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DEPRESSION OF TRANSMITTER RELEASE AT THE NEUROMUSCULAR JUNCTION OF THE FROG

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SUMMARY

1. Depression of transmitter release produced by preceding conditioning stimulation was studied at the frog's neuromuscular junction.

2. Depression occurred when transmitter release was restricted to a short length of nerve terminal, or when release was initiated by electrotonic depolarization of the terminals after action potentials were abolished by tetrodotoxin.

3. Quantitative studies revealed a non-linear relationship between the estimated magnitude of 'zero-time' depression and the amount of transmitter released by conditioning stimulation.

4. Two stimuli separated by 20–200 msec were given and the ratio of the end-plate potential amplitudes (V_2/V_1) was measured. This amplitude ratio increased during depression produced by stimuli preceding the test pair.

5. These observations may be explained by assuming that depression is associated with a reduction in release probability as well as a depletion of transmitter available for release.

INTRODUCTION

At the neuromuscular junction, the second of a pair of stimuli may produce an end-plate potential (e.p.p.) which is smaller than that produced by the first. This depression was first described by Eccles, Katz & Kuffler (1941) in mammals, and by Lundberg & Quilisch (1953) in amphibia. During depression, the sensitivity of the post-synaptic membrane to applied acetylcholine (ACh) is unchanged (Otsuka, Endo & Nonomura, 1962), leading to the conclusion that depression is due to a reduction in the

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amount of transmitter released from the nerve terminal. The magnitude of depression depends on the amount of transmitter released by the conditioning stimulus; depression increases (i.e. test e.p.p.s become relatively smaller) when several conditioning stimuli are given or when the calcium concentration of the bathing solution is raised, thereby increasing the amount of transmitter released by the conditioning stimulation (Liley & North, 1953; Takeuchi, 1958; Thies, 1965). Recovery from depression follows an exponential time course, with a time constant of about 5 sec at 20° C. These observations form the basis of the hypothesis that depression reflects a depletion of transmitter available for release and that recovery from depression reflects a replenishment of this pool of transmitter.

The present study was performed in an attempt to answer several questions raised by these observations. First, is depression a property of the mechanism of transmitter storage and release, or does it result from an alteration in the propagation of impulses through the branched terminals of the motor nerve? Secondly, can a simple one-compartment model based on the 'depletion' hypothesis quantitatively account for e.p.p. amplitudes under different experimental conditions?

METHODS

All experiments were performed on neuromuscular junctions in isolated sartorius muscles of the frog (*Rana pipiens*). The normal saline solution consisted of (mM): NaCl, 115; KCl, 2·0; $NaHCO_3$, 2·4; $CaCl_2$, 1·8. For experiments involving focal recordings, calcium was reduced to about 0·1 mM and 1·0–1·4 mM-$MgCl_2$ was added to reduce transmitter release to very low levels. For most other experiments, transmitter release was increased by the addition of $CaCl_2$ (up to 4 × normal) to the bathing solution, and in some cases by the replacement of up to one third of the NaCl with sucrose or methylamine hydrochloride. E.p.p. amplitudes were reduced to low levels by the addition of (+)-tubocurarine (5–10 μg/ml.). When e.p.p.s were larger than about 5 mV, they were corrected for non-linear summation (Martin, 1955), assuming a reversal potential of −15 mV (Takeuchi & Takeuchi, 1960*a*).

The nerve was lifted into a separate oil-filled compartment and stimulated with supramaximal shocks. In a few experiments, transmitter release was initiated by electrotonic depolarization of nerve terminals after action potentials were abolished by tetrodotoxin (1 μg/ml.) A saline-filled micropipette (15–25 μ tip diameter) was placed over the nerve near the end-plate region and negative pressure was applied to the pipette to draw the axon against the electrode tip. Current passed through the pipette crossed the axon membrane and spread into the terminal region causing transmitter release.

Glass micropipettes filled with 3 M-KCl (15–25 MΩ) were used for intracellular recordings. Signals were fed through a high impedance unity gain amplifier to the oscilloscope. Backing voltages were applied from a potentiometer in series with the bath electrode leading to ground. E.p.p.'s were either recorded photographically or displayed on a storage oscilloscope and measured directly from the screen.

The technique described by del Castillo & Katz (1954*a*) and by Katz & Miledi (1965) was used for external recording of 'focal' e.p.p.s. Glass micropipettes filled

with 0·5–0·8 M-$CaCl_2$ (2–10 MΩ) were used for recording. In the low-calcium solution, the amount of transmitter released by a nerve impulse depended on the diffusion of calcium from the tip of the pipette, which was placed close to the nerve terminal. The optimal location was found by exploring the end-plate region with the electrode until relatively large unit potentials with relatively fast rise times were found. Diffusion of calcium from the electrode tip was controlled by an electrical bias. In this way transmitter release was restricted to a short length of terminal directly beneath the electrode tip. A second electrode filled with 3 M-KCl was used to obtain simultaneous intracellular recordings of e.p.p.s.

RESULTS

Depression in a short length of terminal. As the motor axon reaches the end-plate region, it loses its myelin sheath and splits into about a dozen small branches which make synaptic contact with the muscle fibre. Katz & Miledi (1968) have demonstrated that an action potential normally is propagated regeneratively through the terminals and that electrotonic spread of depolarization beyond a focal nerve block is not very effective in causing transmitter release. One possible explanation of depression is that it is due to a block of action potential propagation into some of the terminal branches of the motor axon, in which case recovery from depression would reflect the recovery of excitability in the terminal branches. It could be that release of large amounts of transmitter somehow interferes with subsequent impulse propagation.

To investigate this possibility, transmitter release was restricted to a short length of terminal (see Methods), and e.p.p.s were recorded simultaneously with focal and intracellular electrodes. Results from a typical experiment are illustrated in Fig. 1. Two conditioning stimuli (Cond.) produced a depression of test e.p.p.s 1 sec later (Test). Depression is expressed quantitatively as

$$D = 1 - V_t/V_1, \tag{1}$$

where V_t = amplitude of the test e.p.p. and V_1 = amplitude of the first conditioning e.p.p. In the experiment illustrated in Fig. 1, the average depression of test e.p.p.s was 0·26 (112 trials, intracellular measurements), and the average number of quanta released by the conditioning stimuli was about 30 (obtained by dividing the mean amplitude of the summed conditioning e.p.p.s by the mean amplitude of the spontaneous miniature e.p.p.s, all measured intracellularly).

From these results, an estimate can be made of the length of nerve terminals which is being activated. At the frog neuromuscular junction, release of about 100 quanta produces about 0·1 depression (Martin, 1955; Takeuchi & Takeuchi, 1960*b*). Assuming that depression varies linearly with the amount of release (which is approximately valid for low values of D_0 (see later)), then to produce a depression value of 0·26 (i.e. that observed in Fig. 1) 260 quanta must be released from the entire terminal. Then the focal recording was from 30/260 or 11·5 % of the entire length of

terminal. This is probably an over-estimate for several reasons. Some recovery from depression occurred during the 1 sec interval between stimuli, so that the magnitude of 'zero-time' depression was greater than 0·26. In addition, the calcium concentration decreases away from the tip of the pipette, and in the more 'remote' areas some release would occur, but not enough to cause depression. This would have the effect of adding units to both conditioning and test e.p.p.s, resulting in an underestimate of depression, and therefore an over-estimate of the length of terminal from

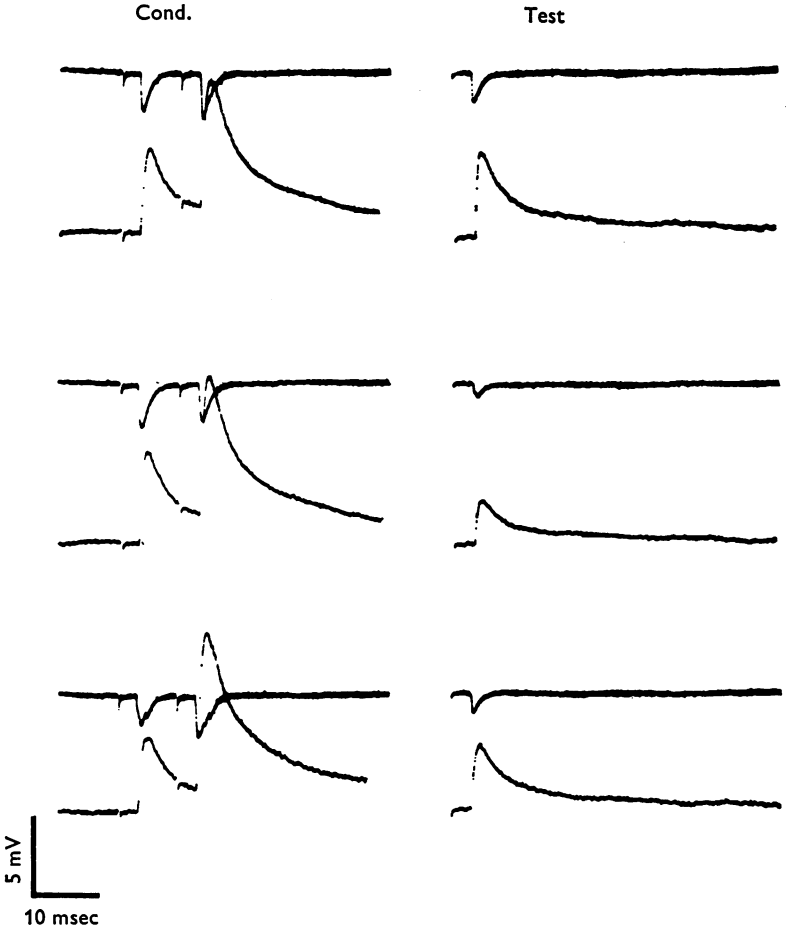

Fig. 1. Simultaneous recordings of focal e.p.p.s (upper traces) and intracellular e.p.p.s (lower traces). Upward deflexion positive. The preparation was bathed in low-calcium saline and transmitter release was controlled by efflux of calcium from the focal pipette. Two conditioning stimuli (Cond.) preceded a single test stimulus (Test) by 1 sec. Mean depression of the intracellular test e.p.p. was 0·26 (112 samples). Two conditioning stimuli were given in order to increase the magnitude of depression. The second conditioning e.p.p. was larger than the first due to facilitation of transmitter release at this short interval (10 msec). The interval was chosen so that recovery from depression between conditioning stimuli was negligible.

which the recording was made. The true length was probably less than 10% of the entire terminal.

In a few experiments, the nerve action potential was recorded with the focal pipette, and it did not appear to change during depression. Although this argues against a total block of the action potential, a small reduction in its amplitude, which might result in a large reduction in the amount of transmitter released, would be difficult to detect with extracellular recording techniques. However, depression could be demonstrated when action potentials were abolished by tetrodotoxin (1 μg/ml.) and the nerve terminals depolarized by electrotonic stimulation (see Methods). Figure 2*A* illustrates the effect of three conditioning pulses on a test e.p.p. evoked 440 msec later. In Fig. 2*B*, amplitude ratios (test e.p.p. amplitude divided by amplitude of first conditioning e.p.p.) are plotted against conditioning-test intervals. Although there is considerable scatter, the initial facilitation, followed by depression, is similar to the sequence of events produced by propagated action potentials (cf. Katz & Miledi, 1967*b*). It thus seems likely that neuromuscular depression is independent of the action potential mechanism and represents some change in the process of transmitter storage in and release from the nerve terminal.

The relation between depression and the amount of transmitter released. According to the 'depletion' hypothesis, depression of e.p.p. amplitudes following conditioning stimulation reflects a reduction in the amount of transmitter available for release. In terms of the quantum hypothesis, the number of quanta released (m) is given by the product of two terms: n, the number of quanta available for release, and p, their average probability of release. In its simplest form the depletion hypothesis states that n is reduced by that number of quanta released by the conditioning stimulation, and predicts that the magnitude of depression will be directly proportional to the amount of transmitter released.

This hypothesis was tested by measuring the magnitude of 'zero-time' depression (D_0) and comparing it to the amount of transmitter released by the conditioning stimulation. As illustrated in Fig, 3, D_0 was estimated by plotting values of depression (as defined by eqn. (1)) against conditioning-test intervals on semi-logarithmic scale, and extrapolating a regression line to zero-time. This procedure was necessary because depression is obscured by a superimposed facilitation at short intervals. Conditioning-test intervals were usually longer than 1 sec, thus avoiding the period of facilitation. The amount of transmitter released, and therefore the magnitude of depression, was varied by changing the number of conditioning stimuli and by changing the calcium concentration in the bathing solution.

In Fig. 4, the values of D_0 from Fig. 3 are plotted against the summed amplitudes of conditioning e.p.p.s, which are assumed to be proportional

to the number of quanta released (see Discussion). 'Release' is expressed in multiples of the smallest conditioning response (amplitude of one e.p.p. in 2·7 mM-Ca). In all fifteen experiments, the relationship deviated from linearity over the range studied (D_0 values from 0·1 to 0·9).

There are several ways of explaining this deviation. It could be argued that a simple one-compartment model is not a valid description of the store of transmitter available for release. However, rate constants of

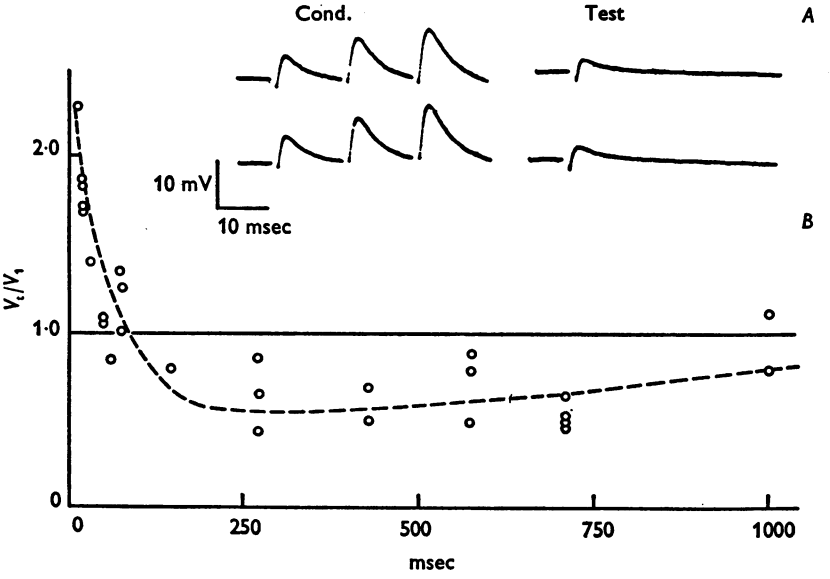

Fig. 2. Depression in the absence of propagated responses. Action potentials were blocked with tetrodotoxin (1 μg/ml.) and transmitter release was initiated by electrotonic depolarization of the nerve. *A*. Examples of e.p.p.s evoked by pulses of equal strength and duration. Three conditioning pulses (Cond.) preceded a test pulse (Test) by 440 msec. Multiple conditioning stimuli were given in order to increase the magnitude of depression; recovery from depression between conditioning stimuli was negligible at this short interval (15 msec). *B*. Amplitude ratios (V_t/V_1, where V_1 = amplitude of first conditioning e.p.p. and V_t = amplitude of test e.p.p.) are plotted against the conditioning-test interval, illustrating that tetrodotoxin does not abolish facilitation or depression. The dashed line was fitted by eye.

recovery from depression showed no correlation with the magnitude of D_0 (Fig. 5). Correlation coefficients for linear regressions of such plots ranged from $-0{\cdot}44$ to $+0{\cdot}24$ (five experiments, six to seven points per experiment). This is consistent with the hypothesis of a homogeneous population of quanta exhibiting first order kinetics. However, this analysis would not detect a 'fast' phase of recovery from depression at short

intervals (i.e. during facilitation), which would invalidate the estimate of D_0 by extrapolation to zero time. Although facilitation may be studied in the absence of depression (by reducing the level of release), unfortunately the reverse is not true. One can, however, compare the time course of the decay of facilitation under control conditions and during depression. In a

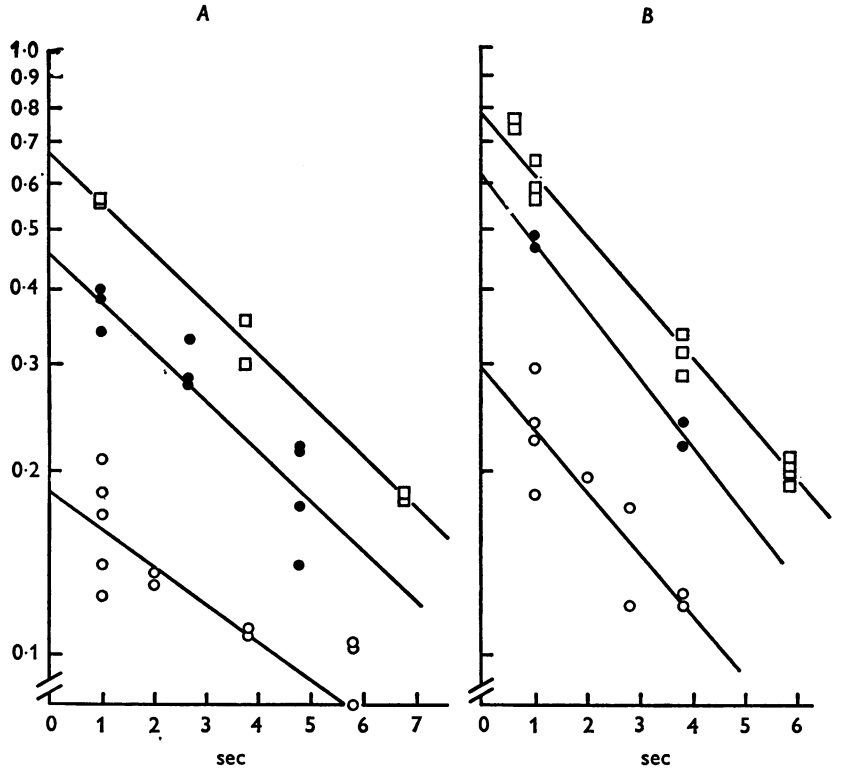

Fig. 3. Depression as defined by eqn. (1) is plotted against the conditioning-test interval (sec). From an experiment on one fibre under different conditions: *A*, 2·7 mM-Ca. *B*, 6·3 mM-Ca. Calculated regression lines extrapolated to zero time give estimates of D_0. The number of conditioning stimuli in each series was 1 (open circles), 2 (filled circles), and 4 (squares). Not shown for purposes of clarity is a series in 6·3 mM-Ca with three conditioning stimuli; D_0 was estimated as $0{\cdot}71 \pm 0{\cdot}03$.

few such experiments, no difference was found, supporting the assumption that recovery from depression follows a single exponential throughout its time course.

An alternative explanation of the deviation from linearity in Fig. 4 is that the release probability changes during depression. In particular, the deviation can be explained by assuming that p decreases during depression,

and that the decrease is proportional to the depletion of n. The relationship between D_0 and release is then as follows:

Assume that p at any time is related to n by

$$p/p_0 = n/n_0, \tag{2}$$

where p_0 and n_0 are control values of release probability and store of transmitter, respectively. By the quantum hypothesis, $m = np$. Solving eqn. (2) for p and substituting,

$$m = p_0 n^2/n_0. \tag{3}$$

For the conditioning stimulus: $m_0 = n_0 p_0$. After this stimulus, the number of quanta available for release (assuming no recovery)

$$n_1 = n_0 - m_0 = n_0(1 - p_0),$$

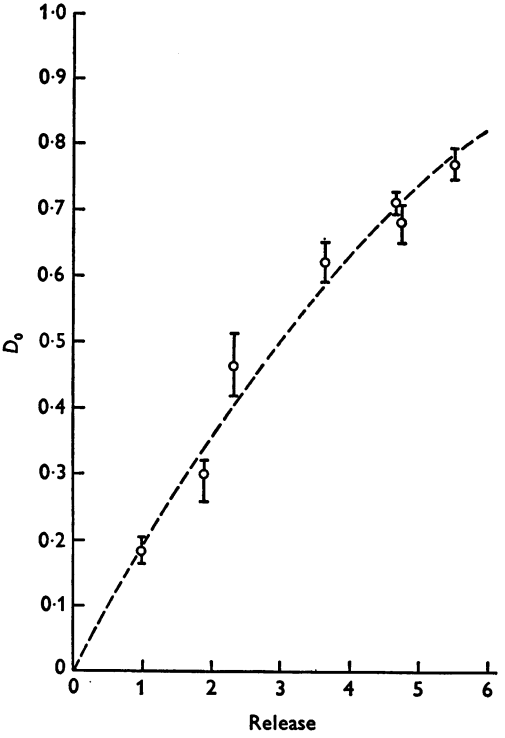

Fig. 4. Values of $D_0 \pm 1$ S.E. of the estimate are plotted against the amount of transmitter released by the conditioning stimuli (from the same experiment as Fig. 3). 'Release' is expressed in multiples of the smallest conditioning response (i.e. the e.p.p. in response to one stimulus in 2·7 mM-Ca). The deviation from linearity, though slight, was a consistent finding (fifteen experiments), and the experimental points are fitted better by a parabola (dashed line). See text for derivation.

and a 'zero-time' test stimulus releases $m_1 = n_0 p_0 (1-p_0)^2$ quanta. Zero-time depression is defined as $D_0 = 1 - m_1/m_0$. Substituting for m_1 and m_0:

$$D_0 = 1-(1-p_0)^2. \tag{4}$$

This parabolic relationship between p_0 and D_0 is shown graphically in Fig. 6. Shown for comparison is the relationship predicted by the linear depletion model (straight line). The two models could be distinguished easily if it were possible to measure p_0 experimentally; unfortunately the best one can do is plot D_0 as a function of the amplitude of conditioning

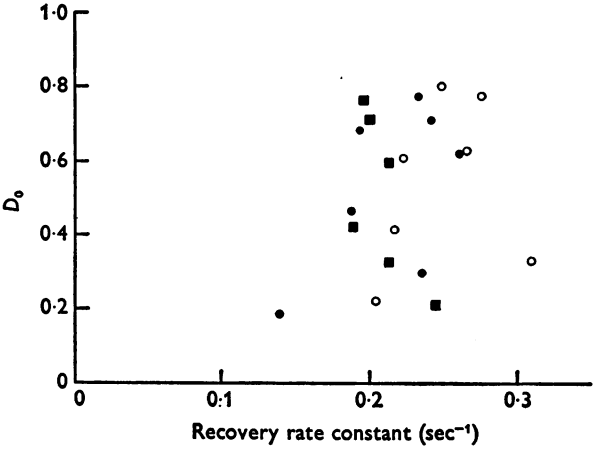

Fig. 5. Rate constants of recovery from depression from three experiments are plotted against corresponding values of D_0. No correlation was observed between these two variables.

e.p.p.s, as in Fig. 4. In eqn. (4), p_0 is the fraction of transmitter released by a single conditioning stimulus. Experimentally, when several conditioning stimuli are given, this is equivalent to $\Sigma m/n_0 = k.\Sigma V_c$, where ΣV_c = summed amplitudes of the conditioning e.p.p.'s and $k = (n_0.v_1)^{-1}$, v_1 being the amplitude of a single quantum. Experimental points were consistently fitted better by a parabolic function than by a linear function. Standard errors of estimated lines were about twice as large for a linear model, and a paired analysis of the differences in standard errors for the two models was significant at the 1 % level (five experiments, six to seven points per experiment). The dashed line in Fig. 4 is such an estimated line. By visual inspection, the deviation from linearity, though consistent, was not large, and a different experimental test was sought which might better distinguish these models.

One test is suggested by the prediction that if $m = p_0 n^2/n_0$ and if n recovers exponentially after a stimulus, then it can be shown that recovery from depression is

determined by the difference between two exponentials, rather than by a single exponential term. The equation is: $D_t = 2p_0.\exp(-kt) - p_0^2.\exp(-2kt)$, where k = rate constant for recovery of n and p. This predicts an initial 'lag' in recovery (due to the second term). The expected deviation from a single exponential is small however (about 5 % when $D_0 = 0{\cdot}85$), and in the same direction as facilitation, for which it might be mistaken. For these reasons, and because of the variation in depression from trial to trial, it was not possible with these techniques to resolve any deviation from exponential recovery beyond the period of facilitation.

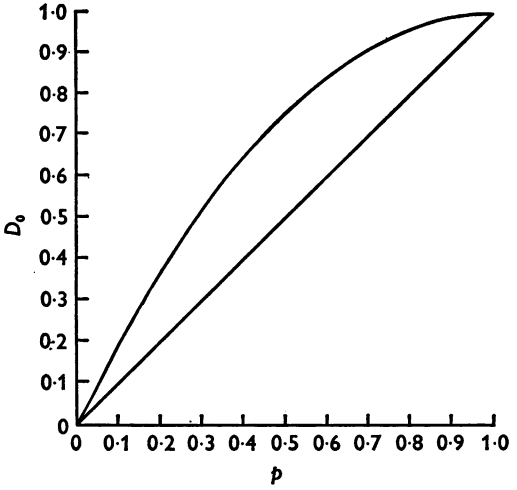

Fig. 6. Theoretical curves showing the relationship between release probability (abscissa) and D_0 for two different models. The linear relationship is predicted by assuming that each stimulus releases a constant fraction of the remaining transmitter available for release; the parabolic relationship is predicted by assuming that p decreases during depression. See text for derivation.

E.p.p. amplitudes during depression. An experiment designed to distinguish more clearly between the possible interpretations of the non-linearity in Fig. 4 involved comparing amplitude ratios of two e.p.p.s after varying amounts of conditioning stimulation. According to the linear depletion model, each stimulus adds a constant fractional increment of depression, and therefore amplitude ratios should be unaffected by preceding conditioning stimulation. However, if p decreases during depression, amplitude ratios should increase during depression. The fractional increment of depression added by a test stimulus would be smaller than that produced by the conditioning stimulus, since depression does not sum linearly with increasing amounts of release (cf. the parabolic line in Fig. 6).

The experimental procedure is illustrated in Fig. 7. Two stimuli were given at an interval of about 100 msec, and the e.p.p. amplitude ratio

(V_2/V_1) was calculated. At this short interval, recovery from depression could be neglected. The procedure was then repeated with one or more conditioning stimuli given 0·5–15 sec before the test pair. Since the test stimuli were separated by about 100 msec, the effects of facilitation had to be taken into account. Specifically, it was assumed that facilitation reflects an increase in release probability, and that facilitation is independent of depression. Evidence in support of the latter assumption was mentioned earlier (the time course of facilitation decay did not appear to be affected by depression produced by conditioning stimulation). In

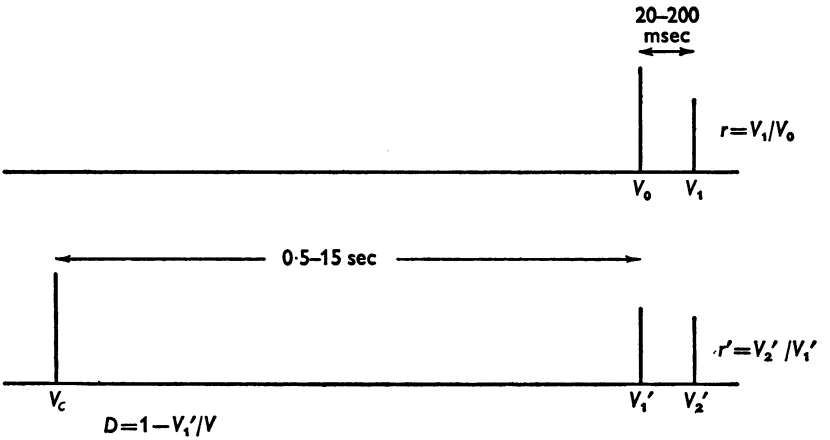

Fig. 7. Experimental method for calculating e.p.p. amplitude ratios. Two stimuli were given separated by 20–200 msec, and the amplitude ratio (r) was calculated. The procedure was then repeated with one or more conditioning e.p.p.s preceding the test pair by 0·5–15 sec. The magnitude of depression of the first test e.p.p. was measured also. In this way, the relationship between R, the fractional change in amplitude ratios produced by conditioning stimulation, and D, the magnitude of depression, was studied.

addition, test stimuli in the present experiments were separated by intervals ranging from 20 to 200 msec, thereby spanning most of the period of facilitation, with no difference in results.

According to the linear depletion model, amplitude ratios should be independent of any preceding conditioning stimulation. The derivation of this relationship is as follows:

Assume $m = np$. 'Primed' expressions (e.g. n') indicate that preceding conditioning stimulation was given. In the absence of conditioning stimulation, $m_0 = n_0 p_0$, and a second stimulus about 100 msec later produces an e.p.p. $m_1 = f p_0 n_1$ where f is a 'facilitation factor' assumed to act on p. Recovery from depression can be ignored at this short interval, so $n_1 = n_0 - m_0 = n_0(1-p_0)$, and therefore $m_1 = f p_0 n_0 (1-p_0)$. The amplitude ratio $r = m_1/m_0 = f(1-p_0)$.

Now consider the effect of conditioning stimulation. The e.p.p. in response to a conditioning stimulus $m_c = n_0 p_0$. The conditioning-test interval is long enough to allow facilitation to decay, so the first test e.p.p. $m_1' = p_0 n_1'$. Now the value of n_1' depends on the amount of conditioning stimulation and on the conditioning-test interval. The magnitude of depression is defined as: $D = 1 - m_1'/m_c = 1 - n_1'/n_0$. Solving for n_1', we obtain $n_1' = n_0(1-D)$, and $m_1' = n_0 p_0 (1-D)$. Now the second test e.p.p. $m_2' = f p_0 n_2'$, where $n_2' = n_1' - m_1' = n_0(1-D)(1-p_0)$. Therefore,

$$m_2' = f p_0 n_0 (1-D)(1-p_0).$$

The amplitude ratio of the test pair $r' = m_2'/m_1' = f(1-p_0)$. Thus $r = r'$, and the linear depletion model predicts that the amplitude ratio should be unaffected by conditioning stimulation.

If p decreases during depression in a manner described in the previous section, it can be shown that amplitude ratios should increase during depression.

The derivation of this relationship is exactly like that for the linear depletion model, except that it is assumed that $m = p_0 n^2/n_0$ (eqn. (3)). The resulting equations for the amplitude ratios are:

$$\text{control amplitude ratio } r = m_1/m_0 = f(1-p_0)^2;$$

$$\text{amplitude ratio during depression } r' = f\{1 - p_0\sqrt{(1-D)}\}^2.$$

Now let $R = r'/r$, which is the ratio of the amplitude ratios under two different conditions.

$$R = \frac{1 - p_0\sqrt{(1-D)}}{1-p_0}. \tag{5}$$

(For the linear depletion model, $R = 1{\cdot}0$ for all values of D and p_0.) Solving eqn. (5) for $\sqrt{(1-D)}$ we obtain an equation which is graphically linear

$$\sqrt{(1-D)} = 1/p_0 + (1 - 1/p_0)\sqrt{R}. \tag{6}$$

This equation predicts that, as depression increases, the amplitude ratio of test e.p.p.s should also increase. Both D and R are experimentally measurable quantities; p_0 can be calculated from eqn. (4), solved for p_0: $p_0 = 1 - \sqrt{(1-D_0)}$. D_0 is estimated by the two-shock extrapolation method described earlier (Fig. 3).

The results from three experiments are shown in Fig. 8. D_0 values after a single stimulus were 0·28, 0·59, and 0·78; calculated values of p_0 were 0·15, 0·36, and 0·53, respectively. The straight lines in Fig. 8 were calculated according to eqn. (6) using these values of p_0. Amplitude ratios were measured without conditioning stimulation, and then 0·5–15 sec after one or more conditioning stimuli. Although there is considerable scatter among experimental points, they fall fairly well along the predicted line, except at low values of p_0. Similar results were found in seven other experiments. These results are clearly distinguishable from the prediction of the linear depletion model, which is simply that all points in Fig. 8 should fall on the ordinate ($R = 1{\cdot}0$).

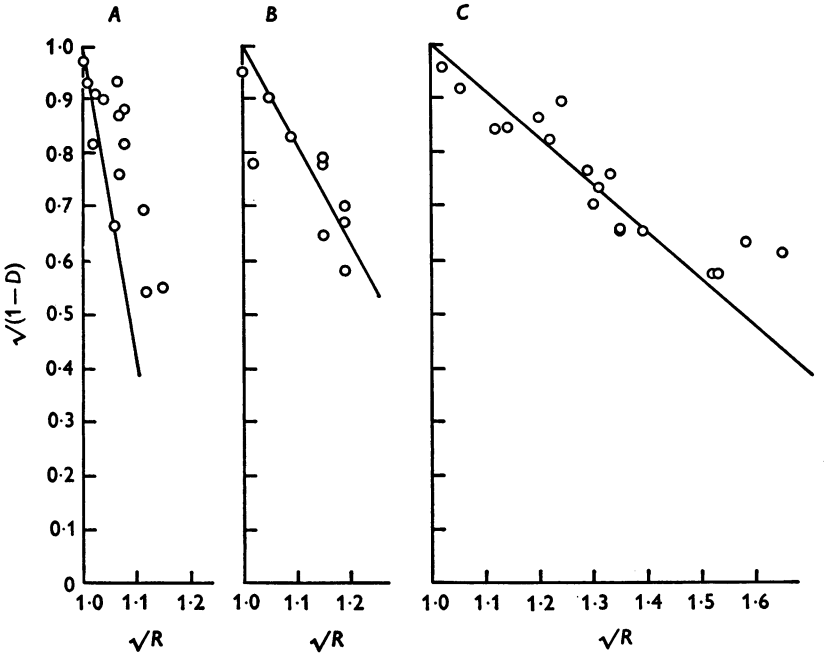

Fig. 8. Results from three experiments in which values of R and D were calculated following the experimental procedure outlined in Fig. 7, and plotted according to eqn. (6). In addition, D_0 was estimated by the two shock extrapolation procedure, and p_0 was then calculated from eqn. (4); the continuous lines were drawn according to eqn. (6), using the calculated values of p_0. Estimates of p_0 were: A, 0·15; B, 0·36; C, 0·53.

DISCUSSION

The experiments demonstrating depression in a short length of terminal and in the absence of propagated action potentials are consistent with the idea that depression is associated with the mechanisms of transmitter storage and release. However, they do not rule out the possibility that an alteration in impulse propagation plays a role as well. A reduction in the size of the action potential during depression would result in a reduction in p, and could account for the results of the present study. More conclusive evidence has been obtained at the squid giant synapse, where it is possible to record the presynaptic action potential with an intracellular electrode. Takeuchi & Takeuchi (1962) showed that the squid presynaptic action potential is unchanged during depression. Depression at the squid giant synapse occurs also when action potentials are abolished by tetrodotoxin and release is initiated by passing currents across the ganglion (Katz & Miledi, 1967*a*).

The lack of correlation between rate constant of recovery from depression and magnitude of D_0 in the present study agrees with similar observations by Thies (1965) on the mammalian neuromuscular junction. However, Takeuchi (1958) reported a small positive relationship between recovery time constant and D_0 in one experiment at the frog neuromuscular junction using extracellular (whole muscle) recording techniques. Small positive and negative correlations were observed on occasion in the present study (cf. Fig. 5, square symbols), but no consistent relationship was observed. When long trains of stimuli are given, recovery from depression is slowed markedly (Feng, 1941; del Castillo & Katz, 1954*b*). This may reflect a depletion of a 'reserve' pool of quanta from which the 'releasable' pool is filled, or an effect of the number of stimuli on the rate of recovery. Attempts to avoid such effects were made in the present study by waiting several minutes between trials and by keeping the number of conditioning stimuli low.

Deviation from a linear depletion model has been noted previously. Takeuchi (1958) found that e.p.p.s during low frequency repetitive stimulation were larger than predicted. He suggested that this deviation arose from a superimposed facilitation, since in a separate experiment in which release was reduced to low levels, he found that e.p.p.s increased in amplitude during low frequency stimulation. Since then, studies by several investigators (Mallart & Martin, 1967; Rahamimoff, 1968) have shown that the duration of facilitation is considerably shorter than the stimulus intervals Takeuchi used. These conflicting observations require further study; the main point is that depressed e.p.p. amplitudes during low frequency stimulation can be accounted for by the conclusion of the present study that p decreases during depression.

Experiments performed by B. N. Christensen and A. R. Martin (personal communication) further support the hypothesis that p decreases during depression. They calculated values of p by assuming a binomial distribution of e.p.p. quantum contents and measuring the coefficient of variation of e.p.p. amplitudes at two different levels of release (cf. Blackman, Ginsborg & Ray, 1963). Binomial p values were smaller than D_0 values calculated by the two shock extrapolation procedure, suggesting that D_0 over-estimates the value of p, and therefore that p decreases during depression.

An explanation not yet considered for the non-linear relationship between D_0 and release (Fig. 4)is that quantum size (i.e. the amount of transmitter per quantum) is reduced during depression. Del Castillo & Katz (1954*b*) found that the high frequency of occurrence of spontaneous miniature end-plate potentials (m.e.p.p.s) obscured individual m.e.p.p amplitudes following prolonged stimulation. But after 'moderate' stimu-

lation, when evoked e.p.p.s were less depressed, no apparent change was noted in m.e.p.p. amplitudes. Similar results were obtained in this laboratory following short trains of stimuli (unpublished observations). The change in quantal size necessary to explain the non-linearity in Fig. 4 would have been detectable easily in these experiments.

There are several possible physical interpretations of a reduction in release probability. A change in impulse propagation could reduce p, although this seems an unlikely explanation for reasons discussed earlier. It is not known whether the release process is described better by stochastic process (whereby the nerve membrane is equipotential in its releasing ability) or by a stoichiometric process (whereby specific 'release sites' in the membrane govern release), but in either case it is not difficult to imagine that an area of nerve membrane or a 'release site' from which a quantum of transmitter is released might be transiently refractory. Alternatively, if p is associated with the quanta themselves, and if different quanta have different release probabilities, then a conditioning stimulus would release those with the highest p values, leading to a depletion of n and a reduction of p. According to the 'vesicular' hypothesis, whereby the contents of each synaptic vesicle seen in electron micrographs correspond to a quantum of transmitter, recovery from depression might involve diffusion of vesicles towards the surface membrane (Takeuchi, 1958). Associated with such a recovery in the number of vesicles available for release might be a recovery of p for the individual quanta as they approach the surface membrane, with the consequence that n and p would have similar time courses of recovery. Finally, the mathematical statement $m = p_0 n^2/n_0$ suggests that a co-operative action between two quanta might be required in the process of transmitter release. In this model, the release probability remains constant during depression.

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