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CHANGES IN MINIATURE END-PLATE POTENTIALS AFTER BRIEF NERVOUS STIMULATION AT THE FROG NEUROMUSCULAR JUNCTION

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SUMMARY

1. The amplitude of miniature end-plate potentials (m.e.p.p.s), recorded at the frog neuromuscular junction in a normal ionic environment and in absence of drugs, was examined following 10–450 nerve impulses using conventional electrophysiological techniques and on-line computational analysis.

2. In both contracting preparations and non-contracting preparations pre-treated with glycerol, 100 or more nerve impulses resulted in a maximal fall in mean amplitude of about 20% with recovery apparent over the next 10–20 min.

3. In an altered ionic environment with a lowered Ca and raised Mg concentration, 450 nerve impulses did not produce a decrease in mean amplitude but a similar reduction was seen following a larger number of impulses.

4. The reduction in amplitude was estimated to follow the release of the order of 5000–10000 quanta at end-plates in a normal ionic environment and on average 17000 quanta in the presence of a lowered Ca and raised Mg concentration.

5. Changes in the mean size of the spontaneous quantal response is considered to be a presynaptic event and to reflect the loss and slow recovery of larger packets of transmitter from a vesicular store that is readily released by nerve impulses.

INTRODUCTION

The amplitude of miniature end-plate potentials arising from the spontaneous release of transmitter at the neuromuscular junction has been reported to remain remarkably constant, in contrast to the lability of frequency under conditions that effect presynaptic release of transmitter (Fatt & Katz, 1952; Katz, 1978). Under conditions of prolonged nervous stimulation, small reductions in mean miniature end-plate potential amplitude have been attributed to a lessened quantal packet size (Jones & Kwanbunbumpen, 1970; Katz, 1978) with pronounced decreases only apparent following an inhibition of choline uptake (Elmqvist & Quastel, 1965; Jones & Kwanbunbumpen, 1970). However, biochemical (Zimmermann & Whittaker, 1977;

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Zimmermann, Stadler & Whittaker, 1981) and physiological evidence (Fatt & Katz, 1952; McLachlan, 1975; Wernig, 1976; Kriebel, Llados & Matteson, 1982; Kelly & Robbins, 1984) is accumulating for a considerable heterogeneity of cholinergic vesicles in relation to transmitter content and/or release properties under physiological conditions. We have studied populations of miniature end-plate potentials (m.e.p.p.s) at the frog neuromuscular junction in a normal ionic environment, in the absence of drugs and using stimulus parameters that do not produce changes in post-synaptic sensitivity (Magleby & Pallotta, 1981) in order to re-investigate the relationship between evoked transmitter release and the size of spontaneous quantal responses. A preliminary account has been reported (Doherty, Hawgood & Smith, 1982).

METHODS

Rana pipiens and *Rana temporaria* were stored at 10 °C and experiments were performed on the isolated cutaneous pectoris nerve–muscle preparation at room temperature (21–23 °C). M.e.p.p.s were recorded by conventional electrophysiological techniques with output both to a Mingograf pen recorder (frequency response 700 Hz) and a CMB 2001 microprocessor in which machine code and BASIC programs allowed on-line voltage sampling every 0·2 ms for recognition of a m.e.p.p. and statistical analysis of populations (Doherty, Hawgood & Smith, 1981). A recognition pulse from the microprocessor to the chart recorder allowed confirmation of m.e.p.p.s. Mean values were obtained from 50–150 m.e.p.p.s and recorded in bins of 2–5 min, after which sampling was interrupted for 1 min to allow computation. In four experiments in which m.e.p.p. amplitudes of control and test samples were measured both on-line and manually, mean amplitude of test as a percentage of control agreed within 2% using the two methods. Inherent errors in the calculation of amplitude for populations sampled at frequencies of up to 300 m.e.p.p.s per minute can be calculated to be about 1% (Doherty, 1983).

End-plates were selected on the basis of large control m.e.p.p. amplitude, a procedure that resulted in the sampling of a population of small diameter fibres exhibiting relatively low basal m.e.p.p. frequencies (Kuno, Turkanis & Weakly, 1970). Mean control parameters were: frequency $23{\cdot}0 \pm 12{\cdot}3$ min^{-1} (17), amplitude $0{\cdot}95 \pm 0{\cdot}23$ mV (30), rise time $1{\cdot}20 \pm 0{\cdot}23$ ms (30), time from peak amplitude to half-decay $3{\cdot}70 \pm 0{\cdot}73$ ms (30) and resting membrane potential $78{\cdot}6 \pm 5{\cdot}5$ mV (30). All values $\pm$S.D. (n). Unless otherwise stated all results refer to the first period of indirect stimulation applied to any fibre, and no more than two fibres were sampled in any preparation.

In contracting preparations, the micro-electrode was withdrawn prior to nerve stimulation and square-wave pulses of 50 μs and 5–10 V were delivered by a suction electrode. Providing that the fibre was seen to contract, the end-plate was re-impaled and recording continued. Results are reported only for those preparations where the resting membrane potential remained within ± 4% of the value measured at electrode withdrawal. In non-contracting preparations, fibres were pre-treated with 400 mM-glycerol–Ringer solution for 40–60 min followed by re-equilibration in normal Ringer solution for 60 min (Howell & Jenden, 1967); such treatment does not interfere with transmitter release parameters (Miyamoto, 1975). As contractions often returned within 1–2 h of treatment, only preparations showing no contraction upon stimulation were included. The micro-electrode remained *in situ* throughout the recording period.

The mean quantal content (m) of transmitter release (Del Castillo & Katz, 1954) was estimated by the method of variance (Martin, 1966; Ginsborg & Jenkinson, 1976) at end-plates where the addition of *d*-tubocurarine blocked muscle contraction. A modified program allowed statistical analysis of e.p.p.s in groups of 50 within a train of 450 impulses delivered at 7·5 Hz. Mean quantal content and quantum size was determined during periods of stable release, usually after the first 50 impulses, by averaging the value obtained from four to six populations of 50 e.p.p.s. Small corrections to the value of m (< 15%) were made for the variance contribution of the base-line noise. End-plate potentials were of amplitude 1–6 mV (less than 10% of the resting membrane potential) and no correction was made for non-linear summation (McLachlan & Martin, 1982). In the presence of high-Mg low-Ca Ringer solution, mean quantal content was determined from the ratio of mean e.p.p. amplitude to mean m.e.p.p. amplitude. Composition of normal Ringer solution was (mM):

KCl, 2·5; NaCl, 111; NaH_2PO_4, 0·45; Na_2HPO_4, 2·55; $CaCl_2$, 1·8, with modification to $CaCl_2$, 0·9 and $MgCl_2$, 5. With two preparations bathed in normal Ringer solution, and three bathed in high-Mg low-Ca Ringer solution, 50–100 mM-sucrose was present throughout the recording period to increase basal m.e.p.p. frequency (Fatt & Katz, 1952). Similar results were obtained with these preparations.

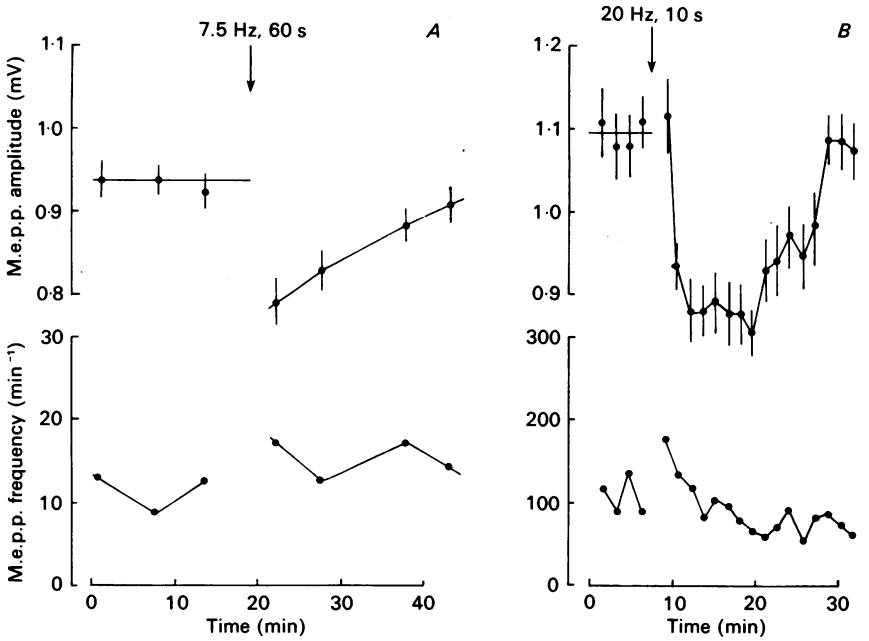

Fig. 1. M.e.p.p. amplitude (upper trace) and frequency (lower trace) as a function of time following a period of indirect stimulation. *A*, a train of 450 stimuli (7·5 Hz) was delivered to the motor end-plate of a preparation bathed in normal Ringer solution. Measurements are from populations of an average 81 m.e.p.p.s. The resting membrane potential on withdrawal, re-impalement and at the end of the recording was measured as 81, 80 and 82 mV respectively. *B*, a train of 200 impulses (20 Hz) (at arrow) was applied to a preparation bathed in Ringer solution containing 100 mM-sucrose. The mean sample size was 115 m.e.p.p.s. The measured resting membrane potential on withdrawal, re-impalement and at the end of the recording was 77, 79 and 80 mV respectively. Bars show ±S.E. of means.

RESULTS

The response to brief trains of indirect stimulation

At end-plates in a normal ionic environment and in the absence of drugs, trains of 100–450 stimuli applied to the motor nerve at frequencies of 2–20 Hz produced a significant fall in mean m.e.p.p. amplitude. The time course of response is illustrated for two end-plates in Fig. 1. In one preparation (Fig. 1*B*), the basal frequency was raised using 100 mM-sucrose–Ringer solution. Results with varying numbers of stimuli are presented in Table 1. Although no significant decrease in mean m.e.p.p. amplitude was seen at any end-plate following <20 stimuli, in four cases tested a significant decrease followed a second train of 450 stimuli. A train of 50 stimuli (7·5 Hz) produced, in three of six preparations, a significant ($P < 0·1$ %) decrease to 82·2 ± 1·2 % control (mean ± S.E. of mean) as calculated from the population of lowest

amplitude sampled 5–10 min post-stimulus. This value is not significantly different from that of 82·8 ± 2·2 % control (mean ± S.E. of mean, $n = 10$) recorded after 450 stimuli delivered at the same frequency. In the other three preparations, no significant amplitude change was recorded (99·0 ± 1·2 % control, mean ± S.E. of mean).

In these experiments, the micro-electrode was withdrawn prior to nerve stimulation. To test the possibility that the change in m.e.p.p. amplitude was a consequence of the preceding muscle contraction and/or re-impalement of the end-plate, a group of

TABLE 1. The effect of number of stimuli on mean m.e.p.p. amplitude

No. of stimuli	Stimulation frequency Hz (n)	Post-stimulation m.e.p.p. amplitude % control (N)
	Untreated preparations	
0	—	99·2 ± 0·8 (4)
10–20	2 (4), 7·5 (3)	99·8 ± 1·2 (7)
50	7·5 (6)	90·6 ± 2·3 (6)[a]**
100–300	2·5 (2), 15 (2), 20 (3)[b]	83·0 ± 1·7 (7)***
450	7·5 (10), 15 (3)	82·1 ± 1·8 (13)***
	Glycerol-treated preparations	
100	10 (5)	81·8 ± 2·9 (5)**

Results are means ± S.E. of means. n is the number of preparations stimulated at the given frequency. N is the total number of preparations subjected to the given number of stimuli. The mean m.e.p.p. amplitude post-stimulus was calculated from the population of least amplitude recorded over the initial 5–10 min period following stimulation. [a]Three preparations showed significant decreases, and three showed no change (see text). [b]Two preparations were bathed in Ringer solution containing 100 mM-sucrose. The group probability was calculated using two-tailed Student's t test. * $P < 5\%$, ** $P < 1\%$, *** $P < 0{\cdot}1\%$.

preparations was pre-treated in 400 mM-glycerol containing Ringer solution to disrupt contraction (see Methods). A similar decrease in mean m.e.p.p. amplitude was observed following 100 stimuli (10 Hz) as shown in Table 1.

The fall in m.e.p.p. amplitude was reversible, with recovery to 96·5 ± 1·5 % control (mean ± S.E., $n = 12$). The increase in amplitude was generally complete in 10–20 min post-stimulus (Fig. 1).

The number of quanta released by evoked stimulation in the normal ionic environment

The mean quantal content of evoked release has been calculated by the method of variance (see Methods) as 191 ± 80 (S.D.) from a total of eleven end-plates in two preparations. The above estimate is based on the Poisson statistical model; however, considerable evidence suggests that the binomial model is more applicable for physiological levels of transmitter output. Data presented for the frog neuromuscular junction (Miyamoto, 1975; Wernig, 1975) imply that values of m of the order of 200, obtained by use of the Poisson statistical model, will be over-estimated by approximately 100 % (assuming a P value of ~0·5). Correcting for this, and noting that the mean amplitude of the first 50 e.p.p.s in the train was 105·4 ± 6·3 % (± S.D., $n = 4$) of that measured during the period from which m was calculated, it can be estimated that 100 stimuli (7·5 Hz) will directly release of the order of 10000 quanta.

The effect of reducing quantum content (m)

To determine the effect of reducing the value of m on the response of m.e.p.p. amplitude to a period of indirect stimulation, six preparations were bathed in Ringer solution containing 0·9 mM-$CaCl_2$ and 5 mM-$MgCl_2$ (Del Castillo & Katz, 1954; Jenkinson, 1957). This reduced the value of m to 5–20 quanta as determined from the ratio of mean e.p.p. to m.e.p.p. amplitude. In contrast to the results reported

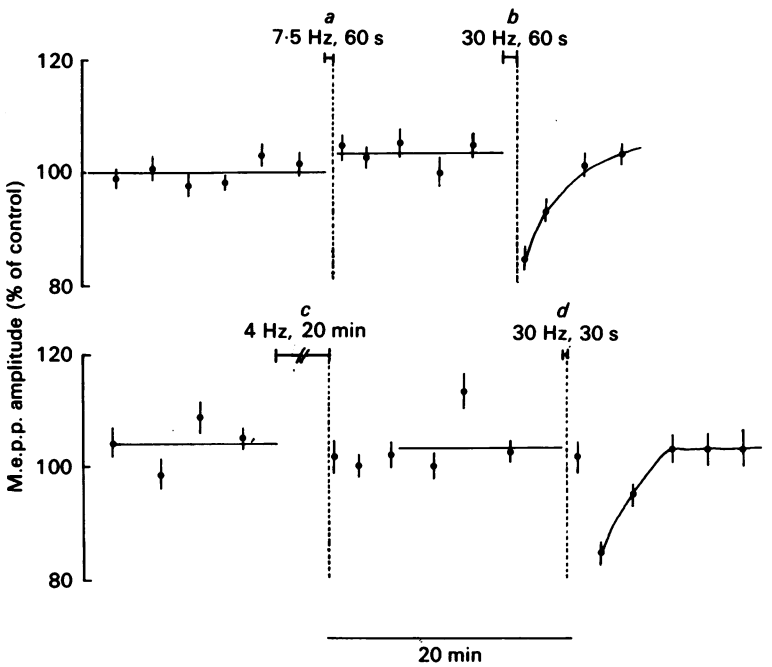

Fig. 2. Continuous record of m.e.p.p. amplitude following indirect stimulation at an end-plate partially blocked by raised Mg and lowered Ca concentration. Indirect stimulation released 2400, 21000, 18000 and 9600 quanta at *a*, *b*, *c* and *d* respectively. 100% control amplitude was 0·64 mV, and the control m.e.p.p. frequency was 65 min^{-1}. The resting membrane potential was measured as 70 mV at the start of recording and 72 mV at the end; there was no change during the periods of m.e.p.p. amplitude change. Points represent the means ± S.E. of means of populations of an average 126 m.e.p.p.s.

for preparations in the normal ionic environment, a train of 450 stimuli (7·5 Hz) produced no decrease in mean m.e.p.p. amplitude for any preparation tested (a typical example is illustrated in Fig. 2). Approximately 10–20 min later each preparation was subjected to a second, more intense period of indirect stimulation. At five of six end-plates, a significant decrease in m.e.p.p. amplitude to a mean value of 86·0 ± 1·3% control (± S.E. of mean, $n = 5$, $P < 2\%$) followed the period of stimulation Fig. 2.) The mean number of quanta released directly by stimulation was calculated as 6500 ± 3900 and 16900 ± 4600 for the first and second periods of stimulation respectively (± S.D., $n = 6$). On three occasions when a large number of quanta (approx. 20000) were released over a longer period (> 10 min), no decrease in mean m.e.p.p. amplitude was observed post-stimulus (Fig. 2). A full recovery

of mean amplitude (to 99·4 ± 1·1 %, mean ± S.E., $n = 5$) followed over a 10–15 min period, after which a second period of indirect stimulation could elicit a reduction in amplitude (Fig. 2).

Stimulation-associated changes in m.e.p.p. amplitude distributions

At all end-plates, m.e.p.p. samples showed normal unimodal distributions. Ringer solution containing 50–100 mM-sucrose was used to increase the basal m.e.p.p. frequency and thus allow the sampling of larger populations of m.e.p.p.s during the period of decreased m.e.p.p. amplitude. Distributions of m.e.p.p. amplitudes recorded during this period generally showed a discriminate suppression of the larger quantal responses (Fig. 3*A* and *B*). The changes are illustrated for both a preparation stimulated in a normal ionic environment and one stimulated in high-Mg low-Ca Ringer solution. The cumulative mean amplitude distributions show that divergence from the pre-stimulation controls occurred only after the modal amplitude. Return to control amplitude was associated with the reappearance of the larger quantal responses (Fig. 3*A* and *B*).

DISCUSSION

The results of these experiments show the average size of the m.e.p.p. to be transiently reduced following the evoked release of an estimated 5000–10000 quantal packets at end-plates in the normal ionic environment. A similar reduction of mean m.e.p.p. amplitude has previously been reported at both the frog (Kriebel & Gross, 1974) and the rat (Jones & Kwanbunbumpen, 1970) neuromuscular junctions; however, in both cases the response was consequential to greater periods of indirect stimulation (600 impulses and ~20000 respectively), and may therefore have been secondary to a generalized reduction in the amount of acetylcholine available for transmitter release (Katz, 1978). Considering the small numbers of quanta released by indirect stimulation in the present study, the decreases in mean m.e.p.p. amplitude reported are unlikely to reflect a significant decrease in the total content of acetylcholine in the nerve terminal.

Transmitter-induced desensitization of the acetylcholine receptor, which is maximal at the moment of cessation of indirect stimulation, has been reported at the non-eserinized neuromuscular junction of the frog for stimuli applied at intervals of less than 25 ms (Magleby & Pallota, 1981). In the present study the interval between impulses at end-plates bathed in the normal ionic environment was greater than 50 ms, and the maximal amplitude decrease was not always apparent immediately following the period of indirect stimulation (see Fig. 1*B*). Furthermore, the half-time of recovery of the acetylcholine receptor from desensitization is of the order of seconds (Magleby & Pallota, 1981), whereas in the present study m.e.p.p. amplitude returned to the control value over a 10–20 min period. Thus the decrease in amplitude reported in the present study cannot readily be attributed to desensitization of the acetylcholine receptor.

No reduction in m.e.p.p. amplitude occurred following up to 450 (7·5 Hz) impulses at end-plates where the quantal content had been considerably reduced by the presence of high-Mg low-Ca Ringer solution. However, a decrease was observed

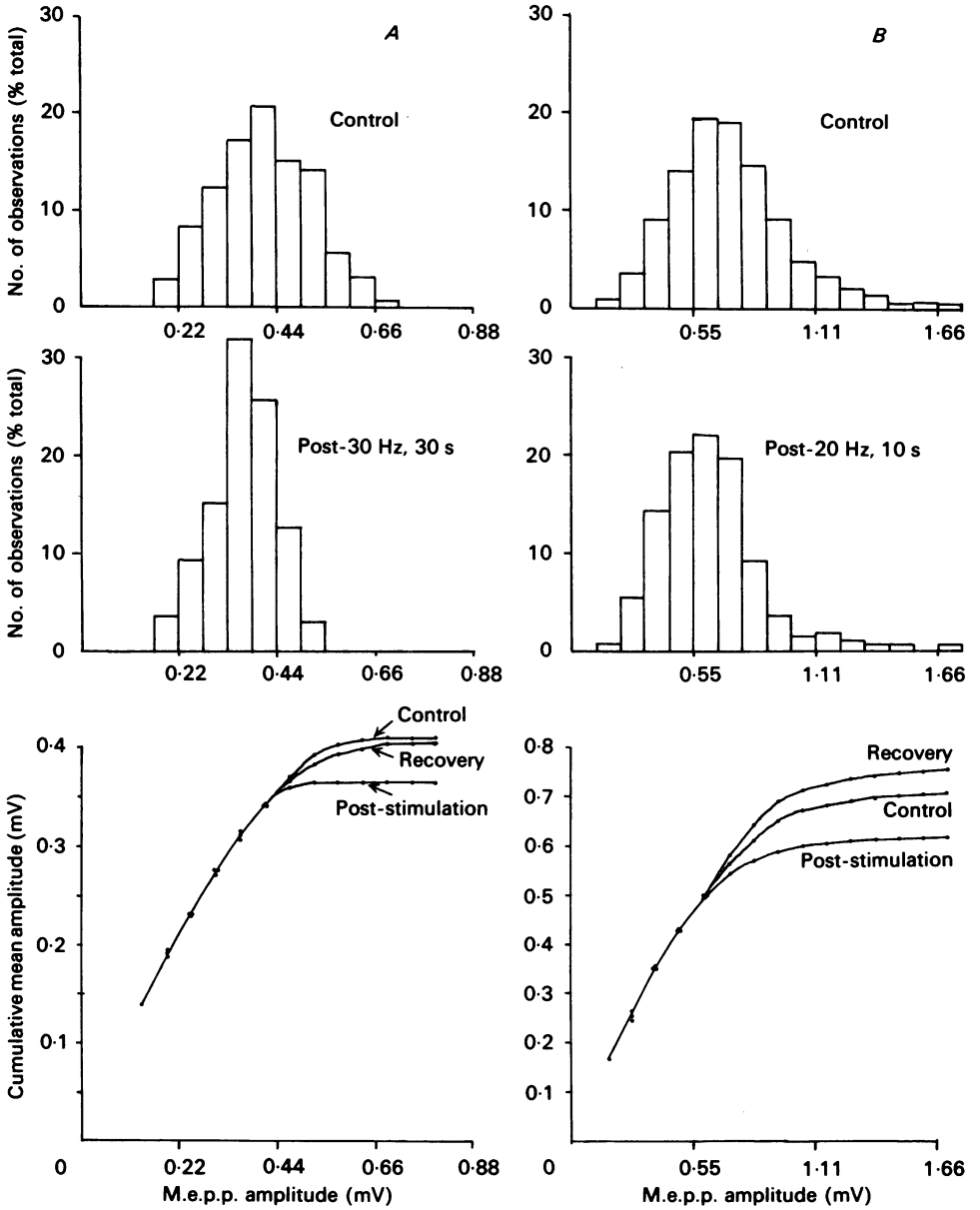

Fig. 3. The amplitude (top and centre) and cumulative mean amplitude (bottom) distributions of m.e.p.p.s sampled before and after indirect stimulation. *A*, contraction was blocked by high-Mg low-Ca Ringer solution and the micro-electrode remained *in situ* during the period of indirect stimulation (30 Hz, 30 s), which was estimated to have evoked the release of 14400 quanta. M.e.p.p.s were sampled over a 10 min period immediately prior to stimulation (mean amplitude 0·41 mV, $n = 768$), and over the 2–5 min period post-stimulation (mean amplitude 0·37 mV, $n = 284$) and the 20–25 min period post-stimulation (mean amplitude 0·40 mV, $n = 454$). 50 mM-sucrose was present throughout. *B*, the micro-electrode was removed during the period of indirect stimulation (20 Hz, 10 s). M.e.p.p.s were sampled over an 8 min period immediately prior to stimulation (mean amplitude 0·70 mV, $n = 1024$), and over the 8–9 min period post-stimulation (mean amplitude 0·62 mV, $n = 512$) and the 21–24 min period post-stimulation (mean amplitude 0·76 mV, $n = 631$). 100 mM-sucrose was present throughout.

following further stimulation that evoked the release of, on average, 17000 quanta. Thus the reduction in mean m.e.p.p. amplitude appears to be directly related to the number of quantal packets released during the train of indirect stimulation, which suggests that it is a consequence of presynaptic changes in size of the spontaneously released quantal packets of transmitter.

The period of reduced mean m.e.p.p. amplitude was associated with discriminate changes in the shape of the m.e.p.p. amplitude distribution. As the variation in the size of m.e.p.p.s recorded with an intracellular electrode reflects the differing sizes of the intraterminal population of quantal packets (Katz & Thesleff, 1957; Wernig, 1976) the above changes can be interpreted classically as due to an alteration in the size distribution of those quanta immediately available for spontaneous release. Kriebel & Gross (1974) reported a similar suppression of the right-hand shoulder of the major mode of m.e.p.p. amplitude following 600 stimuli, as well as the appearance of increasing numbers of a discrete population of small m.e.p.p.s. In the present study, small m.e.p.p.s did not contribute to the decreased response.

At present it is still not clear as to how a quantal packet of transmitter is formed. The majority of accumulated evidence remains in support of the general concept of the vesicle hypothesis (Del Castillo & Katz, 1954; Katz, 1978; Heuser, Reese, Dennis, Jan, Jan & Evans, 1979; Zimmerman, 1979). Studies on the heterogeneity of transmitter content found within differing populations of vesicles isolated from the electric organ of *Torpedo marmorata* have led to the hypothesis that a small population of readily releasable synaptic vesicles may be preferentially filled with newly synthesized transmitter (Zimmermann & Whittaker, 1977; Zimmermann *et al.* 1981). At the frog neuromuscular junction a morphological correlate of such a population is the 10000–20000 synaptic vesicles aligned at the active zone of the nerve terminal (Couteaux & Pécot-Dechavassine, 1974; Katz, 1978; Heuser *et al.* 1979). The results of this study show a change in the mean amplitude and size distribution of m.e.p.p.s recorded shortly after the evoked release of a similar number of quanta. The results therefore support the hypothesis that a small population of readily releasable synaptic vesicles may contain on average more transmitter than in those vesicles mobilized to replace them. The time course of m.e.p.p. amplitude recovery, which is similar to that reported at junctions transiently depleted of transmitter (Elmqvist & Quastel, 1965; Jones & Kwanbunbumpen, 1970), suggests that over a 10–20 min period this latter population may undergo a second period of transmitter uptake to restore the *status quo*.

The size differential, as regards transmitter content, between quanta of the differing vesicular stores cannot be ascertained from the results of this study. Several factors, including the distribution of release probabilities within the population of vesicles available for spontaneous release, together with the rate of transmitter uptake into those vesicles, may combine to limit the indirect stimulation-associated decrease in mean m.e.p.p. amplitude to a fall of around 20%.

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