J. Physiol. (1974), **241**, *pp.* 91–110
With 5 text-figures
Printed in Great Britain

CHARACTERISTICS OF CRAYFISH NEUROMUSCULAR FACILITATION AND THEIR CALCIUM DEPENDENCE

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(*Received 15 November 1973*)

SUMMARY

1. A quantitative description of facilitation in the crayfish claw opener muscle is presented. The facilitation of a test response following one or more conditioning stimuli, and the growth of facilitation during a tetanus, are measured.

2. In superficial central fibres facilitation following one or more impulses can be described as the sum of two components which are both maximum at the end of the conditioning train and decline simultaneously and exponentially with different time constants thereafter.

3. During a tetanus, facilitation to successive stimuli grows more rapidly than is predicted by assuming that each impulse adds a constant facilitative effect to an accumulating total state of facilitation.

4. Sufficiently large values of tetanic facilitation are predicted by a model which assumes that transmitter release is proportional to the nth power of a substance or factor accumulating in nerve terminals. But no single value of n predicts the correct rise of facilitation in a tetanus and the time course of its subsequent decline from the facilitation following a single spike.

5. A model which assumes that the facilitative effects of successive spikes multiply in a tetanus predicts responses that are larger than those observed.

6. The effects of varying the calcium concentration ($[Ca^{2+}]$) on transmitter release and facilitation were studied. When a magnesium-EDTA buffering system is used to vary $[Ca^{2+}]$, transmitter release is found to be nearly linearly related to $[Ca^{2+}]$ in the range 0·1–13·5 mM.

7. The magnitude and time course of facilitation during and following a tetanus are unaffected by varying $[Ca^{2+}]$ between 1·0 and 40 mM.

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8. The relation between 'steady-state' facilitation and stimulus frequency is also unaffected by changing [Ca^{2+}], except that in high [Ca^{2+}] transmitter release appears to saturate at high frequencies (above 30 Hz).

9. The results are discussed in terms of the 'calcium accumulation' hypothesis of facilitation. The findings in crayfish appear to be qualitatively consistent with this hypothesis if certain modifications are made in the hypothesis.

INTRODUCTION

During repetitive motor neurone stimulation, the neuromuscular responses may grow during the tetanus until a steady state is reached in which each action potential releases on average a quantity of transmitter much greater than that released by the first impulse (del Castillo & Katz, 1954; Dudel & Kuffler, 1961). At crayfish neuromuscular junctions the nerve terminal action potentials remain relatively constant in size and shape during the tetanus (Zucker, 1974*a*). This fact, plus the growth of facilitation during a tetanus, suggest that there is accumulating in the nerve terminals some substance which enhances transmitter release by nerve spikes. Since calcium, or a calcium complex, is thought to enter the nerve terminal during the action potential and subsequently cause transmitter release (Katz & Miledi, 1967*a*, *c*), it is natural to suppose that calcium complex accumulates following one or more nerve spikes (Katz & Miledi, 1965, 1968; Rahamimoff, 1968; Miledi & Thies, 1971) and that this calcium is responsible for facilitation.

It has been shown in frog that the facilitation caused by a nerve spike is increased if calcium is present in the external medium during the spike (Katz & Miledi, 1968). Moreover, in rat external calcium prolongs post-tetanic potentiation – a long-lasting form of facilitation – only if it is present during the tetanus (Rosenthal, 1969; Weinreich, 1971). These results support the calcium accumulation hypothesis of facilitation.

In this study the growth of facilitation during a tetanus and its decay following the tetanus have been measured as well as the facilitation following a single stimulus. The data are compared to the predictions of several models of the accumulation of some activating substance. In addition, the calcium dependence of transmitter release and facilitation were studied with a view to testing the calcium hypothesis of facilitation at crayfish neuromuscular junctions.

METHODS

All experiments were performed on fibres on the dorsal surface of the claw opener muscle of the cheliped of *Procambarus clarkii*. The dissection, preparation chamber, electrodes, stimulation and recording apparatus, and methods for averaging responses have all been described (Zucker, 1973, 1974*a*).

The usual bathing medium was composed of (mM): NaCl 195, KCl 5·4, $CaCl_2$ 13·5,

$MgCl_2$ 2·5, and Tris maleate buffer 10, adjusted with NaOH 10 to pH 7·1. Most experiments were performed at 20° C.

In experiments with low calcium (< 3 mM) it was found necessary to buffer the Ca^{2+} in order to obtain consistent effects. A buffer system using magnesium and ethylenediamine tetra-acetic acid (EDTA) was devised. The various reactions of EDTA with Ca^{2+}, Mg^{2+}, and H^+ are equivalent to two competing reactions (Portzehl, Caldwell & Rüegg, 1964):

$$Ca^{2+} + EDTA^{2-} \rightleftharpoons CaEDTA \quad K_1 = 3 \times 10^7,$$

$$Mg^{2+} + EDTA^{2-} \rightleftharpoons MgEDTA \quad K_2 = 3 \times 10^5,$$

where K_1 and K_2 are dependent on pH. The values given are for pH = 7·1. Applying the mass action law to the above equations,

$$\frac{[CaEDTA]}{[MgEDTA]} = \frac{[Ca^{2+}]\ K_1}{[Mg^{2+}]\ K_2}.$$

So long as the total calcium ($[Ca^{2+}]+[CaEDTA]$) and the total magnesium exceed the total EDTA, very little EDTA will be free, and

$$[CaEDTA]+[MgEDTA] = \text{total EDTA}.$$

The above equations were solved, setting total EDTA = 10 mM, $[Mg^{2+}]$ = 2·5 mM, and $[Ca^{2+}]$ equal to the desired final free calcium concentration. Then the solution was prepared with a total magnesium concentration equal to $[Mg^{2+}]+[MgEDTA]$ and a total calcium concentration of $[Ca^{2+}]+[CaEDTA]$. Table 1 shows the composition of solutions used to obtain free $[Ca^{2+}]$ of 0·01, 0·1, 0·3 and 1·0 mM. Free $[Mg^{2+}]$ was always 2·5 mM. The remaining ionic constituents were the same as in normal solution. All cations were added as chloride salts. The final solutions were carefully titrated to pH = 7·1 with NaOH.

TABLE 1. Total concentrations of divalent ions in buffered solutions with different concentrations of free $[Ca^{2+}]$ ions

Solution	Free $[Ca^{2+}]$ (mM)	Total $[Ca^{2+}]$ (mM)	Total $[Mg^{2+}]$ (mM)	Total EDTA (mM)
1	0·01	2·87	9·63	10
2	0·10	8·10	4·50	10
3	0·30	9·53	3·27	10
4	1·00	10·76	2·74	10

When a solution was changed to one with a higher calcium concentration, the old solution was washed out and the claw superfused with 500 ml. of the new solution. Electrodes could be left in place throughout the procedure. Before changing to a solution with a lower calcium concentration, the claw was first rinsed in calcium-free medium plus 2 mM ethyleneglycol bis (β-amino-ethyl ether)-N,N′-tetra-acetic acid (EGTA). Low $[Ca^{2+}]$ often led to spontaneous firing in the motor neurone, which lasted about 5–30 min. No data were collected until this activity subsided.

Facilitation was measured for the average responses to paired stimuli, or during a tetanus, or to a test stimulus following a tetanus. Facilitation was computed as one less than the excitatory junctional potential (e.j.p.) amplitude divided by the unfacilitated e.j.p. amplitude (Mallart & Martin, 1967; Zucker, 1974*a*). The response to the first stimulus of the pair or the tetanus was designated the unfacilitated response. The interval between pairs or tetani was usually 10 sec. This was the

minimum interval necessary such that the first response was, as nearly as practicable, truly unfacilitated. In each experiment this was tested by varying the interval between tetani and choosing the minimum interval such that the first response was at most 20 % larger than single stimuli delivered once per 20 sec.

Some problems arose in a few experiments where this unfacilitated response changed during the experiment (in the same medium). The 'unfacilitated response' could either wax or wane during the period of 1 to several hours. Since these changes bore no obvious relationship to the stimulus regimen, it appears unlikely that the state of facilitation was changing, but rather that some other pre- or post-synaptic factor governing e.j.p. amplitude changed. When this occurred facilitation was always calculated relative to the unfacilitated response of the same response series.

Standard errors of estimates of facilitation (F) were calculated from the formula (derived similarly to Rahamimoff, 1967):

$$\text{S.E.}_F = F\sqrt{1{\cdot}1a\left(\frac{1}{N_F R_F}+\frac{1}{N_0 R_0}\right)},$$

where a is the amplitude of spontaneous miniature e.j.p.s and N_F, N_0, R_F, and R_0 are the number and average amplitude of facilitated and unfacilitated e.j.p.s. R_F was the average of 32, 64 or 128 (N_F) responses, while usually $N_0 = 256$ unfacilitated responses were averaged to obtain R_0. The above formula assumes in its derivation that transmitter release follows Poisson statistics, and thus tends to overestimate the standard errors slightly when F is large (Zucker, 1973).

Whenever an e.j.p. amplitude exceeded 2 mV, it was corrected for the non-linear relation between post-synaptic conductance changes and e.j.p. amplitude (Martin, 1955): $R' = R/(1-R/C)$, where R is the observed e.j.p. amplitude, R' is the corrected e.j.p. amplitude, and C is the difference between the membrane potential at the beginning of the e.j.p. and the equilibrium potential for the e.j.p. The latter was taken as 0 mV (Taraskevich, 1971). The membrane potential at the time of an e.j.p. was not necessarily the resting potential, since the membrane may still be depolarized by a previous e.j.p. Since the single quantum (spontaneous) e.j.p. is very small in this muscle (Bittner & Kennedy, 1970), this correction could be applied directly to the averaged e.j.p. amplitude (see Hubbard, Llanás & Quastel, 1969, p. 135).

Computations of the predictions of several models for facilitation were performed automatically on a Wang 700 C desk computer.

The individual fibres of the crayfish claw opener display varying degrees of facilitation. Superficial fibres near the centre of the muscle exhibit the largest facilitation (Bittner, 1968) and were used in all experiments, with a few exceptions indicated in the text.

RESULTS

Characteristics of facilitation

When paired stimuli are applied several times to the exciter axon and the responses averaged, the second excitatory junctional potential (e.j.p.) is always larger than the first. This facilitation exhibited by the second response is at a maximum for the shortest separation between the stimuli. As the separation is increased the second response declines gradually until it returns to the same level as the first. There is never seen any period of depression following one stimulus. Fig. 1 presents an example of the time course of facilitation following a single stimulus.

If the motor neurone is stimulated repetitively the averaged responses to successive stimuli grow until a steady state is reached. Immediately following a tetanus, the nerve terminals are in a highly facilitated state,

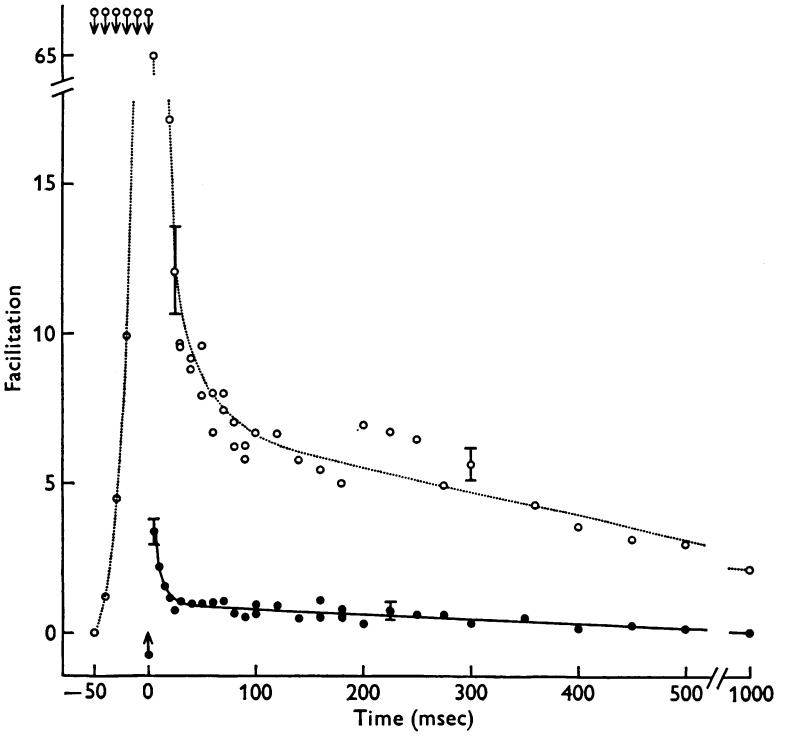

Fig. 1. Time course of facilitation of averaged e.j.p.s in one muscle fibre. The filled circles show the facilitation exhibited by the second response to paired stimulation as a function of the separation between stimuli. The continuous line is drawn through the points by eye. Open circles indicate facilitation to successive stimuli of a 6 spike, 100 Hz tetanus, and the facilitation exhibited by responses to test stimuli at various intervals following the tetanus. The dotted line was fitted to these points by eye. The responses to the 5th and 6th tetanic stimuli and to several test responses following the tetanus at brief intervals had facilitation values between 8 and 64, and do not appear on the graph. In this and other Figures, S.E. for a few points are shown to indicate the accuracy of the estimates of the averaged responses.

such that test stimuli evoke e.j.p.s much larger than the unfacilitated response at the beginning of the tetanus. As with single conditioning stimuli, the average amplitude of e.j.p.s following a tetanus gradually declines as the interval between the tetanus and the test stimulus is

lengthened, until single responses have the same amplitude as before the tetanus. A period of depression is never observed in this muscle. This behaviour of facilitation during and after a tetanus is also illustrated in Fig. 1 for a fibre which exhibited an unusually large facilitation.

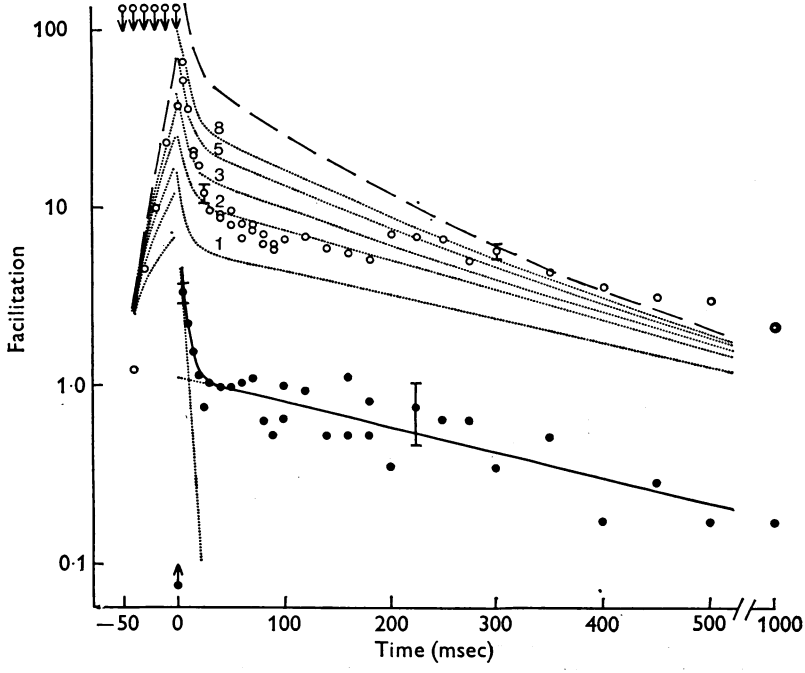

Fig. 2. The same data as in Fig. 1, replotted as $\log_{10}$ facilitation *vs.* time. The two straight dotted lines are exponentials fitted to the test responses following one impulse (filled circles) as described in the text. The continuous line is the sum of these two exponentials. The dotted lines in the upper part of the figure are the 'power law' model predictions for the facilitation expected during and following the tetanus. The numerals represent n, the assumed power of the relationship between transmitter release and an accumulating substance. The lowest line ($n = 1$) is the prediction of the 'linear summation' model. The dashed line is the prediction of the 'multiplicative' model for the accumulation of facilitation.

When facilitation is plotted on linear co-ordinates as in Fig. 1, it is frequently difficult to plot the facilitation induced by a single spike and by a tetanus on the same scale, since the magnitude of the latter is so much greater than the former. This difficulty is alleviated by plotting the logarithm of facilitation *vs.* time during or following a conditioning tetanus or single stimulus. In Fig. 2 the same data as in Fig. 1 are plotted in this fashion.

The curve describing the logarithm of facilitation as a function of time following a single impulse suggests that the time course of facilitation can be described as the sum of two components, each starting at the moment of the conditioning spike and declining thereafter exponentially. These two hypothetical components can be extracted from the data in the following way. The points indicating the responses following a single impulse by more than 30 msec appear to lie on a straight line. A least-squares line (dotted line in Fig. 2) was fitted to these points, and the ordinates of this line at early intervals were subtracted from the points that deviate from the line. A second dotted line was fitted to these points representing the difference between the first dotted line and the data points with larger facilitation values. Each of the dotted lines is an exponential with different peak magnitude at the moment of the conditioning impulse and different time constant of decay rate. The continuous line is the sum of these two components, and it provides a very satisfactory description of the time course of facilitation.

Data from five fibres were analysed in this fashion, and it was found that the time course of facilitation following one spike could generally be described adequately as the sum of two exponentials,

$$F(t) = f_1 e^{-t/\tau_1} + f_2 e^{-t/\tau_2}.$$

The average values of the parameters f_1, f_2, τ_1, and τ_2 are given in Table 2. There was a good deal of variation in the facilitation parameters of the various fibres. In one fibre, facilitation following one spike (and also a tetanus) could be adequately described by a single exponential, whose time constant was 165 msec.

TABLE 2. Parameters of facilitation in crayfish claw opener superficial central muscle fibres. f_1 and f_2 are the 'zero-time' peaks of facilitation of the first and second components, whose decay time constants are τ_1 and τ_2. The 'steady-state' facilitation (F) reached after 10 sec of stimulation at 5 and 20 Hz is also shown. Figures give mean ± S.D. of the number of fibres shown in parentheses

f_1	τ_1 (msec)	f_2	τ_2 (msec)	F at 5 Hz	F at 20 Hz
3·32 ± 0·78 (4)	19·6 ± 10·2 (4)	1·78 ± 0·80 (5)	412 ± 219 (5)	4·79 ± 2·02 (5)	24·9 ± 30·0 (5)

In a few experiments facilitation was studied in superficial fibres near the distal or proximal ends of the muscle. These fibres have larger unfacilitated responses and facilitate less, and at lower frequencies, than the central fibres (Bittner, 1968). The time course of the facilitation following one impulse or a brief tetanus could not be described as the sum of two exponentials. Rather, the time course featured an early decay, followed by a flat region or even a secondary increase in facilitation, and then a final slow decay. This behaviour is much more difficult to analyse and model than

that of central fibres. Furthermore, reduced external calcium concentrations sometimes enhanced facilitation in these fibres, and changed its time course. Facilitation may well be confounded by depression in these fibres. Since the central fibres were relatively free of these complications, and displayed much larger values of facilitation, they were used exclusively in all further experiments.

Another way of measuring facilitation is to stimulate the motor neurone with a long tetanus and measure the 'steady-state' response attained after several seconds of stimulation. Then facilitation may be plotted as a function of frequency. An example of such a measure of facilitation is given in Fig. 5. Table 2 includes the average level of facilitation reached in five fibres at stimulus frequencies of 5 and 20 Hz.

TABLE 3. Comparison of facilitation observed in one fibre to the prediction of several models. The first row gives the facilitation exhibited by each spike in a tetanus (50 Hz, 6 spikes) and following the tetanus at intervals of 10, 40 and 150 msec, and to 10 sec of repetitive stimulation at 5 and 20 Hz. The remaining figures are the predicted values of facilitation, assuming various models of the process

	Facilitation									
	During tetanus (Response no.)					Following tetanus (msec)			To a steady frequency (Hz)	
	2	3	4	5	6	10	40	150	5	20
Observed	2·00	3·00	7·81	16·6	18·2	26·3	21·5	13·4	4·52	18·71
Linear summation	1·62	2·88	4·06	5·20	6·30	8·14	6·79	5·64	3·63	16·33
Power law $n = 3$	1·62	3·84	6·86	10·8	15·7	25·02	19·12	14·48	7·60	179
Power law $n = 4$	1·62	4·04	7·63	12·67	19·46	33·15	24·56	17·99	8·89	381
Multiplicative	1·62	4·93	11·9	26·6	57·0	159	92·3	53·5	19·0	$4{\cdot}2 \times 10^5$

It is of interest to see whether the facilitation observed during and following a tetanus can be predicted from the facilitation evoked by a single impulse. It has been shown in frog that the effects of individual stimuli during a tetanus summate linearly (Mallart & Martin, 1967). Thus tetanic facilitation can be predicted accurately by adding the facilitative effects of each impulse. Each impulse elicits an increase in facilitation equal to the same percentage of an unfacilitated response as one impulse alone, and the effect of each impulse declines with the same time course.

The predictions of the 'linear summation' model of facilitation are shown for one experiment as the lowest dotted line in the upper part of Fig. 2. Clearly, the facilitation observed in a tetanus, and following it, is much larger than this prediction (note the logarithmic ordinate). Similar results were obtained in the other four fibres studied. This discrepancy was present for facilitation evoked by tetani of different parameters (5 or 6 spikes at frequencies of 20–100 Hz). A comparison of the facilitation

observed in a different fibre from that shown in Figs. 1 and 2, and the predictions of the 'linear summation' model, are given in Table 3.

Another model for the tetanic summation of facilitation supposes that transmitter release is proportional to the nth power of the concentration of a substance which accumulates during a tetanus (Katz & Miledi, 1968; Rahamimoff, 1968; Miledi & Thies, 1971). Each spike contributes a constant amount to the pool of this substance (perhaps calcium ions or a calcium complex), and each increment to the pool decays with the same time course as that from a single impulse (Rahamimoff, 1968). Then the time course of decay of each increment from the pool is (Linder, 1973)

$$B(t) = A[(1+F(t))^{1/n}-1], \tag{1}$$

where A is the amount of the substance entering the nerve terminal during each spike. The facilitation at time t following a tetanus of N spikes at frequency $1/\Delta t$ is then given by (Linder, 1973)

$$F_N(t) = \left\{\sum_{i=1}^{N}[(F([N-i]\,\Delta t+t)+1)^{1/n}-1]+1\right\}^{n}-1. \tag{2}$$

Facilitation for the jth spike in the tetanus is predicted from the same equation by setting $t = \Delta t$ and $N = j-1$.

The predictions of this 'power law' model for various powers (values of n) are plotted in the upper part of Fig. 2. For $n = 1$, this model reduces to the 'linear summation' model. Although the predictions of the 'power law' model for values of $n > 1$ are closer to the observed facilitation, they are still not satisfactory. The best fit to the data illustrated in Fig. 2 for facilitation following a tetanus is obtained with $n = 2$ or 3. However, the shape of the decay curve is not correct. The predicted values of F are too small at short (< 50 msec) and long (> 300 msec) intervals following the tetanus, and too large in between. Thus a larger value of n is needed to predict facilitation at short than at intermediate latencies. Similarly, a larger value of n ($n = 8$) provides the best fit to the rise of facilitation during a tetanus than the value of n (2 or 3) which provides the best fit to the decay. Furthermore, a value of $n = 13$ was needed to predict the steady-state responses in this fibre to 20 Hz continuous stimulation. It is remarkable that in all fibres the 'linear summation' model provides the best description of the time course of decay following a tetanus, but all the values are too small.

Similar difficulties were encountered in attempting to fit this model to the data from each of the five fibres studied in detail. As is evident from Table 3, in this fibre the best fit for the decay of facilitation after a tetanus requires $n = 3$, while during the tetanus $n = 4$ provides the best fit. The steady-state facilitation, on the other hand, was better predicted by the 'linear summation' model ($n = 1$).

Yet another model of facilitation has recently been proposed by Cook & Quastel (1973) and by Linder (1973). They suggested that each spike independently conditions the presynaptic terminals by *multiplying* release to succeeding impulses by a factor. The multiplication or 'activation' caused by each impulse reaches a maximum immediately after the spike and declines gradually to unity. The magnitude of this factor at time t following one spike is then $1+F(t)$, and the facilitation at time t following a tetanus of N spikes at frequency $1/\Delta t$ is (Linder, 1973)

$$F_N(t) = \prod_{i=1}^{N} [F([N-i]\Delta t+t)+1]-1. \quad (3)$$

As with equation (2), this formula can be used to predict the growth of facilitation during a tetanus and its subsequent decay. This prediction is shown as the dashed line in Fig. 2 for one fibre, and as the last row in Table 3 for another. In these two fibres, as well as the remaining three, the predictions of this model were much too large.

The calcium dependence of transmission and facilitation

As a prelude to studying the effects of varying the external calcium concentration on facilitation, the calcium dependence of unfacilitated transmission was explored. The results of such experiments had already been reported (Bracho & Orkand, 1970; Ortiz & Bracho, 1972), so the outcome was anticipated. At the outset, however, a difficulty was encountered which was unexpected. When the normal bathing medium was exchanged for one which was nominally calcium-free, the e.j.p. amplitude in surface fibres often dropped by only 50 %. After vigorous and prolonged washing of the muscle (which often dislodged electrodes), the e.j.p. amplitude was reduced to zero, but it gradually recovered to about 20 % full size. Responses could be abolished by adding 1 mM-EGTA, however, and transmission remained blocked until calcium was added to the medium.

These results suggest that there exists some diffusion barrier between the extracellular space of the neuromuscular junctions and the bathing medium, and that calcium can leak into this extracellular space. Experiments with γ-methyl glutamate (Zucker, 1974*a*), a post-synaptic antagonist of glutamate receptors (Lowagie & Gerschenfeld, 1973), and on nerve terminal excitability (Zucker, 1974*b*) lead to a similar conclusion regarding the extracellular space around nerve terminals. Apparently, when a calcium-chelating agent is used to buffer external $[Ca^{2+}]$, the calcium concentration in this restricted region can be more readily controlled. Hence all further experiments with altered calcium concentrations were conducted using the magnesium-EDTA calcium buffering system described

in the Methods section. In all experiments the magnesium concentration was set to 2·5 mM.

Fig. 3*A* shows the calcium dependence of nearly unfacilitated (1 Hz) and facilitated (10 Hz) transmission in two muscle fibres. Each line is a least-squares fit to the points. There is a great deal of scatter, and the points do not fall neatly on a smooth curve. This is due to the long periods required to collect data for unfacilitated responses in low $[Ca^{2+}]$. Since the miniature e.j.p. amplitude in these fibres was roughly 100 μV, the responses in 0·1 mM-Ca^{2+} had an average quantal content (m) of about 0·1. Since, approximately $\text{S.E.}_m = \sqrt{m/N}$ (Martin, 1966), $N = 100$ responses must be averaged just to obtain a standard error that is one third the response. Thus these experiments with unfacilitated responses in low $[Ca^{2+}]$ lasted a long time, and deterioration of the preparation was inevitable. This is clear from Fig. 3*A*, in which the order of solutions used was $[Ca^{2+}]$ = 13·5 mM (normal), 0·01 mM, 0·1 mM, 1·0 mM, 6 mM, and finally 13·5 mM again. Non-zero responses in 0·01 mM-Ca^{2+} were so infrequent that they were indistinguishable from spontaneous e.j.p.s. These results were therefore discarded. The arrows demarcate the averaged responses for the first exposure to 13·5 mM-Ca^{2+}. These are significantly larger than the same responses at this concentration obtained at the end of the experiment, and used for fitting the lines.

Fig. 3*A* also shows that the slope of the regression line relating the logarithm of e.j.p. amplitude to $\log_{10}[Ca^{2+}]$ was the same for 1 and 10 Hz responses in both fibres. Thus the calcium dependence of transmitter release may be safely estimated from 10 Hz responses, which are larger, and so require fewer measurements, and take less time. These preparations are more stable and yield smooth curves like those in Fig. 3*B* for the relationship between e.j.p. amplitude and external calcium concentration. In ten experiments, the average slope of the best-fitting regression line was $0{\cdot}76 \pm 0{\cdot}16$ (mean $\pm$ S.D.), and the slope was usually higher (about 1·0) at the lowest calcium concentrations. The results are fully comparable to those of Bracho & Orkand (1970). These authors and Ortiz & Bracho (1972) also showed that when the post-synaptic effects of calcium are taken into account, the relationship between transmitter release and external calcium concentration is more nearly linear. The present work extends their findings to lower calcium concentrations and unfacilitated e.j.p.s.

Having thus developed and tested a reliable method for controlling the external calcium concentration, the effect of changes in $[Ca^{2+}]$ on facilitation was explored. The facilitation during and following a tetanus in two fibres is shown in Fig. 4. Facilitation was measured with respect to the unfacilitated (first tetanic) response in each calcium concentration (1 and

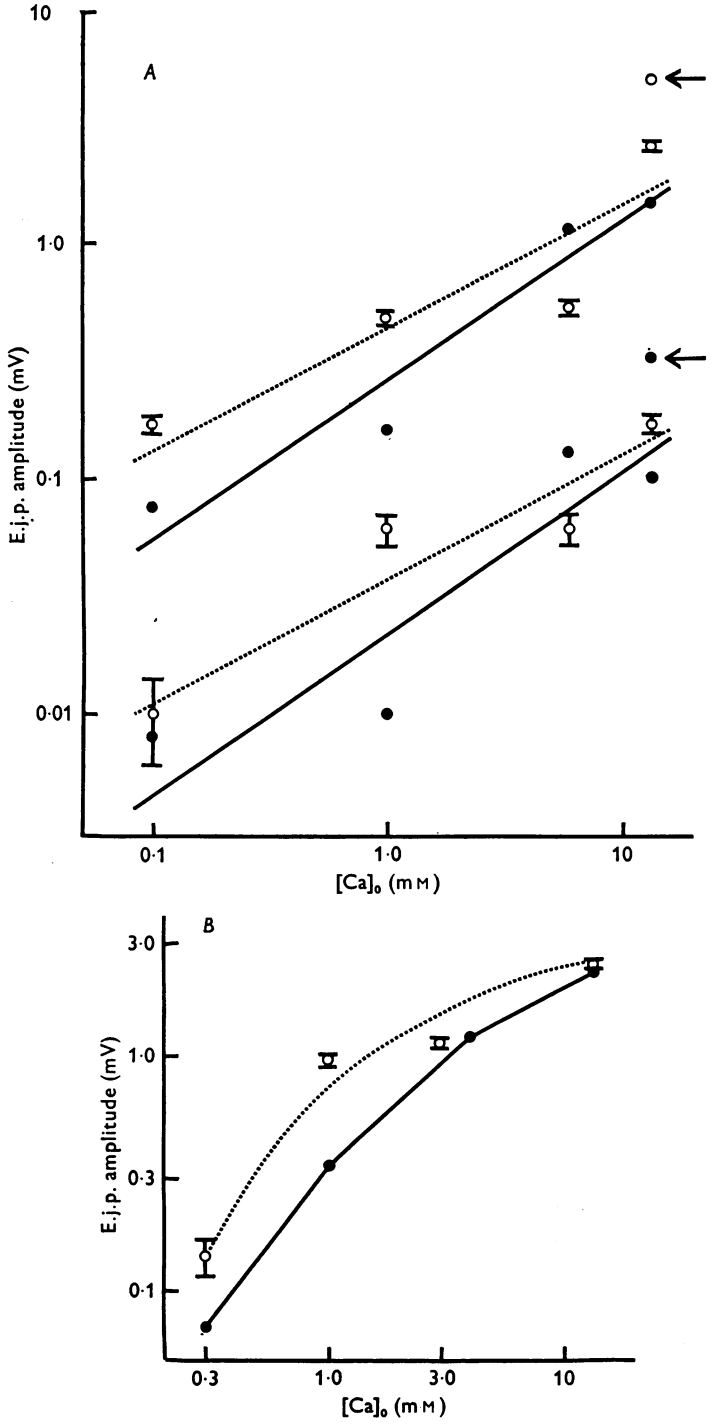

Fig. 3*A* and *B*. For legend see facing page.

40 mM in the upper graph, 13·5 and 40 mM in the lower graph). Evidently, changes in calcium concentration over a wide range have no significant effects on the time course or magnitude of facilitation. Similar results have been obtained with two other fibres.

This lack of dependence of facilitation on external calcium concentration was already suggested by Fig. 3*A*, in which the effects of calcium on 1 and 10 Hz responses were similar. Another measure of facilitation, shown in Fig. 5*A*, is the relation between 'steady-state' facilitation achieved after several seconds' stimulation at various frequencies. When the external calcium concentration was increased from 1·0 to 13·5 mM, this relation remained virtually unchanged for frequencies up to 30 Hz. At higher frequencies, however, facilitation reached a 'plateau' in normal calcium, which was not observed in the presence of low calcium. When the results are replotted in terms of the actual e.j.p. amplitude (Fig. 5*B*), it appears that transmission reaches about the same maximum level at the highest frequency (140 Hz) in both calcium concentrations. Similar results were obtained in two other experiments.

Two explanations can readily account for these results. First, it might be that at very high frequencies in normal calcium a depression sets in which partially masks the true level of facilitation. If depression is caused primarily by a depletion of transmitter available for release (Liley & North, 1953; Betz, 1970), then this depression would be alleviated by reducing transmitter release with low external [Ca^{2+}]. Furthermore, such a depression ought to appear as a progressive reduction in e.j.p. amplitude during a tetanus following the early phase of facilitation. On the contrary, successive responses continued to increase gradually throughout long tetani.

This continued increase in facilitation may be an example of the long-term facilitation described in crayfish neuromuscular junctions by Sherman & Atwood (1971). The contribution of each stimulus in the tetanus to such a component of facilitation must be vanishingly small and very prolonged, and would not appear in the present measurements of facilitation following one or a few spikes. Such a late progressive phase of facilitation may be one reason for the failure of all models of the accumulation of facilitation to account for 'steady-state' facilitation.

An alternative explanation for the plateau in Fig. 5 is that transmission

Fig. 3. Relation between average e.j.p. amplitude and external calcium concentration. *A* shows data from two fibres (filled circles, fibre 1; open circles, fibre 2). The lower points are responses to 1 Hz stimulation, the upper points to 10 Hz. Solutions were changed to test the effects of external calcium concentration in the following order (mM-[Ca^{2+}]): 13·5, 0·1, 1·0, 6·0, 13·5. The continuous lines are regression lines for fibre 1, the dotted lines for fibre 2. The first measurement in 13·5 mM-[Ca^{2+}] (arrows) was omitted in fitting the regression lines. *B* gives the average e.j.p. amplitudes in two different fibres, stimulated at 10 Hz, as a function of calcium concentration. The lines were fitted to the data by eye.

may be 'saturated' at high frequencies in normal calcium, because each spike releases all the immediately available transmitter. A similar interpretation (Zucker, 1974*a*) has been suggested to explain the differential effects of caesium ion on crab synapses with different facilitative properties (Atwood & Lang, 1973). Furthermore, if transmitter release is governed by a binomial process with low n (Johnson & Wernig, 1971) and if both facilitation and high $[Ca^{2+}]$ increase transmitter release by increasing p (Wernig, 1972*a*, *b*; Zucker, 1973), then transmitter release should be limited at high frequencies in high external $[Ca^{2+}]$ by a maximum value of $p = 1$.

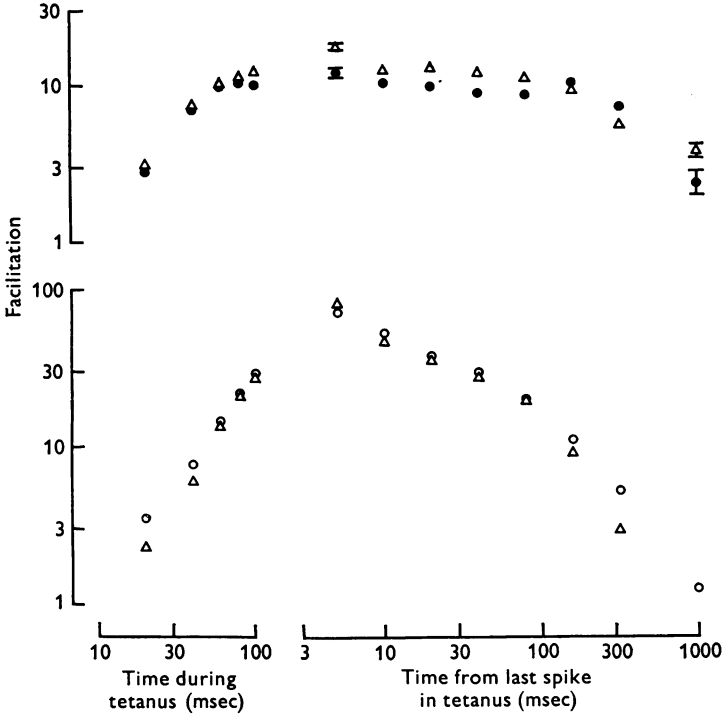

Fig. 4. Facilitation during and following a tetanus (6 spikes, 50 Hz) at different external calcium concentrations. The upper and lower graphs are from two different fibres. Calcium concentrations were 1 mM (filled circles), 13·5 mM (open circles), and 40 mM (triangles).

In conclusion, then, facilitation evoked by both long and short tetani is essentially independent of the level of external calcium. Similar results have been reported in rat (Hubbard, Jones & Landau, 1971) and crab (Linder, 1973), but not frog (Rahamimoff, 1968).

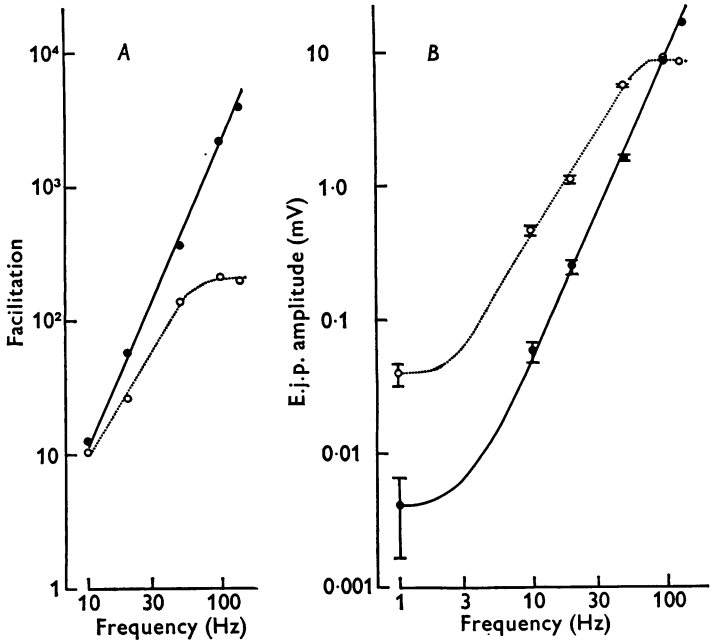

Fig. 5. Relation between facilitation (A) or e.j.p. amplitude (B) and stimulus frequency in one fibre. The external calcium concentration was 1 mM for the filled circles and 13·5 mM for the open circles. Straight parts of lines are regression lines for the data lying on them; the curved parts of the lines are fitted by eye.

DISCUSSION

A current hypothesis for facilitation states that facilitation arises from an accumulation of calcium or a calcium complex, called 'active calcium', Ca*, inside nerve terminals (Katz & Miledi, 1965, 1968). In its current form the hypothesis states that each spike in a tetanus admits a constant increment of active calcium (A) proportional to the external calcium concentration, that spike-evoked transmitter release (R) is proportional to some power (greater than one) of the total amount of active calcium present just following the spike, and that active calcium is removed from its pool by non-linear high-order rate kinetics. Facilitation occurs because the total level of active calcium reached following a spike is the sum of that entering during the spike (A) and the 'residual' active calcium (B) left in the pool. Thus a facilitated response (R') occurring in the presence of residual calcium (B) is larger than an unfacilitated response (R) according to

$$1+F=\frac{R'}{R}=\frac{(A+B)^n}{A^n},$$

where F (the facilitation shown by R'), R' and B are functions of time. The power nature of this relation also accommodates the non-linear relation between unfacilitated release (R) and external calcium concentration (Dodge & Rahamimoff, 1967), since A is proportional to $[Ca^{2+}]$. In its most comprehensive form (Miledi & Thies, 1971), the hypothesis supposes that the residual active calcium following a spike or tetanus is also responsible for the increase in miniature end-plate potential (m.e.p.p.) frequency. If removal of Ca* follows high-order rate kinetics, then B is relatively less sensitive to changes in $[Ca^{2+}]$ than A, and the model predicts an observed (Rahamimoff, 1968) reduction of facilitation in high external calcium, and a low calcium sensitivity of the post-tetanic increase in m.e.p.p. frequency (Miledi & Thies, 1971). However, the model fails to predict quantitatively the relation between increase in m.e.p.p. frequency and facilitation (Barrett & Stevens, 1972; Cooke & Quastel, 1973) and the linear summation of facilitation in frog (Mallart & Martin, 1967; Magleby, 1973).

In crayfish, this model runs into more serious difficulties. The linear relation between transmitter release and $[Ca^{2+}]$ sets $n = 1$, and under the calcium accumulation model, the maximum possible facilitation following N spikes is N. This prediction is very clearly contradicted by the results (Figs. 1 and 2, Tables 2 and 3). Beyond this, there is no single value of n which predicts correctly the time course and magnitude of facilitation during and following a tetanus from that following a single spike. A possible reason for this failure is that the time course for removal of active calcium was assumed to follow linear rate kinetics in the 'power law' models considered in the Results section. However, if non-linear rate kinetics are involved, facilitation following a tetanus should resemble that following one impulse shifted to the right, with a more rapid initial decay, even for $n = 1$. This was not observed.

The calcium-independence of facilitation in crayfish also contradicts the above model, but only because $B(t)$ is thought to be insensitive to changes in external calcium. This provision, which follows from non-linear rate kinetics for the removal of active calcium, was inserted in the model to account for the rapid decline of transmitter release following a spike (Katz & Miledi, 1968) and the calcium-insensitivity of the post-tetanic potentiation of m.e.p.p. frequency (Miledi & Thies, 1971). No quantitative data are available for crayfish on the increase in miniature e.j.p. frequency during the brief period of intense facilitation following a short tetanus. Such data are difficult to collect in this preparation, where miniature e.j.p. amplitude and frequency are low. A simple modification of the model would be to suppose that each element of active calcium is removed by linear kinetics formally described as a sum of exponentials (Rahamimoff,

1968). Then the amplitude of $B(t)$ at any time (t) following a spike or tetanus would be proportional to A entering during each spike, and facilitation would be calcium-independent because the calcium-dependence of A and B would be identical.

Rahamimoff (1968) proposed a slightly different model for facilitation in which accumulated active calcium caused increasing *fractions* of unactivated transmitter release sites to be activated. This model behaves identically to that of Katz & Miledi (1968) and Miledi & Thies (1971), except at high [Ca^{2+}] and high levels of facilitation, where transmitter release saturates. This modification is consistent with the observations of Fig. 5 and the binomial nature of transmitter release in crayfish (Johnson & Wernig, 1971), which also predicts a saturation of transmission in high [Ca^{2+}] (Wernig, 1972*b*) during facilitation (Wernig, 1972*a*; Zucker, 1973). However, the above objections to the Katz & Miledi (1968) model apply equally to that of Rahamimoff (1968).

If these models fail to account for the characteristics of facilitation in crayfish, how can we explain the phenomenon? Recent experiments with *Aplysia* somata (Stinnakre & Tauc, 1973) suggest one possibility. These authors found that during successive spikes in a tetanus, each spike was apparently accompanied by a greater calcium entry into the cell than previous spikes. Perhaps a similar mechanism at motor neurone terminals is responsible for facilitation. Successive spikes in a tetanus may admit larger amounts of calcium or a calcium complex, and it is this *additional calcium entry* to each spike which evokes additional transmitter release and leads to facilitation.

How does this increased calcium entry to successive spikes come about? One possibility is that calcium does accumulate during a tetanus, and this internal calcium sensitizes the membrane calcium channels to respond to an impulse by admitting additional calcium. Thus facilitation may well be due to calcium accumulating in some pool, but instead of the accumulated calcium adding to a constant amount of calcium admitted by each spike, it enhances the calcium admitted by successive spikes. Now facilitation does not require a highly non-linear relationship between active internal calcium and transmitter release. This circumvents the remaining difficulty in applying a calcium accumulation model to crayfish. Moreover, increased external [Ca^{2+}] would multiply all responses by a constant factor, and leave facilitation unaffected. If accumulating calcium activates increasing fractions of membrane calcium channels or carriers, synaptic transmission would also display a saturation.

The detailed behaviour of such a model depends on the form of the relationship between accumulated calcium and enhanced calcium entry during a spike, and on the dependence of the kinetics of removing accumulated

calcium on the amount of calcium in the pool. With so many variables to set, it seems premature to attempt a detailed exploration of such a system without further experimental support for the general mechanism.

The modified calcium accumulation hypothesis proposed here still requires calcium in the external medium for a presynaptic spike to have a facilitative effect. This requirement should be testable by the method developed in the frog by Katz & Miledi (1965, 1967*b*, 1968), in which a calcium pipette is used to rapidly change the calcium concentration at a synapse between conditioning and test stimuli. Unfortunately, this technique fails to restore transmission locally in calcium-free medium in crayfish, because transmitter release requires much more calcium than in the frog; more, apparently, than a calcium pipette can provide. Even this experiment would not prove that calcium accumulation or enhanced calcium entry is responsible for facilitation, since the mechanism might still reside in some step in the release process either subsequent to calcium entry or otherwise dependent on external calcium. A critical test of this hypothesis requires, therefore, the development of new techniques, or the discovery of a more favourable preparation.

I wish to thank Professors B. Katz and R. Miledi for providing the opportunity to work in their laboratory, and for their many helpful discussions and suggestions. The author was supported by the Helen Hay Whitney Foundation.

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