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CHANGES IN STATISTICAL RELEASE PARAMETERS DURING PROLONGED STIMULATION OF PREGANGLIONIC NERVE TERMINALS

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SUMMARY

1. An analysis has been made of the release of acetylcholine (ACh) from the preganglionic nerve terminals of the isolated superior cervical ganglion of the guinea-pig during prolonged repetitive stimulation (10 Hz), using the amplitude of the excitatory post-synaptic potential (e.p.s.p.) as a measure of the amount of ACh released.

2. The decline in the mean amount of ACh released by each impulse over 20 min of continuous stimulation was accompanied by a small decrease in the mean miniature (min.) e.p.s.p. amplitude ($< 20\%$), both in the presence and absence of supplementary choline (3×10^{-5} M). During stimulation in the presence of hemicholinium-3 (HC-3) (2×10^{-5} M), the fall in min.e.p.s.p. amplitude was significantly larger.

3. Amplitude–frequency histograms of e.p.s.p.s evoked at different times after the beginning of stimulation were usually well predicted by binomial statistics, using the min.e.p.s.p.s released during each sample period as a measure of the quantal unit. In the other cases, histograms could be predicted using Poisson's Law.

4. A decline in quantal content, m, occurred in all experiments. In the presence of synthesis, with or without added choline, this was always associated with a decrease in the binomial parameter, n, and, in many cases, with a decrease in the binomial parameter, p. During stimulation in the presence of HC-3, a larger fall in p was observed in all experiments.

5. The results suggest that depletion of the ACh stored in the terminal decreases both the size and number of quanta available for release, and that the decrease in the amount of ACh in each quantum reduces the probability of its release.

INTRODUCTION

The application of binomial statistics to the study of quantal release of transmitter substances from nerve terminals (Johnson & Wernig, 1971; Bennett & Florin, 1974; McLachlan, 1975*a*; Wernig, 1975) has allowed direct evaluation to be made of the release parameters, n, the number of quanta available for release, and p, their average probability of release; the mean number of quanta released, m, is given by the product of these parameters, as originally defined in the model of del Castillo & Katz (1954). Changes in these parameters have been reported as a result of changes in Ca^{2+} and Mg^{2+} ion concentrations (Wernig, 1972*b*; Bennett & Florin, 1974), and at the beginning of trains of impulses (Wernig, 1972*a*; Zucker, 1973; Bennett & Florin, 1974; McLachlan, 1975*a*); although a number of models have been proposed (see, for example, Zucker, 1973; Wernig, 1975), it is still not clear what the physical correlates of n and p might be.

It has been suggested that n is closely associated with the releasable store of transmitter in the terminal, and that p is independent of n and of changes in the store (Bennett & Florin, 1974). In the present experiments, the relationship between the amount of acetylcholine (ACh) stored in preganglionic nerve terminals and the values of the binomial release parameters has been examined further. An analysis has been made of the excitatory post-synaptic potentials (e.p.s.p.s) recorded in sympathetic ganglion cells during prolonged stimulation of individual preganglionic axons. Estimates of m, p and n during depletion of preformed ACh have been determined, both under conditions in which synthesis of new ACh can replenish the store, and during inhibition of synthesis in the presence of hemicholinium-3 (HC-3). The results suggest that both p and n are affected by a decrease in the amount of transmitter available for the formation of quanta.

METHODS

Isolated superior cervical ganglia from guinea-pigs (150–250 g) were used in all experiments. The experimental arrangement and techniques and the statistical treatment were the same as has been described previously (Bennett & McLachlan, 1972*a*; McLachlan, 1975*a*; Robinson, 1975). The strength of the stimulus to part or all of the cervical sympathetic trunk was adjusted so that individual preganglionic axons were excited, repetitive stimulation at 10 Hz being commenced not less than 5 min after the preliminary examination of the synaptic input to the impaled ganglion cell. Samples of evoked and spontaneous min.e.p.s.p.s were recorded from 15 to 30 sec after the beginning of stimulation (initial steady-state, see McLachlan, 1975*a*); subsequently, samples of about 10 sec duration were recorded every few minutes during prolonged stimulation of each axon. Cells were sometimes hyperpolarized with currents of up to 0·3 nA to block action potentials or local responses.

Synapses were rejected if they did not satisfy the following criteria: (i) observed resting membrane potential > 55 mV throughout stimulation; (ii) largest evoked e.p.s.p. amplitude < 10 mV during repetitive stimulation; (iii) sufficient numbers of min.e.p.s.p.s recorded to provide a good fit to a Γ-distribution (χ^2 test, $P > 0{\cdot}1$) for each sample period during stimulation (usually $n > 25$); (iv) each sample of evoked e.p.s.p.s satisfied conditions of steady-state release (determined by comparison of the first and last ten responses using Student's t test); (v) binomial analysis gave $p > 0{\cdot}5$ during initial steady-state of release, so that meaningful estimates of p and n could be made (McLachlan, 1975*a*).

Either choline chloride (3×10^{-5} M) or HC-3 (Aldrich Chemical Co.) (2×10^{-5} M) was present in the bathing solution in some experiments.

RESULTS

In all experiments, stimulation of individual preganglionic axons at 10 Hz evoked e.p.s.p.s which increased in amplitude until a steady state of release was reached by about 30 sec after the beginning of stimulation (Bennett & McLachlan, 1972*a*, *b*; McLachlan, 1975*a*). The frequency of min.e.p.s.p.s also increased during this period, and these were assumed to originate from the stimulated axon (Blackman & Purves, 1969). It was therefore possible to determine the mean ($\bar{x}$) and variance (σ^2) of the min.e.p.s.p.s as well as the mean ($\bar{X}$) and variance (S^2) of the evoked e.p.s.p.s for each axon. The mean values of $\bar{X}$ at this time were similar for each group of experiments (normal solution, $\bar{X} = 3{\cdot}61 \pm 0{\cdot}96$ mV, S.E. of mean, $n = 7$; added choline, $\bar{X} = 4{\cdot}81 \pm 0{\cdot}81$ mV, S.E. of mean, $n = 7$; added HC-3, $\bar{X} = 5{\cdot}40 \pm 0{\cdot}72$ mV, S.E. of mean, $n = 8$). The initial period of steady-state release was taken as the control period, and the results for all subsequent samples were normalized to those at this time before pooling data for each group of experiments.

During continuous repetitive stimulation, the evoked e.p.s.p.s gradually declined in amplitude over 5–15 min until a maintained level of ACh output (about half of the control value) was reached if synthesis was not blocked (Fig. 1*A*). In the presence of HC-3, a faster exponential decline in evoked e.p.s.p. amplitude was observed (Fig. 1*A*); pooled data for eight experiments in HC-3 gave a mean time constant of decline of amplitude of 3·34 min (cf. 3·6 min, Bennett & McLachlan, 1972*a*). These changes in ACh output per impulse from the population of terminals selected in this study (see Methods) were thus indistinguishable from those described previously for terminals not selected by any special criteria (Bennett & McLachlan, 1972*a*, *b*).

Changes in quantal size during prolonged stimulation

The mean values of $\bar{x}$ were the same in each group of experiments (normal solution, $\bar{x} = 0{\cdot}51 \pm 0{\cdot}04$ mV, S.E. of mean, $n = 7$; added choline, $\bar{x} = 0{\cdot}54 \pm 0{\cdot}06$ mV, S.E. of mean, $n = 7$; added HC-3, $\bar{x} = 0{\cdot}54 \pm 0{\cdot}04$ mV,

S.E. of mean, $n = 8$). It therefore seems unlikely that HC-3 at this concentration (2×10^{-5} M) had a curare-like activity on the post-synaptic receptors (Martin & Orkand, 1961).

In most experiments, $\bar{x}$ decreased during continuous stimulation, but over a slower time course than the decline in $\bar{X}$ (Fig. 1*B*). The change in $\bar{x}$ was the same in normal solutions and in solutions containing added choline. In one experiment in normal solution, and in two in added

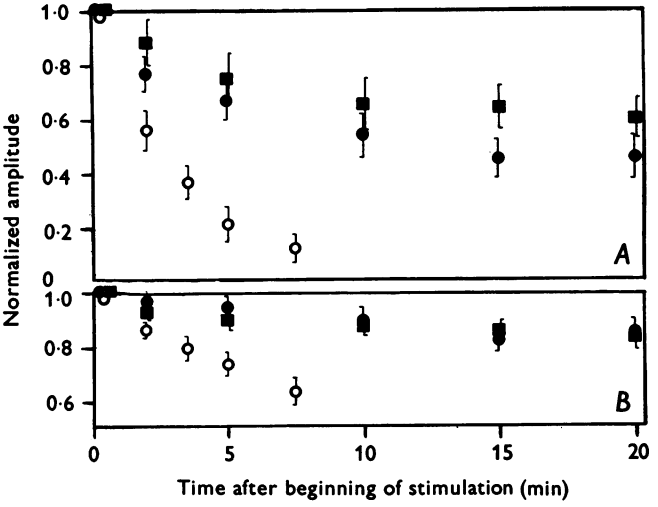

Fig. 1. The changes in amplitude of (*A*) evoked and (*B*) min.e.p.s.p.s during continuous stimulation of preganglionic nerve terminals at 10 Hz. The mean e.p.s.p. amplitudes for each axon have been normalized to the value at half a minute after the beginning of stimulation. The filled circles represent the mean results for axons stimulated in normal bathing solutions ($n = 7$), the filled squares those obtained in solutions containing choline (3×10^{-5} M) ($n = 7$), and the open circles those obtained in solutions containing HC-3 (2×10^{-5} M) ($n = 8$). Bars indicate S.E.S of the means. There was little difference between the changes in either evoked or min.e.p.s.p. amplitudes except during inhibition of ACh synthesis.

choline, no significant change in $\bar{x}$ was observed. However, a significantly greater decrease in $\bar{x}$ was observed in all axons examined during stimulation in the presence of HC-3 (Fig. 1*B*), although the fall was smaller (about 40 %) than the corresponding decrease in $\bar{X}$ (> 90 %) after 8 min of stimulation.

In three experiments carried out in the presence of HC-3, stimulation was stopped after about 10 min when $\bar{X}$ was very small, and $\bar{x}$ had decreased by about 40 %. Min.e.p.s.p. frequency subsequently decreased until a normal resting frequency was observed after about 15 min. During this time, $\bar{x}$ increased towards control

values, presumably as a consequence of the residual synthesis of ACh at this concentration of HC-3 (MacIntosh, Birks & Sastry, 1956; see also Jones & Kwan-bunbumpen, 1970).

It has been shown that the amplitude distribution of the population of min.e.p.s.p.s released during stimulation of a single preganglionic axon can be described using the Γ-distribution (McLachlan, 1975*a*). During the decline in $\bar{x}$ in all synapses which were included for analysis, no divergence of fit of min.e.p.s.p. amplitude histograms to Γ-distributions was observed (P usually $> 0{\cdot}3$), even when the min.e.p.s.p.s became quite small in the presence of HC-3 (Fig. 2). The shape of the Γ-distribution is described by the parameter $k\ (= \bar{x}^2/\sigma^2)$; although the value of k for a given axon varied slightly ($\pm 25\%$) between sample periods, it did not change progressively during stimulation. The possible loss of small potentials in the noise ($\simeq 150\ \mu$V) at later times during stimulation might have been expected to increase the observed value of k progressively. As this was not observed, the shift in the min.e.p.s.p. amplitude distributions indicates that a decrease in quantal size had occurred.

Changes in binomial release parameters during prolonged stimulation in normal solutions

Using the values of $\bar{x}$ and σ^2, and of $\bar{X}$ and S^2, for each sample period, m, p, and n were calculated assuming that release is a binomial process (McLachlan, 1975*a*). An example of this analysis is shown in Fig. 3, and data from all axons studied in normal solutions are shown in Table 1. Observed evoked e.p.s.p. amplitude histograms were compared with the binomial distributions predicted from these estimates, and the χ^2 test always gave $P > 0{\cdot}1$, except when small ($< 0{\cdot}2$) or negative values of p were derived. In these cases, good fits with a predicted Poisson distribution were obtained.

In two samples (each from a different axon), taken more than 10 min after the beginning of stimulation, neither binomial nor Poisson predictions described the observed data, too many large and small e.p.s.p.s being recorded. $\bar{X}$ in each case was consistent with the normal pattern of decline in ACh output. Inspection of the recordings showed bursts of large and small e.p.s.p.s (see Bennett & McLachlan, 1972*b*). It appears that, during prolonged stimulation of some axons, there is intermittent disruption of the binomial release process, although the mean ACh output per impulse over a period is not necessarily altered.

As the observed change in $\bar{x}$ was small ($< 20\%$), m always fell progressively during prolonged stimulation, paralleling the change in $\bar{X}$. The decrease in m was always accompanied by a decrease in n, while a progressive decrease in p was observed in all but one of the axons studied. The size of the change in p varied between axons, and in some cases the fall was not significant (see, for example, Fig. 3). However, in one case

(axon V in Table 1), an initial control value of $p = 0{\cdot}8$ had decreased to such an extent by 10 min after the beginning of stimulation that the Poisson prediction was applicable for each later sample. The decrease in n was significant in all axons, except when the value of p became small, when the S.E. of the estimate of n was large (Johnson & Wernig, 1971). There was no correlation between control values of $\bar{X}$, $\bar{x}$, m or p for a given axon and the relative changes in p or n. However, in the case in which p did not change, no decrease in $\bar{x}$ was observed for the duration of the experiment.

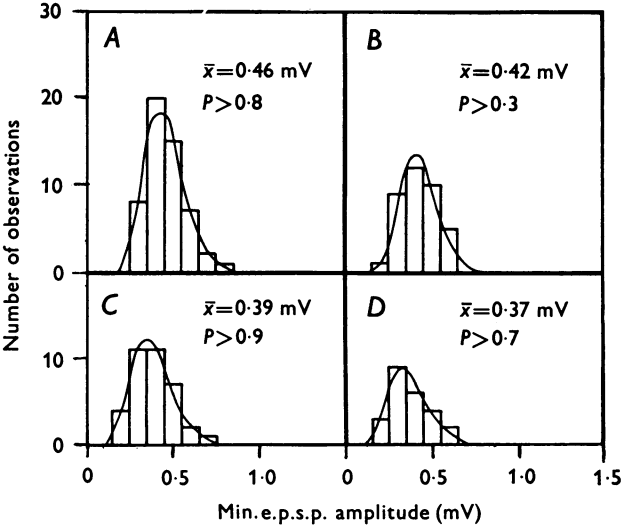

Fig. 2. Changes in quantal size during stimulation in the absence of ACh synthesis. Amplitude–frequency histograms of min.e.p.s.p.s recorded at 0·5 min (*A*), 2 min (*B*), 5 min (*C*) and 7·5 min (*D*) after the beginning of stimulation at 10 Hz in the presence of HC-3 (2×10^{-5} M). Continuous lines represent the Γ-distributions calculated from each sample. $\bar{x}$, mean min.e.p.s.p. amplitude; P, probability that the population could be a sample from the predicted Γ-distribution. After 7·5 min of stimulation, the mean evoked e.p.s.p. amplitude was 0·36 of the value at 0·5 min after the beginning of stimulation, but $\bar{x}$ was reduced by $< 0{\cdot}20$.

During the decline in ACh output per impulse, as the ACh stored in the terminal is partially depleted during prolonged stimulation (Birks & MacIntosh, 1961; Bennett & McLachlan, 1972*b*), there is a progressive decrease in the number of quanta released by each impulse, as a consequence of decreases in both of the parameters p and n.

The effect of supplementary choline on the changes in statistical release parameters

The maintained level of ACh output per impulse during prolonged stimulation of preganglionic nerve terminals *in vitro* is not dependent on an external supply of choline, unlike the case in perfused ganglia (Birks & MacIntosh, 1961; Bennett & MacLachlan, 1972*b*), suggesting that the main source of substrate for resynthesis is the choline derived from the

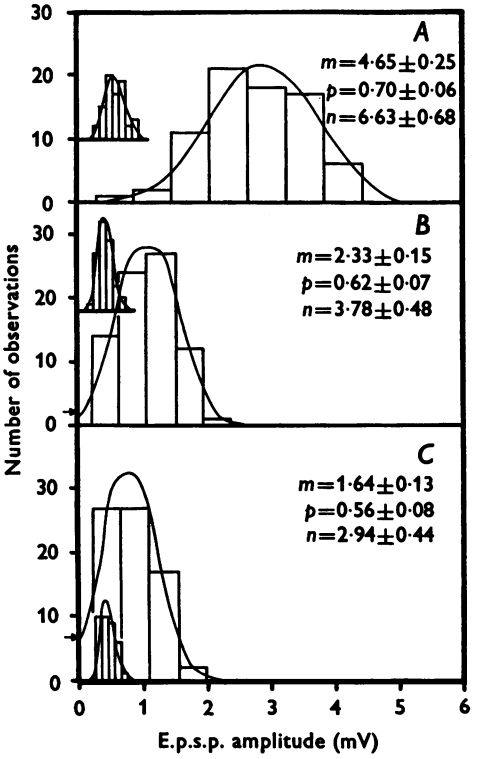

Fig. 3. Binomial analysis of release from an axon (axon IV in Table 1) stimulated at 10 Hz in normal solution. Amplitude–frequency histograms of e.p.s.p.s recorded at 0·5 min (*A*), 5 min (*B*), and 20 min (*C*) after the beginning of stimulation. Min.e.p.s.p. histograms with bin-widths of 0·1 mV have been displaced upwards from the abscissa for clarity in *A* and *B*. Evoked e.p.s.p. histograms have bin-widths = mean min.e.p.s.p. amplitude for each sample. Continuous lines superimposed on min.e.p.s.p. histograms represent Γ-distributions, and those on evoked e.p.s.p. histograms binomial distributions, predicted from these data. Arrows indicate the observed number of failures of release. $P > 0·3$ for all distributions shown (χ^2-test). The decrease in m in this axon during prolonged stimulation is mainly due to a decrease in n. Note that in (*C*), the min.e.p.s.p. histogram corresponds to the unit evoked response.

degradation of released ACh by acetylcholinesterase. It has been suggested however, on the basis of ultrastructural studies of perfused ganglia (Parducz, Feher & Joo, 1971), that choline incorporated in intracellular membranes may be mobilized for ACh synthesis in the absence of supplementary extracellular substrate: thus, following prolonged stimulation, few synaptic vesicles were observed in the presynaptic axons, unless choline at plasma concentrations was present in the perfusion medium. Similar results have been obtained for preganglionic terminals stimulated *in vitro* (E. M. McLachlan, unpublished observations), although in these conditions ACh output is normally maintained. If either p or n is associated in some way with the synaptic vesicles, it might be expected that these parameters would vary independently of the level of ACh output, in conditions in which variations in the vesicle numbers may occur.

TABLE 1. The effect of prolonged stimulation on estimates of statistical release parameters

Axon	Time (min)	$m \pm$ S.E.$_m$	$p \pm$ S.E.$_p$	$n \pm$ S.E.$_n$	P
I	0·5	12·71 ± 0·40	0·78 ± 0·04	16·29 ± 1·04	> 0·2
	15	3·37 ± 0·23	0·63 ± 0·07	5·32 ± 0·76	> 0·5
II	0·5	2·91 ± 0·16	0·75 ± 0·05	3·87 ± 0·34	> 0·1
	15	2·54 ± 0·19	0·67 ± 0·07	3·80 ± 0·51	> 0·3
III	0·5	12·34 ± 1·20	0·75 ± 0·06	16·38 ± 2·59	> 0·1
	15	8·01 ± 0·66	0·62 ± 0·07	12·92 ± 2·12	> 0·9
IV	0·5	4·65 ± 0·25	0·70 ± 0·06	6·63 ± 0·68	> 0·3
	15	1·96 ± 0·15	0·50 ± 0·09	3·95 ± 0·73	> 0·3
V	0·5	6·98 ± 0·34	0·81 ± 0·04	8·66 ± 0·65	> 0·5
	15	3·56 ± 0·26	−0·18	—	> 0·5*
VI	0·5	2·74 ± 0·17	0·51 ± 0·09	5·41 ± 0·99	> 0·2
	15	1·43 ± 0·12	0·27 ± 0·11	5·32 ± 2·21	> 0·3
VII	0·5	5·68 ± 0·34	0·72 ± 0·07	7·91 ± 0·92	> 0·2
	15	2·88 ± 0·17	0·70 ± 0·05	4·13 ± 0·41	> 0·5

'Time' indicates the time after the beginning of stimulation at 10 Hz in normal solution at which each sample was taken. P, probability that the population could be a sample from the predicted binomial distribution of Γ-distributed units, except at *, which refers to a predicted Poisson distribution.

As shown in Fig. 1, the decline in ACh output per impulse from seven axons stimulated in the presence of choline (3×10^{-5} M) was the same as that observed in normal bathing solutions, although output was maintained at a slightly higher level. This might be expected if some choline is washed out of the ganglion into the organ bath, and the result is similar to that reported previously (Bennett & McLachlan, 1972*b*).

The change in quantal size was identical to that seen in the absence

of choline (Fig. 1*B*); thus the changes in $\bar{X}$ were mainly due to a decrease in m (see Fig. 5). Binomial release parameters were calculated and examples of results from all axons studied are given in Table 2. There was no significant difference between the groups for the mean control values of m, p or n (normal solutions, $m = 6{\cdot}86 \pm 1{\cdot}57$, $p = 0{\cdot}72 \pm 0{\cdot}04$, $n = 9{\cdot}31 \pm 1{\cdot}91$, S.E.s of means, $n = 7$; cf. added choline, $m = 8{\cdot}83 \pm 0{\cdot}91$, $p = 0{\cdot}68 \pm 0{\cdot}06$, $n = 13{\cdot}53 \pm 1{\cdot}79$, S.E.s of means, $n = 7$). The changes in the release parameters were substantially the same as those obtained in the absence of choline. The decrease in m always resulted from a decrease in n, while a smaller fall in p was usually observed.

TABLE 2. The effect of added choline on estimates of statistical release parameters during prolonged stimulation

Axon	Time (min)	$m \pm$ S.E.$_m$	$p \pm$ S.E.$_p$	$n \pm$ S.E.$_n$	P
I	0·5	9·63 ± 0·52	0·62 ± 0·07	15·60 ± 2·20	> 0·2
	15	4·81 ± 0·30	0·72 ± 0·05	6·65 ± 0·66	> 0·3
II	0·5	5·35 ± 0·27	0·54 ± 0·08	9·91 ± 1·63	> 0·3
	15	4·44 ± 0·21	0·52 ± 0·07	8·53 ± 1·25	> 0·3
III	0·5	6·12 ± 0·38	0·65 ± 0·06	9·35 ± 1·18	> 0·2
	15	6·06 ± 0·49	0·58 ± 0·08	10·39 ± 1·87	> 0·5
IV	0·5	10·69 ± 0·50	0·92 ± 0·03	11·57 ± 0·73	> 0·1
	15	9·59 ± 0·59	0·64 ± 0·07	14·87 ± 2·03	> 0·5
V	0·5	12·22 ± 0·74	0·55 ± 0·07	22·37 ± 3·77	> 0·9
	15	8·26 ± 0·58	0·38 ± 0·10	21·99 ± 6·55	> 0·7
VI	0·5	8·77 ± 0·47	0·55 ± 0·07	15·89 ± 2·41	> 0·3
	15	9·52 ± 0·56	0·67 ± 0·06	14·20 ± 1·72	> 0·9
VII	0·5	9·04 ± 0·46	0·90 ± 0·03	10·05 ± 0·67	> 0·2
	15	4·18 ± 0·26	0·11 ± 0·14	39·08 ± 49·86	> 0·7*

'Time' indicates the time after the beginning of stimulation at 10 Hz in solution containing choline (3×10^{-5} M) at which each sample was taken. P, probability that the population could be a sample from the predicted binomial distribution of Γ-distributed units, except at *, which refers to a predicted Poisson distribution.

In two of three axons, there was no significant change in p, and in these cases, no change in $\bar{x}$ could be detected during stimulation. In one other case (axon VII of Table 2), a shift to a release pattern described by Poisson's Law occurred, indicating a substantial fall in p had occurred.

The decrease in ACh output was again mainly due to a decrease in the number of quanta released, and this was a consequence of decreases in both p and n. All parameters were affected less than in the absence of added choline; however, while the decrease in p was not significantly different between the groups ($P > 0{\cdot}1$, t test), the fall in n may have been rather smaller in the presence of supplementary choline (see Fig. 5),

although insufficient data have been obtained to test this point conclusively. If this partial maintenance of n were real, it seems possible that changes in the numbers of synaptic vesicles in the nerve terminals may affect n.

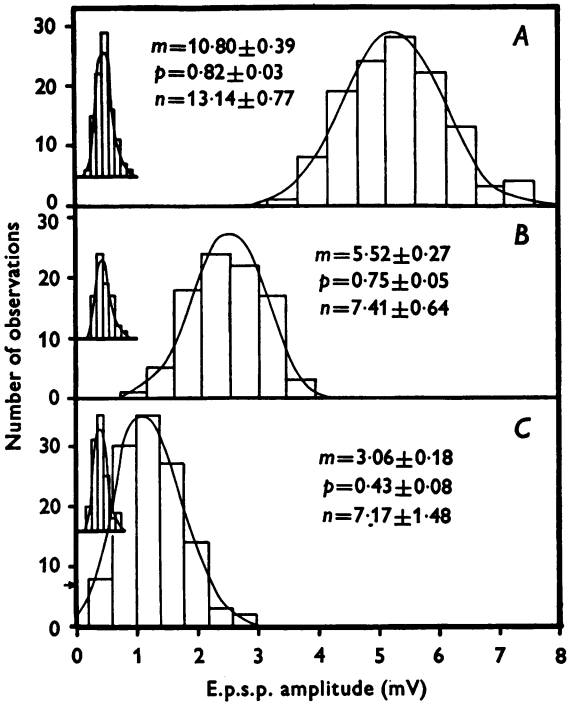

Fig. 4. Binomial analysis of release from an axon (axon III in Table 3) stimulated at 10 Hz in solution containing HC-3 (2×10^{-5} M). Amplitude–frequency histograms of e.p.s.p.s recorded at 0·5 min (*A*), 3·5 min (*B*) and 7·5 min (*C*) after the beginning of stimulation. Conventions used for drawing histograms as in Fig. 3. $P > 0{\cdot}3$ for all distributions shown (χ^2-test). A decrease in m in the absence of synthesis results from decreases in both p and n.

The effect of depletion of preformed ACh on the statistical release parameters

HC-3 blocks choline uptake and thus inhibits the synthesis of new ACh (MacIntosh, 1959) so that the ACh stored in the terminal is depleted during continuous stimulation (Birks & MacIntosh, 1961; Bennett & McLachlan, 1972*a*). Despite the decrease in quantal size (see above), the exponential decrease in ACh output during stimulation in HC-3 (2×10^{-5} M) was accompanied by a decrease in m, although the time course of this decline was slower ($\tau_m = 4{\cdot}6$ min, cf. $\tau_{\bar{x}} = 3{\cdot}3$ min). The results of the binomial analysis for one axon are shown in Fig. 4, and examples of data from all eight axons studied are given in Table 3. There was no

significant difference between the control values of m, p and n ($m = 10{\cdot}22 \pm 1{\cdot}47$, $p = 0{\cdot}75 \pm 0{\cdot}05$, $n = 13{\cdot}59 \pm 1{\cdot}54$, S.E.S of means, $n = 8$), as compared with those obtained in the absence of HC-3 (see above).

TABLE 3. Changes in the estimates of statistical release parameters during stimulation in the absence of ACh synthesis

Axon	Time (min)	$m \pm$ S.E.$_m$	$p \pm$ S.E.$_p$	$n \pm$ S.E.$_n$	P
I	0·5	11·21 ± 0·56	0·63 ± 0·07	17·68 ± 2·34	> 0·5
	3·5	3·73 ± 0·36	0·23 ± 0·15	16·12 ± 11·08	> 0·5
II	0·5	13·94 ± 0·51	0·94 ± 0·03	14·83 ± 0·76	> 0·2
	3·5	3·22 ± 0·36	0·18 ± 0·17	17·89 ± 17·90	> 0·5
III	0·5	10·80 ± 0·39	0·82 ± 0·03	13·14 ± 0·77	> 0·5
	3·5	5·52 ± 0·27	0·75 ± 0·05	7·41 ± 0·64	> 0·3
IV	0·5	5·27 ± 0·34	0·64 ± 0·06	8·19 ± 1·11	> 0·2
	3·5	3·59 ± 0·27	0·36 ± 0·10	9·88 ± 2·93	> 0·8
V	0·5	10·24 ± 0·48	0·55 ± 0·11	18·64 ± 4·04	> 0·3
	3·5	6·40 ± 0·49	0·34 ± 0·15	18·81 ± 8·91	> 0·5
VI	0·5	17·35 ± 0·89	0·98 ± 0·02	17·72 ± 1·01	> 0·1
	3·5	3·82 ± 0·27	0·98 ± 0·02	3·91 ± 0·29	> 0·1
VII	0·5	5·58 ± 0·23	0·72 ± 0·05	7·71 ± 0·60	> 0·1
	3·5	3·90 ± 0·20	0·76 ± 0·05	5·12 ± 0·41	> 0·2
VIII	0·5	7·36 ± 0·35	0·68 ± 0·06	10·79 ± 1·14	> 0·8
	3·5	1·30 ± 0·15	0·42 ± 0·11	3·10 ± 0·87	> 0·1

'Time' indicates the time after the beginning of stimulation at 10 Hz in solution containing HC-3 (2×10^{-5} M) at which each sample was taken. P, probability that the population could be a sample from the predicted binomial distribution of Γ-distributed units.

The decrease in m was accompanied by decreases in both parameters, p and n. After 5 min of stimulation, p was low ($< 0{\cdot}5$) in all axons studied, and estimates of n therefore became somewhat inaccurate (see Table 3). The standard error of the estimate of n becomes large when p is small (see Johnson & Wernig, 1971; McLachlan, 1975*a*), and is also increased if the estimate of n itself is large. However, the data were never incompatible with a concurrent decrease in this parameter.

The pooled results for all axons studied in HC-3 are shown in Fig. 5, together with the pooled data for those studied without inhibition of ACh synthesis. Only those estimates of n with S.E.$_n$ < 20% have been included, and these always fell during the decrease in m. In normal solutions and in solutions containing choline, decreases in p of similar magnitude were observed, while this parameter fell more markedly in the presence of HC-3. The decrease in m in all cases resulted from decreases in both p and n.

DISCUSSION

The decrease in the amount of ACh released by each impulse from preganglionic nerve terminals during prolonged high frequency stimulation is primarily due to a decrease in the number of quanta of ACh released, as the concurrent decline in quantal size is small (< 20%); this has also been reported for the mammalian neuromuscular junction (Elmqvist & Quastel, 1965*b*; Jones & Kwanbunbumpen, 1970; Bennett & Florin, 1974). During inhibition of ACh synthesis in the presence of HC-3, both the number of quanta released and their size decreased more quickly until evoked release was barely detectable. Similar changes have been

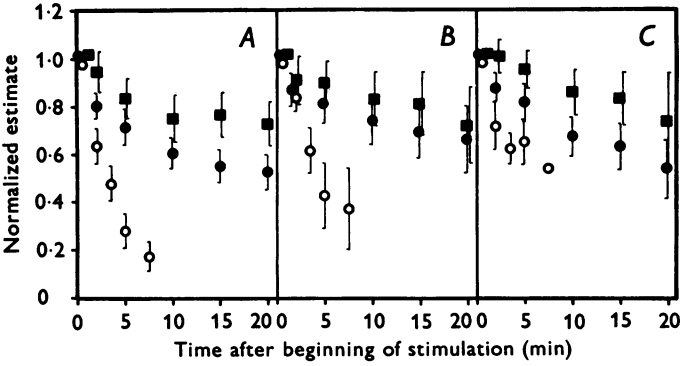

Fig. 5. Changes in the release parameters m (*A*), p (*B*) and n (*C*), during prolonged stimulation at 10 Hz. Pooled normalized data for axons stimulated in normal solutions (filled circles, $n = 7$), in the presence of added choline (3×10^{-5} M) (filled squares, $n = 7$), and in the presence of HC-3 (2×10^{-5} M) (open circles, $n = 8$). Bars indicate S.E.s of the means. Data for n include only values with $S.E._n < 20\%$, i.e. do not show changes in n for samples with low p. The falls in p observed in the presence or absence of added choline were not significantly different; however, p decreased further in the absence of synthesis. The change in n was least when choline was present in the bathing solution.

observed at the neuromuscular junction (Elmqvist & Quastel, 1965*a*; Jones & Kwanbunbumpen, 1970); it is not clear why Sacchi & Perri (1973) were unable to detect any change in quantal size in similar experiments on the ganglionic synapse.

It is possible that the observed decrease in quantal size represents a decrease in sensitivity of the post-synaptic receptors during repeated activation by ACh. However, no change in post-synaptic sensitivity to externally applied agonists has been observed following trains of impulses (Otsuka, Endo & Nonomura, 1962), even in the presence of HC-3 (Elmqvist & Quastel, 1965*a*). In addition, at a time when the size of

the min.e.p.s.p.s arising from stimulated axons is significantly reduced in the presence of HC-3, those derived from unstimulated axons on the same ganglion cell are unchanged in amplitude (McLachlan, 1975*b*). Although it is possible that HC-3 causes a specific exaggeration of desensitization only of those receptors which are activated during repetitive stimulation (see, for example, Rang & Ritter, 1969), it seems easier to interpret the changes in terms of a reduction in the amount of ACh in each quantum as the amount of transmitter in the terminal decreases. This idea is supported by the close parallel between the observed changes in post-synaptic potential amplitudes, and the independent measure of ACh output from preganglionic terminals provided by bio-assay techniques (Birks & MacIntosh, 1961).

The application of binomial statistics, using the population of min.e.p.s.p.s as a measure of the quantal size, has provided direct estimates of the release parameters, p and n, at all stages during prolonged stimulation of preganglionic nerve terminals. It has been shown that, for most preganglionic axons, the results are similar to those reported for regenerating neuromuscular junctions (Bennett & Florin, 1974), in that under normal conditions of ACh turnover there is always a decline in n. The absolute size of the changes in n shown in Fig. 5*C* may be misleading, some of the estimates of n having been omitted because of their large standard errors. When p fell, there was an increase not only in the standard error of the estimate of n, but also in the derived value of the estimate iself (see Tables). It therefore seems possible that the relative change in n may really be greater than that indicated in Fig. 5*C*.In the present experiments, however, a decrease in p has also been detected (Fig. 5), this being the parameter more markedly affected when synthesis is inhibited with HC-3. The addition of supplementary choline to ensure optimum conditions for synthesis does not prevent the change in p, so that it seems that the fall in p in normal solutions is not simply due to inadequate supplies of substrate. An additional action of HC-3 in reducing the amplitude of nerve action potentials during prolonged high frequency stimulation has been demonstrated (Frazier, 1968), although the concentration of HC-3 and the duration and frequency of stimulation which produced this effect were considerably greater than has been used in the present experiments. Furthermore, it has recently been shown that nerve terminal action potentials recorded extracellularly from rat phrenic nerve terminals are unaffected by 90 min of stimulation at 11 Hz in HC-3 (5×10^{-5} M) (Silinsky, 1975). It seems more likely that the larger decrease in p in HC-3 is a consequence of its action in inhibiting ACh synthesis.

It may therefore be concluded that the release parameters, p and n, are both reduced when the stores of ACh are depleted during repetitive

stimulation of preganglionic nerve terminals. The fall in n appears to be less in conditions which preserve the numbers of synaptic vesicles in the terminal axoplasm. This observation is consistent with the hypothesis that the vesicles represent the quanta of transmitter; when these are depleted by prolonged stimulation (Parducz *et al.* 1971; Ceccarelli, Hurlbut & Mauro, 1972), the number of quanta available for release is also reduced. It has been suggested that n is related to the size of the nerve terminal (Bennett & Florin, 1974), and corresponds to the number of activated 'release sites', seen as 'active zones' in the electron microscope (Zucker, 1973; Wernig, 1975). However, it seems possible that n may also be influenced by the number of synaptic vesicles present in the nerve terminal.

The conditions which resulted in changes in p are remarkably similar to those which affected quantal size (cf. Figs. 1 and 5); it is not clear why no fall in p was detected at regenerating motor nerve terminals (Bennett & Florin, 1974) concurrently with a fall in quantal size comparable to that observed in the present study. However, the correlation between changes in p and changes in quantal size reported here suggests that the probability of release of a quantum may be decreased as its size is reduced. It seems possible that quanta are normally only released if they contain more than some limiting amount of transmitter. This concept is consistent with the observations in this and the previous study (McLachlan, 1975*a*) that the amplitude distributions of min.e.p.s.p.s are well fitted by Γ-distributions. The skewed shape of these distributions implies that the probability of release of a quantum decreases to a greater extent if the quantum is smaller rather than larger than the modal size.

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