctions (*A*, *B* and *C*, *D*). The binomial prediction for the e.p.p.s for each impulse is given by a continuous line. The values of $\overline{m}$, p and n for the first and second impulses at one junction (*A*. *B*) were *A*, $6{\cdot}75 \pm 0{\cdot}18$, $0{\cdot}530 \pm 0{\cdot}064$, $12{\cdot}73 \pm 1{\cdot}52$ and *B*, $8{\cdot}92 \pm 0{\cdot}21$, $0{\cdot}564 \pm 0{\cdot}060$, $15{\cdot}80 \pm 1{\cdot}67$ respectively and for the other junction (*C*, *D*) the values of $\overline{m}$, p and n for the first and ninth impulses were *C*, $8{\cdot}00 \pm 0{\cdot}28$, $0{\cdot}618 \pm 0{\cdot}081$, $12{\cdot}94 \pm 1{\cdot}68$ and *D*, $10{\cdot}46 \pm 0{\cdot}35$, $0{\cdot}602 \pm 0{\cdot}089$, $17{\cdot}37 \pm 2{\cdot}57$ respectively. $[Ca]_o$, 1·8 mM; $[Mg]_o$, 6·35 mM.

the values of p change along a monotonic curve, whereas those for $\overline{m}$ and n each change along two curves, one for values at mature synapses and the other for those at newly formed synapses. The only change in eqn. (1) necessary to generate each of these two curves was to decrease L by about

50 % for the newly formed synapses, suggesting that these possessed about half as many X receptors as mature synapses.

When the values of $\overline{m}$, p and n in different $[Mg]_o$ were plotted against $\left(1+\frac{[Ca]_o}{K_1}+\frac{[Mg]_o}{K_2}\right)$ on log–log co-ordinates (Fig. 5B) they fell on lines with slopes of 4·3, 1·1 and 3·3 respectively, as expected from eqn. (1) and the results in Fig. 2.

TABLE 1. Statistical parameters of transmitter release during facilitation. The values of $\overline{m}$, p and n for the first and the xth impulse ($2 \leqslant x \leqslant 10$) in a short train at either 40 or 50 Hz are shown, for four normal synapses in high $[Mg]_o$ (6·2 mM $\leqslant [Mg]_o \leqslant$ 12·2 mM). The standard errors of $\overline{m}$, p and n were calculated using eqns. (3), (4) and (5) in Bennett & Florin (1974)

$\overline{m}$		p		n	
1	x	1	x	1	x
1·53 ± 0·13	2·07 ± 0·16	0·716 ± 0·079	0·669 ± 0·089	2·13 ± 0·21	3·10 ± 0·38
3·09 ± 0·17	5·03 ± 0·26	0·521 ± 0·087	0·375 ± 0·116	5·92 ± 0·98	13·40 ± 4·14
6·75 ± 0·18	8·92 ± 0·21	0·530 ± 0·064	0·564 ± 0·060	12·73 ± 1·52	15·80 ± 1·67
8·00 ± 0·28	10·46 ± 0·35	0·618 ± 0·081	0·602 ± 0·090	12·94 ± 1·68	17·37 ± 2·57

Changes in the statistical release parameters during facilitation

In order to test the suggestion (see Introduction) that facilitation is due to the existence of residual CaX at the time of the test impulse, and therefore to an increase in n, we studied the changes in p and n that accompany the facilitation which occurs during short trains of up to ten impulses at frequencies between 10 and 100 Hz.

The amplitude–frequency histograms of the quantal content of the facilitated e.p.p.s were well described by binomial statistics (Fig. 6). In all cases where increases in quantal content of the facilitated e.p.p.s were large enough to achieve significant changes in the statistical release parameters, facilitation was due to an increase in n (Table 1), as it is at newly formed synapses (Bennett & Florin, 1974).

Changes in the statistical release parameters during post-tetanic potentiation

To study whether post-tetanic potentiation, which has a multiplicative effect on facilitation (Magleby, 1973*a*, *b*) and therefore according to the above result should be due to an increase in p, both mature and newly formed synapses were stimulated with long trains lasting up to 60s at high frequencies (10–100 Hz); the values of p and n for e.p.p.s collected early in the train (0·5 sec after the beginning when facilitation is complete) were compared with those collected at the end (0·3–1·0 min later) of the train, when post-tetanic potentiation is considerable. The amplitude-

frequency histograms of the quantal contents of the e.p.p.s collected both early and late during continual stimulation were well described by binomial statistics (Fig. 7) for both mature and newly formed synapses. When the increases in quantal content during a train due to post-tetanic potentiation were large, the potentiation was in all cases (but one) due to an increase in p (Table 2). Although depression of transmitter release which is accompanied by a decrease in n (Bennett & Florin, 1974) was avoided as far as possible during these long trains by lowering the quantal

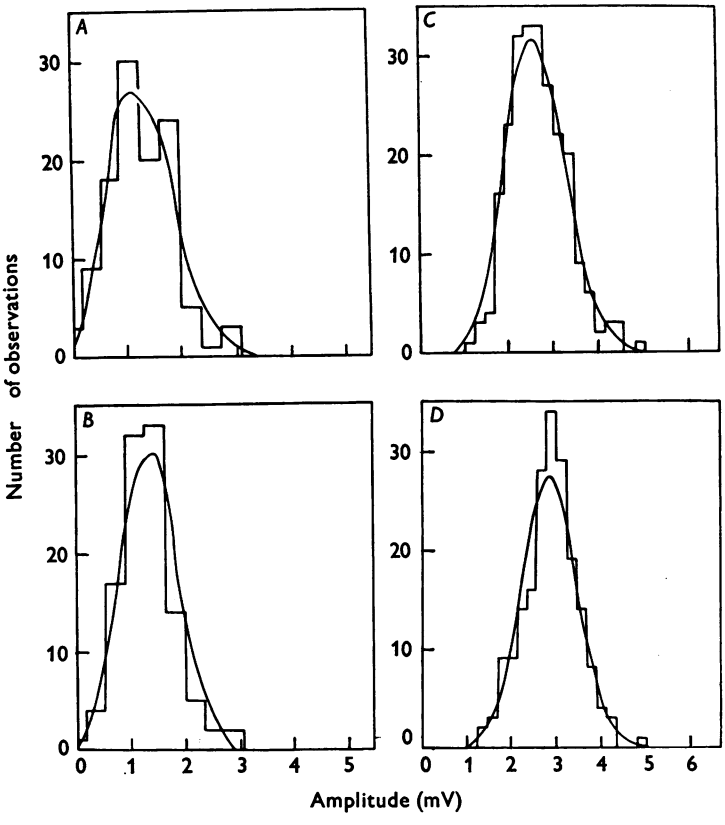

Fig. 7. Amplitude-frequency histograms of e.p.p.s at the beginning and end of a long train of impulses during post-tetanic potentiation at a mature synapse (A, B) and a newly formed synapse (C, D). The binomial prediction for the 200 e.p.p.s collected immediately after facilitation was complete (A, C) and 0·5 minutes later (B, D) is given by the continuous lines. The values of $\overline{m}$, p and n respectively are: A, $3{\cdot}56 \pm 0{\cdot}18$, $0{\cdot}238 \pm 0{\cdot}107$, $14{\cdot}95 \pm 6{\cdot}77$; B, $3{\cdot}63 \pm 0{\cdot}16$, $0{\cdot}472 \pm 0{\cdot}075$, $7{\cdot}68 \pm 1{\cdot}23$; C, $11{\cdot}85 \pm 0{\cdot}30$, $0{\cdot}303 \pm 0{\cdot}070$, $39{\cdot}14 \pm 9{\cdot}21$; D, $12{\cdot}80 \pm 0{\cdot}31$, $0{\cdot}459 \pm 0{\cdot}061$, $27{\cdot}88 \pm 3{\cdot}83$. A, B: 10 Hz with $[Ca]_o$ = 1·8 mM, $[Mg]_o$ = 11·2 mM. C, D: 20 Hz with $[Ca]_o$ = 1·8 mM, $[Mg]_o$ = 1·2 mM.

content of the e.p.p. by adjusting the $[Ca]_0$ to $[Mg]_0$ ratio, a decrease in n generally accompanied the increase in p late in the trains (Table 2 and Fig. 7).

TABLE 2. Statistical parameters of transmitter release during potentiation. The values of $\overline{m}$, p and n at 0 and t s ($1 < t < 60$ s) during continual stimulation at high frequency (10–100 Hz) are shown, for nine normal synapses (A) and five re-innervated synapses (B). Seven of the normal synapses and all the re-innervated synapses were in high $[Mg]_0$ (6·2 mM $\leqslant [Mg]_0 \leqslant$ 13·2 mM) and the remaining two normal synapses in low $[Ca]_0$ (0·3 mM $\leqslant [Ca]_0 \leqslant$ 0·4 mM). The standard errors of $\overline{m}$, p and n were calculated using eqns. (3), (4) and (5) in Bennett & Florin (1974)

	$\overline{m}$		p		n	
	0″	t″	0″	t″	0″	t″
A	5·90 ± 0·20	6·38 ± 0·20	0·330 ± 0·072	0·486 ± 0·057	17·88 ± 3·99	13·13 ± 1·61
	3·34 ± 0·20	4·28 ± 0·20	0·192 ± 0·134	0·419 ± 0·089	17·41 ± 12·21	10·22 ± 2·21
	3·56 ± 0·18	3·63 ± 0·16	0·238 ± 0·107	0·472 ± 0·075	14·95 ± 6·77	7·68 ± 1·23
	9·08 ± 0·31	9·39 ± 0·29	0·444 ± 0·081	0·622 ± 0·057	20·44 ± 3·79	15·10 ± 1·45
	5·52 ± 0·20	6·16 ± 0·23	0·223 ± 0·078	0·290 ± 0·079	24·75 ± 8·81	21·23 ± 5·89
	4·02 ± 0·12	4·69 ± 0·14	0·435 ± 0·052	0·492 ± 0·053	9·25 ± 1·14	9·54 ± 1·06
	7·22 ± 0·26	8·07 ± 0·29	0·384 ± 0·097	0·459 ± 0·093	18·82 ± 4·77	17·56 ± 3·85
	2·02 ± 0·14	2·41 ± 0·15	0·315 ± 0·099	0·478 ± 0·078	6·42 ± 2·06	5·03 ± 0·85
	8·37 ± 0·17	8·78 ± 0·15	0·723 ± 0·034	0·750 ± 0·024	11·58 ± 0·56	11·71 ± 0·41
B	11·85 ± 0·30	12·80 ± 0·31	0·303 ± 0·070	0·459 ± 0·061	39·14 ± 9·21	27·88 ± 3·83
	4·84 ± 0·18	5·69 ± 0·20	0·584 ± 0·066	0·577 ± 0·066	8·29 ± 0·94	9·87 ± 1·14
	1·66 ± 0·09	2·66 ± 0·11	0·377 ± 0·072	0·409 ± 0·064	4·40 ± 0·84	6·51 ± 1·03
	2·69 ± 0·12	3·44 ± 0·15	0·519 ± 0·065	0·537 ± 0·073	5·18 ± 0·66	6·41 ± 0·86
	6·29 ± 0·18	7·59 ± 0·19	0·786 ± 0·039	0·815 ± 0·031	8·00 ± 0·41	9·31 ± 0·40

DISCUSSION

The effect of changes in $[Ca]_0$ and $[Mg]_0$ on statistical release parameters

The fourth power increase in quantal content with an increase in $[Ca]_0$ in low $[Ca]_0$ solutions (Dodge & Rahamimoff 1967; Hubbard, Jones & Landau, 1968) is primarily due to a third power dependence of n on $[Ca]_0$. As raising the $[Ca]_I$ releases transmitter (Miledi, 1973), and the $[Ca]_I$ following an impulse is momentarily raised by an amount linearly related to $[Ca]_0$ (Hodgkin & Keynes 1957; Baker, Hodgkin & Ridgway 1971), the third power relationship between n and $[Ca]_0$ suggests that n must be determined by events inside the nerve terminal.

The antagonism by Mg of the action of Ca in increasing n does not necessarily occur on the inside of the nerve terminal. Mg antagonizes the entry of Ca into axons which accompanies the nerve impulse (Baker *et al.* 1971, Table 3), as well as itself entering the axon during the impulse (Baker & Crawford, 1972). The few values determined for the decrease in the influx of Ca accompanying the action potential in different high $[Mg]_0$ (Baker

et al. 1971, Table 3) are consistent with a competitive action between the two ion species for the Ca channel; furthermore the fact that both Ca and Mg entry during the action potential are blocked by Mn ions (Baker *et al.* 1971; Baker & Crawford, 1972) indicates that any Mg entry is through the Ca channel. These observations suggest that the drop in n in high $[Mg]_o$ is primarily due to a decrease in $[Ca]_I$ (see also below) rather than because Mg competitively blocks the binding of Ca to receptors.

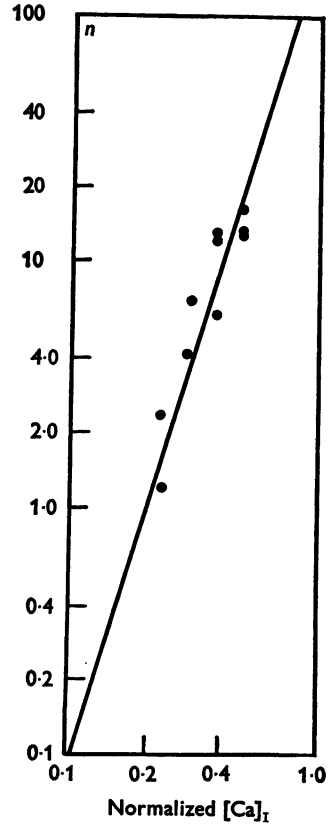

Fig. 8. Theoretical dependence of the statistical parameter n on the increase in internal Ca concentration $[Ca]_I$ which accompanies the action potential. The experimental values of n at different $[Mg]_o$ (Fig. 4*A*) have been transposed to the co-ordinates log n-log (normalized $[Ca]_I$), using the curve of p against $[Mg]_o$ on the basis that p is linearly related to $[Ca]_o$ (Fig. 2*Ab*) and therefore to $[Ca]_I$ (see text). The points now follow a straight line on log–log co-ordinates with slope 3·3, the same as in Fig. 2*A*.

The antagonism by Mg of the action of Ca in increasing p may also be due to Mg blocking Ca entry. The similar size of the dissociation constant K_2 for both p and n is consistent with the argument that the action of Mg

is primarily on the outside of the nerve terminal membrane where it competitively blocks the entry of Ca. If this is so, and as p is linearly dependent on $(Ca)_I$ (because of the linear relationship between Ca entry during the impulse and $[Ca]_o$), then the graph of p against $[Mg]_o$ (Fig. 5*c*) can be read as a graph of normalized $[Ca]_I$ against $[Mg]_o$; this then allows the graph of $\log n - \log [Mg]_o$ to be transposed to the co-ordinates $\log n - \log$ (normalized $[Ca]_I$). When this is done (Fig. 8), the experimental points fall on a line of slope 3·3, the same as that for the $\log n - \log [Ca]_o$ curve. The present results are therefore consistent with Mg having primarily one action at synapses, namely to inhibit the entry of Ca into the terminal.

Changes in statistical release parameters during facilitation

The observation that facilitation is due to an increase in n, which is in turn dependent on the third power of $[Ca]_o$, is consistent with the idea that the facilitatory process is directly due to conditioning impulses leaving some residual CaX in the nerve terminal for a short time. If this explanation for facilitation is correct, then it can be shown that residual CaX formed as a consequence of an impulse is removed at three different rates (Younkin, 1974; Fig. 3) which have time constants similar to those of the three facilitatory components observed at the neuromuscular junction (Katz & Miledi, 1968; Mallart & Martin, 1967). Younkin (1974) has analyzed the facilitation which occurs during short trains of impulses on the basis that quantal release is proportional to the fourth power of CaX and he has found that facilitation occurring at different frequencies with different numbers of impulses can be quantitatively predicted on this basis. This seems to contradict the present analysis, in which the quantal release parameter n is only proportional to the third power of CaX. However if the Younkin analysis is repeated on the data presented in that paper using a third rather than a fourth power relationship, the quantitative predictions are equally good; this is not the case if only the action of one CaX is required. It should also be noted that Barrett & Stevens (1972) found, using a method for measuring facilitation which was more sensitive to changes in the power relationship between release and CaX than that used by Younkin (1974), that a third power relationship gave a best fit to their results.

Changes in statistical release parameters during post-tetanic potentiation

Post-tetanic potentiation was due to an increase in p, as would be anticipated on the basis of the observations that facilitation is due to an increase in n and that post-tetanic potentiation has a multiplicative effect on facilitation (Magleby, 1973*a*, *b*). As p increases linearly with the increase in $[Ca]_I$ which accompanies the action potential, and post-tetanic potentia-

tion is due to a slow increase of p during continual high-frequency stimulation, there should be a small increase in the basal level of $[Ca]_I$ during continual high-frequency stimulation. This is the case (Baker *et al.* 1971; Llinas, Blinks & Nicholson, 1972), and explains why post-tetanic potentiation is solely dependent on Ca (Weinreich, 1971), and why during potentiation there is an increase in the basal level of transmitter release (Magleby, 1973*a*).

We have found it important to carry out a rigorous error analysis of our experimental results (see Bennett & Florin, 1974; Robinson, 1975) as often non-significant changes in p and n were obtained when ionic solutions were changed or during facilitation or post-tetanic potentiation. If these values had been included in our results erroneous conclusions would have been reached concerning the roles of p and n in the release process (especially at low p values when the errors are particularly severe). Furthermore we have found it particularly important to distinguish between facilitation and post-tetanic potentiation and to use experimental procedures (such as those of Magleby (1973*a*, *b*)) in which changes in p and n were not due to mixtures of both effects. Both of these criteria have been applied in a binomial statistic analysis of acetylcholine release in sympathetic ganglia (McLachlan, 1974), where facilitation was shown to be due to an increase in n and tetanic stimulation was accompanied by an increase in p, results which agree with the present and previous (Bennett & Florin, 1974) studies. However it is not clear that both of these criteria have been satisfied in the work of Wernig (1972*a*, *b*) and Zucker (1973), suggesting that this may be responsible for the differences between their results and ours; alternatively the statistics of transmitter release may be different at the invertebrate neuromuscular junctions studied by these authors.

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