*J. Physiol.* (1974), **241**, *pp.* 111–126
*With 4 text-figures*
*Printed in Great Britain*



# EXCITABILITY CHANGES IN CRAYFISH MOTOR NEURONE TERMINALS

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(*Received 15 November 1973*)

SUMMARY

1. Changes in the post-activation excitability of crayfish motor nerve terminals were used to measure afterpotentials that might be related to facilitation of transmitter release.

2. The refractory period is followed by a period of supernormal excitability in which the threshold of nerve terminals drops to about 70 % of its pre-activation level at about 15 msec following an impulse. The threshold returns exponentially to its pre-activation level with a time constant of about 25 msec at 13° C. Such a supernormal excitability is rarely seen in pre-terminal nerve branches or in the main axon.

3. Following a brief high-frequency tetanus the peak of the supernormal excitability is greater than that following a single impulse. At low temperature this peak is reduced and delayed, and the decay rate of the supernormal excitability is prolonged with a $Q_{10}$ of about 2·5.

4. Depolarization of nerve terminals decreases, and hyperpolarization increases, the magnitude of the post-activation supernormal excitability.

5. The magnitude of the supernormal excitability depends on the external potassium concentration, but not on sodium. In low calcium the peak supernormal excitability is often reduced. High calcium concentration and manganese ions have no effect, but cobalt abolishes the supernormal excitability, and its effects are only slightly reversible. Both cobalt and manganese reversibly block neuromuscular transmission.

6. Strophanthidin has no effect on the post-activation supernormal excitability, but proteolytic enzymes reduce or abolish it, and hyperosmotic solutions also affect it.

7. It is suggested that the action potential is followed by a depolarizing afterpotential in nerve terminals which is caused by a transient increase in the potassium concentration around the terminals. There is no evidence

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that afterpotentials in nerve terminals are related to facilitation in any way.

## INTRODUCTION

One mechanism that has been proposed to account for facilitation and post-tetanic potentiation of neuromuscular transmission is that nerve impulses may be followed by afterpotentials which increase the amount of transmitter released by a later nerve impulse (Lloyd, 1949; Curtis & Eccles, 1960; Hubbard & Willis, 1962; Gage & Hubbard, 1966; Hubbard, 1970; Dudel, 1971; Lang & Atwood, 1973). It has been shown that hyperpolarization of the presynaptic terminals enhances the amount of transmitter released by action potentials (del Castillo & Katz, 1954; Hubbard & Willis, 1962; Takeuchi & Takeuchi, 1962; Dudel, 1971), perhaps by increasing the size of the action potential, by 'mobilizing' transmitter into a more readily releasable store, or by otherwise sensitizing the phasic release mechanism. Thus hyperpolarizing afterpotentials could be responsible for facilitation.

One way to test for the presence of afterpotentials in a nerve terminal is to compare the threshold for spike initiation from an external electrode before and after a conditioning orthodromic stimulus (Graham, 1934; Wall, 1958). It has recently been found possible to excite motor neurone terminals in the opener muscle of the crayfish claw (Zucker, 1974*a*). The measurement of terminal excitability changes following orthodromic invasion is used here to test for the presence of afterpotentials. Evidence is presented for a depolarizing afterpotential which is caused by a transient increase in the local potassium concentration around nerve terminals. It is unlikely that facilitation is related in any way to these afterpotentials.

## METHODS

The preparation of the claw opener muscle of *Procambarus clarkii* has been described (Zucker, 1973, 1974*a*, *b*). Presynaptic terminals were located on the dorsal surface of the muscle by recording with a micro-electrode a nerve terminal potential followed by an extracellularly recorded junctional potential in response to orthodromic stimulation of the exciter axon. Most terminals could be excited via the micro-electrode: antidromic axon spikes were recorded from a suction electrode on the axon. An intracellular micro-electrode also recorded an excitatory junctional potential from a muscle fibre. Control experiments to show that the antidromic response was actually due to local excitation of the terminals, and not to current spread to larger nerve branches, have already been described (Zucker, 1974*a*).

The threshold of a nerve terminal was measured as the minimum cathodal voltage which must be applied to the external micro-electrode in order to evoke an antidromic impulse. Brief pulses ($< 1$ msec) of high intensity were preferred as these gave the most consistent results. The current through the electrode was monitored across a resistor in series with the reference lead, and it was ascertained

that a linear relationship existed between the applied voltage and the current, for brief pulses.

Naturally, the threshold so measured depends on the pulse duration. Furthermore, the threshold was subject to large variations during long recording periods and especially when the bathing solution was changed. Similarly, the *difference* between the threshold before and after orthodromic spike invasion of the terminal was also subject to random fluctuations. These measurements are not, therefore, suitable indications of the 'intrinsic' state of the nerve terminals, especially in prolonged experiments. On the other hand, the *ratio* of the threshold following orthodromic invasion to that preceding invasion was found to be a very stable index, little affected by changes in stimulus duration or small movements of the electrode. The inverse of this ratio is called the 'relative excitability', and is the usual index of changes in nerve excitability used in this study. A value of relative excitability equal to one at a certain time following a nerve spike indicates that at this time the threshold is the same as it was before the spike. A relative excitability of 1·4 indicates that the nerve terminal is '40 % more excitable' than before invasion, or that the threshold is 1/1·4 or 0·715 times its preactivation level.

In some experiments triple-barrel micro-electrodes were used to test the effect of steady nerve-terminal polarization on excitability changes. One barrel was used for passing d.c. current, one barrel was used to excite the terminal and measure its threshold, and the middle barrel was connected to earth. Tests were made to check that the polarizing current had no effect on the voltage–current characteristic of the barrel used for measuring threshold.

When the effect of different ions or other agents on the excitability of nerve terminals was tested, the claw was superfused with 300–500 ml. of the new solution (about 6–10 times the volume of fluid in the bath). In solutions where the potassium or calcium concentration was raised above normal, the solutions were prepared by mixing isotonic concentrations of calcium chloride or potassium chloride with the normal bathing solution (Zucker, 1973) so as to keep the osmotic pressure unchanged. Calcium-free solutions contained 1 mM-EGTA, and magnesium was substituted for the calcium. This was necessary in order to prevent spontaneous firing of the motor neurone which led to muscle movement and dislodged the external micro-electrode (Wright & Tomita, 1965). Potassium-free solutions were prepared by omitting the normal potassium (5·4 mM) from the bathing medium. No substitution was made, as the reduction in osmotic pressure ($\sim 2$ %) was negligible. In solutions with low sodium, choline chloride was substituted for sodium chloride on an isomolar basis. To study the effect of osmotic pressure, 394 mM sucrose was added to the bathing medium to make a hyperosmotic solution with an osmotic pressure twice that of the normal medium. To study the effects of proteolytic enzymes, collagenase (Type I, Sigma Chemicals Ltd) and protease (Sigma Type VII) were used. Strophanthidin (Sigma) was used by dissolving 50 mg in 10 ml. ethanol and diluting this to 100 $\mu$M with 1240 ml. normal medium. The final solution contained 0·8 % ethanol, and its effects were always compared to those of control solutions of normal medium containing 0·8 % ethanol.

## RESULTS

For the first few msec following the invasion of terminals of the crayfish claw opener exciter motor neurone by an orthodromic impulse, the terminals are inexcitable. As this refractory period subsides, the terminals become excitable again, although the threshold is initially higher than its level before invasion. The threshold does not simply return to its pre-

activation level, however, but invariably drops to a level substantially below its control value, and only gradually rises from this low level to its pre-excitation value. In each of more than fifty terminals studied, this period of supernormal excitability followed the post-activation period of refractoriness.

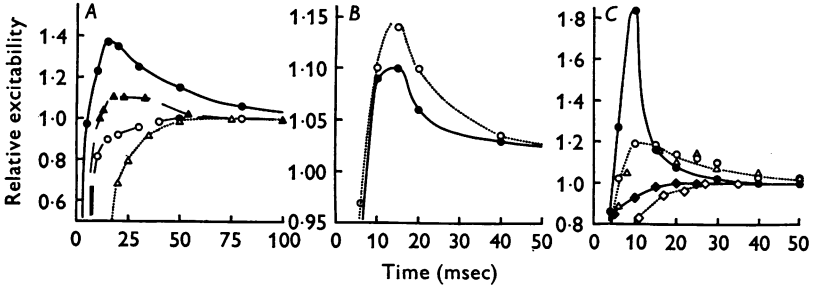


Fig. 1. *A*, post-activation changes in relative excitability in a typical nerve terminal (●), a typical nerve branch (○), and a main axon (△) of the exciter motor neurone to the claw opener. An exceptional supernormal period recorded from a visible nerve branch is also illustrated (▲). Temperatures: 10° C for ●, △; 6° C for ○, ▲. *B*, post-activation relative excitability changes in a nerve terminal following a single impulse (●) and a brief tetanus (○). Temperature: 11° C. *C*, post-activation changes in relative excitability in a nerve terminal, measured first by 11° C (○), then at 21° C (●), and finally again at 11° C (△), and in the main axon at 11° C (◇) and 21° C (◆). In all these graphs, time is measured from the negative peak of the orthodromic nerve terminal potential to the time of the test stimulus. Relative excitability is the inverse of the ratio of the post-activation threshold to the pre-activation threshold. The lines are fitted to the points by eye.

Fig. 1*A* gives an example of the time course of these changes in post-activation terminal excitability. Fig. 2*A* illustrates the same phenomenon in another terminal, where the changes in post-activation threshold are compared to the pre-activation threshold. The exact time course and peak magnitude of these excitability changes vary from terminal to terminal. However, the general form of the curve is quite constant. As refractoriness subsides, the excitability rises rapidly to a fairly broad peak, then declines slowly to its pre-activation level. When the logarithm of the increase beyond unity of the relative excitability is plotted *vs.* the time following invasion of the terminal, the final decay is found to fall on a straight line, and is therefore exponential. The over-all average of the peak supernormal excitability in fifty-four terminals in normal medium was $1{\cdot}41 \pm 0{\cdot}66$ (mean $\pm$ S.D.). The values ranged from 1·10 to 5·30. The average time of occurrence of this peak following a presynaptic impulse was at $14{\cdot}0 \pm 9{\cdot}1$ msec (range 3–60 msec). The average time constant of the exponential

return of the excitability to its control value was 26·6 ± 18·6 msec (range 6–110 msec). These results were obtained in experiments conducted at temperatures from 5 to 23° C. There was never seen any period of subnormal excitability following the supernormal excitability. Results similar to those were also obtained in a few experiments on the terminals of the peripheral inhibitor to the opener muscle.

*Effect of repetitive stimulation.* The peak supernormal excitability following a brief tetanus was always slightly but significantly larger than that following a single impulse (Fig. 1 *B*). In six experiments, the peak excitability following a tetanus of six spikes at 50 Hz was 0·03 ± 0·009 (mean ± S.E.) greater than that following a single spike. The latency to the peak of the supernormal excitability and the time constant of its decline were the same following a brief tetanus as after a single impulse. Longer tetani caused muscle movements and variable changes in the supernormal excitability. When the relative excitability following one impulse was measured at different frequencies of stimulation, the post-activation excitability cycle was unchanged for frequencies up to about 10 Hz, beyond which muscle movements interfered with the measurement of threshold. These results suggest that the peak supernormal excitability following an impulse increases during a tetanus so long as the interval between spikes in the tetanus is less than the time it takes for the supernormal excitability following each spike to subside completely.

*Effect of temperature.* The post-activation excitability cycle was measured in five terminals at temperatures of 21 and 11° C. In each case cooling the preparation had three effects: (i) the peak of the supernormal excitability shifted to a longer latency following invasion (from 5·6 ± 1·8 (S.D.) msec at 21° C to 10·8 ± 4·9 msec at 11° C); (ii) its magnitude was decreased (from 1·75 ± 0·93 at 21° C to 1·20 ± 0·08 at 11° C); and (iii) the time constant for decay of the supernormal excitability was prolonged (from 10·6 ± 4·1 msec at 21° C to 22·0 ± 5·1 msec at 11° C). The large S.D.s reflect the wide differences between terminals. When the *changes* in each parameter are considered, cooling from 21 to 11° C increases the latency to peak by 5·2 ± 2·3 (S.E.) msec, decreases the peak magnitude by 0·35 ± 0·22 (S.E.), and prolongs the decay time constant by 11·4 ± 2·8 (S.E.) msec. Thus all of the changes are significant. An example of these effects of temperature is given in Fig. 1*C*.

It seemed that some of these results might be due to the known effects of temperature on refractoriness (Adrian, 1914). If the refractory period is prolonged at low temperature, it will mask the early part of the supernormal excitability, thus delaying and reducing its peak. Such an effect of temperature on the refractory period of the main axon of the motor neurone has been observed and is illustrated in Fig. 1*C*. It is also evident from the

Figure that the refractoriness at 11° C is sufficiently long to interfere with the measurement of the decay rate of the supernormal excitability. Nevertheless, at least part of the effect of temperature on the decay of supernormal excitability appears to be independent of the effect on refractoriness. The $Q_{10}$ of the decay time constant was calculated for each of the five terminals discussed above, and the average value was $2{\cdot}8 \pm 0{\cdot}7$ (S.E.). Another estimate of this $Q_{10}$ was obtained by plotting the logarithm of the

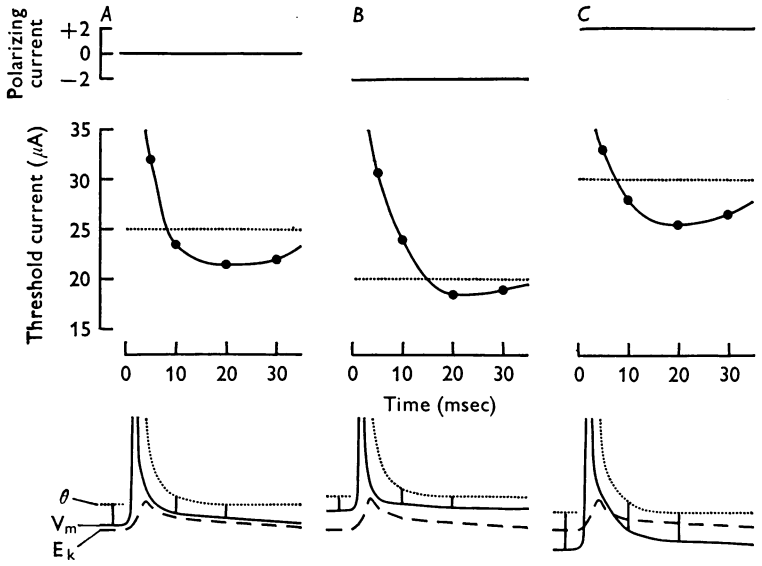


Fig. 2. Effect of nerve polarization post-activation threshold changes in a nerve terminal. The terminal is depolarized in *B*, and hyperpolarized in *C*. *A* is the control, in the absence of polarizing current. Top line shows polarizing current, middle line the pre-activation threshold (dotted lines) and post-activation changes in threshold (●). The bottom line shows how depolarizing currents would affect the internal voltage threshold ($\theta$) and the membrane potential ($V_m$) following a nerve impulse. The impulse is shown with a depolarizing afterpotential caused by a transient shift in the potassium equilibrium potential ($E_k$). The effects of polarizing current on excitability are due to its effects on the difference between $V_m$ and $\theta$, indicated by the vertical lines. Temperature: 8° C.

decay time constant against temperature for each of the thirty-nine terminals for which these data were available. The slope of the regression line for this data gave a $Q_{10}$ for the decay time constant of 2·4, with 95 % confidence limits of 1·65–3·51.

*Effect of nerve polarization.* The purpose of measuring the excitability changes following terminal activation was to obtain evidence for afterpotentials that might contribute to facilitation of transmitter release. The

change in threshold following refractoriness suggests that a terminal action potential is followed by a depolarizing afterpotential. The following experiments on the effects of nerve polarization lend support to this explanation of the supernormal excitability period.

Multibarrel micro-electrodes were used to polarize the nerve terminal with a constant d.c. electric field while independently measuring the post-activation changes in threshold. The results of one experiment are illustrated in Fig. 2. In *A*, the terminal threshold is plotted as a function of time following an orthodromic impulse, and compared to the threshold before activation. In *B*, a long pulse (0·5 sec) of cathodal current (2 $\mu$A) is applied through the polarizing barrel, beginning 300 msec before the orthodromic stimulus to the motor axon. It is evident that the pre-invasion threshold of the terminal is reduced, as is the peak reduction in threshold following an impulse. Part *C* of the Figure shows that hyperpolarizing (anodal) current has the opposite effect, increasing both the pre-invasion threshold and its post-invasion reduction. Similar results were obtained in five other experiments. In each case the magnitude of the effect of polarizing current was larger with larger currents. At one terminal it was possible to depolarize the terminal sufficiently, before reaching rheobase and exciting the terminal, to abolish the supernormal excitability completely.

These results can be understood in terms of the diagram in Fig. 2. Suppose there is a depolarizing afterpotential (d.a.p.) following an impulse. The diagram indicates that this is due to an increase in the potassium equilibrium potential ($E_k$) caused by a transient increase in the external potassium concentration, but any d.a.p. which is due to an approach of the nerve membrane potential to some equilibrium potential would behave similarly. Then depolarization of the nerve will reduce the change in the membrane potential ($V_m$) during the d.a.p. and hyperpolarization will increase it (cf. Yamasaki & Narahashi, 1959). Since the externally measured threshold is roughly proportional to the difference between $V_m$ and the internal voltage threshold ($\theta$), these effects of polarization on the d.a.p. will be revealed as similar effects on the supernormal excitability.

*The ionic basis of the d.a.p.* The above interpretation of the effects of nerve polarization suggests that the depolarizing afterpotential is caused by a tendency of the membrane potential to be drawn toward some depolarized equilibrium level following an impulse. This could arise from an increase in the nerve terminal permeability to some ion whose equilibrium potential is more depolarized than the membrane potential (i.e. sodium or calcium), or from a shift in the equilibrium potential of an ion whose concentration gradient determines the resting potential (i.e. potassium). A series of experiments in different ionic media was designed to test these possibilities.

The effect of varying the potassium concentration was tested in five experiments. The results of one are shown in Fig. 3*A*. When the potassium concentration was tripled (changed from the normal level of 5·4 to 16 mM), the post-activation supernormal excitability of exciter motor neurone terminals was reduced in every experiment (average change in peak $0{\cdot}17 \pm 0{\cdot}05$ (S.E.).) In two of the five terminals, the supernormal excitability was abolished. The effects of potassium were fully reversible, so that the

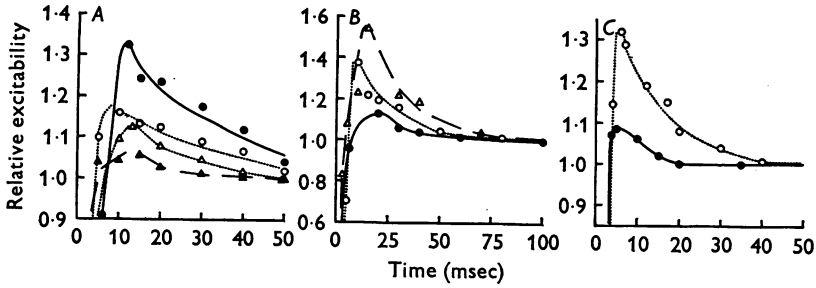


Fig. 3. *A*, post-activation excitability cycles from a nerve terminal exposed to various potassium concentrations: ○, first measurement in normal medium (5·4 mM-$K^+$); ●, potassium-free medium; ▲, high-potassium (16 mM) medium; △, final measurement in normal medium. *B*, effects of low-calcium on post-activation excitability changes: ○, normal medium; ●, calcium-free medium; △, return to normal medium. In seven experiments, the average reduction in excitability was not significant. *C*, effect of proteolytic enzymes (●) on excitability cycle; ○, before enzyme treatment. Temperatures: 11° C in *A* and *B*, 21° C in *C*.

peak of the supernormal excitability returned to very near its original level when the potassium concentration was returned to normal. When potassium was removed from the external medium, the supernormal excitability was always increased (average increase in peak $0{\cdot}11 \pm 0{\cdot}04$ (S.E.)), and this change was also fully reversible. No effect of potassium on the time course of the post-activation excitability cycle was detected.

Changes in the sodium concentration had little effect on terminal excitability changes following an impulse. In four experiments, reducing the sodium concentration to half normal caused an insignificantly small reduction in the peak supernormal excitability ($0{\cdot}019 \pm 0{\cdot}015$ (S.E.)). Doubling the sodium concentration, however, had variable effects on the peak supernormality, so that the mean change was small (an increase of 0·012) while the S.E. was large (0·077). These effects are probably related to the hyperosmolarity of the high-sodium solution.

Doubling the calcium concentration also had no effect on the supernormal excitability (average decrease of $0{\cdot}008 \pm 0{\cdot}018$ (S.E.)). In

calcium-free medium, containing 15–30 mM-$MgCl_2$ to prevent spontaneous firing of the motor neurone, the supernormal excitability may be reduced slightly. A reduction occurred in four out of seven experiments, and only one showed an increase. The average reduction ($0{\cdot}034 \pm 0{\cdot}027$ (S.E.)) was not significant, however ($n = 7$, $t = 1{\cdot}26$, $0{\cdot}2 > P > 0{\cdot}1$). This reduction may be due to the effect of reduced calcium on nerve permeability (see Discussion). Fig. 3*B* shows the results of one experiment with low calcium.

In order to further test the possibility that calcium is involved in generating the d.a.p., the effects of manganese and cobalt were studied. These ions block active calcium permeability increases in a variety of tissues (Hagiwara & Nakajima, 1966; Hagiwara & Takahashi, 1967; Geduldig & Junge, 1968; Brading, Bülbring & Tomita, 1969; Ito, Kuriyama & Tashiro, 1970; Hagiwara & Kidokoro, 1971; Washio, 1972). When 5 mM manganese was added to the bathing medium (Fig. 4*A*), there was no effect on the supernormal excitability (three experiments, average reduction of peak by $0{\cdot}01 \pm 0{\cdot}06$ (S.E.)), although synaptic transmission was completely (and reversibly) blocked. Cobalt ions, on the contrary, had a very striking effect (Fig. 4*B*). 5–10 mM cobalt abolished the supernormal excitability in seven out of eight experiments, and reduced it significantly in the eighth. The average peak of the supernormal excitability in these terminals was 1·146 before treating with cobalt, and 0·932 afterwards (the latter measurement is the average relative excitability at the time of the previous peak supernormality). The reduction was significant ($0{\cdot}48 \pm 0{\cdot}23$, $t = 2{\cdot}11$, $n = 8$, $P < 0{\cdot}05$). Synaptic transmission was also blocked (Fig. 4*D*). When the cobalt was washed out transmission and facilitation returned within 30 min to very near the original levels (compare Fig. 4*C* and *E*). However, even after 1 hr the post-activation excitability remained depressed. After 1 hr the average level of relative excitability at the moment of the original peak supernormality was 0·995. Thus there was only a small partial recovery seen after this time (average recovery from cobalt of $0{\cdot}068 \pm 0{\cdot}021$ (S.E.)).

It might be supposed that the effects of potassium, sodium, calcium and cobalt ions on the supernormal excitability actually reflect some general influence of these ions on the post-activation changes in nerve responsiveness, and not specific effects on d.a.p. Such an interpretation is especially suggested by the finding that some of the effects of temperature are mediated by changes in the refractory period, as measured in the axon. To test this idea the effects of raised and lowered potassium, sodium and calcium, and of the presence of cobalt ions, on the post-activation excitability of the exciter axon were measured in at least two of the previously described experiments with each ion. No consistent change in the time course or magnitude of relative refractoriness in axons was observed that

could account for the effects of these ions on the terminal post-activation excitability cycle.

*Effects of strophanthidin, proteolytic enzymes and sucrose.* It might be thought that the d.a.p. is due in part to the activity of an electrogenic

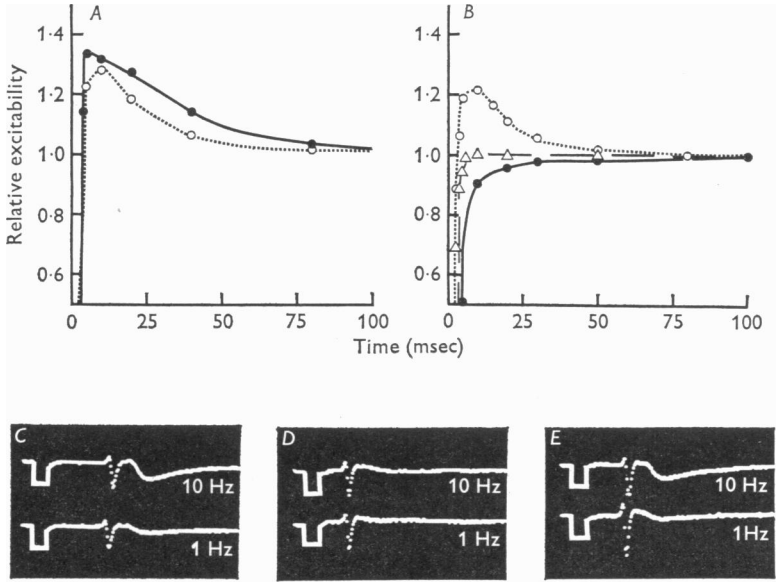


Fig. 4. Effects of manganese and cobalt on nerve terminal post-activation excitability cycles, and synaptic transmission and facilitation. *A*, excitability cycle of a terminal before (○) and after (●) adding 5 mM manganese to the bath. *B*, excitability cycle of another terminal before (○) and after (●) adding 5 mM cobalt to the bath, and 1 hr after washing out the cobalt (△). *C–E*, synaptic transmission and facilitation measured at the synapse formed by the same terminal whose relative excitability changes are shown in *B*. The computer-averaged recordings show the orthodromic nerve terminal potential followed by an extracellularly recorded junctional potential. The axon was stimulated at the frequencies indicated. The records in *C* were obtained in normal medium, concurrently with points ○ in *B*. A facilitation of 1·9 is induced by 10 Hz stimulation. *D* shows responses in the presence of 5 mM cobalt, obtained concurrently with points ● in *B*. Transmission is blocked. The records in *E* were obtained half an hour after returning to normal medium, concurrently with points △ in *B*. Transmission has recovered, and the 10 Hz facilitation (1·5) is close to its initial level.

pump, or that a hyperpolarization caused by such a pump partially masks the d.a.p. These possibilities were tested in two experiments with an inhibitor of the sodium–potassium pump. Strophanthidin (100 $\mu$M) was found to be without effect on the post-activation excitability changes

(average decrease in peak of 0·01). Moreover, in three experiments strophanthidin was found to have no effect on unfacilitated synaptic transmission, or on the facilitation of synaptic transmission following one impulse, or the steady level of facilitation reached during several seconds of stimulation at 10 Hz.

So far, all of the results point to the idea that the d.a.p. in nerve terminals may be caused by an increase in the external potassium concentration. This requires that the nerve terminals be surrounded by a very restricted extracellular space from which ions cannot readily diffuse away. Electron micrographs of nerve terminals in synaptic contact with surface muscle fibres (Atwood & Morin, 1970) reveal that the terminals are always located beneath the sarcolemma, and usually enclosed in invaginations in the muscle surface.

Betz & Sakmann (1973) have recently described a 'disjunction' of neuromuscular synapses following treatment with proteolytic enzymes. The enzymes digested the connective tissue binding the nerve terminals to the muscle at synapses, and the nerve–muscle junctions separated. Using their procedure, four muscles were treated at room temperature with 0·1 % collagenase for 1 hr followed by 0·01 % protease. After about 30 min in the protease the post-activation supernormal excitability rapidly declined in nerve terminals in all four experiments, and transmission failed concurrently. The supernormal excitability was totally abolished in one terminal, and the average reduction in peak supernormal excitability was $1{\cdot}45 \pm 0{\cdot}95$ (S.E.). Fig. 3*C* shows the results of one of these experiments. An additional effect, evident in the Figure, was consistently observed in the three experiments in which a small supernormal excitability persisted after half an hour in protease: the time constant of its decay was reduced markedly. The average time constant was 7·2 msec before enzyme treatment and 2·0 msec following it; the average change was significant ($5{\cdot}2 \pm 0{\cdot}88$ (S.E.) msec $t = 5{\cdot}9$ $n = 3$ $P < 0{\cdot}005$). In one muscle the protease was washed out; its effects were irreversible.

Another way to influence the extracellular space around nerve terminals might be to induce cell shrinkage with hyperosmotic solutions. In five experiments the effect of doubling the osmotic pressure with sucrose on terminal excitability changes was explored. The effects, like those of doubling the sodium concentration, were quite variable. At some terminals the supernormal excitability was enhanced and at others reduced. Such results suggest that on altering the osmotic pressure, the extracellular space around nerve terminals may expand or contract depending on the differential shrinkage occurring in nerve terminals and the surrounding muscle.

Finally control experiments showed that proteolytic enzymes and

sucrose had no effect on the axon refractory period. Like the changes in ionic constituents of the medium, these agents probably operate exclusively by changing the d.a.p. in nerve terminals.

## DISCUSSION

It has been shown that motor nerve terminals in the crayfish claw opener have a period of post-activation supernormal excitability following refractoriness. The effects of nerve polarization suggest that this is due to a depolarizing after-potential (d.a.p.) following nerve impulses in the terminals. The persistence of this supernormal excitability in low sodium makes it unlikely that the d.a.p. is due to a prolonged sodium activation, such as that induced by veratridine (Ulbricht & Flacke, 1965). Nerve terminals in squid and frog have a latent ability to generate calcium-dependent 'spikes' (Katz & Miledi 1969*a*, *b*). Nevertheless, the ineffectiveness of manganese ions and of doubling the calcium concentration mitigate against a prolonged calcium activation as the mechanism of the d.a.p.

The effects of changed potassium concentration are, however, just what are expected if the d.a.p. is due to a transient increase in the local extracellular potassium concentration ($[K^+]$) around nerve terminals. If each impulse leaves in its wake a constant increment in $[K^+]$, then the change in membrane potential (the d.a.p.) caused by this increment in $[K^+]$ will be reduced in high-potassium solutions, and inversely. The effects of a tetanus suggest that the d.a.p.s of single impulses summate, as would be expected if the residual increments in $[K^+]$ accumulate. The effects of proteolytic enzymes and elevated osmotic pressure are also most easily understood in terms of effects on the extracellular space around nerve terminals, where an increased space will reduce the post-activation increment in $[K^+]$ and will hasten its diffusion-related decay. In addition, the fact that the post-activation supernormal excitability is nearly exclusively restricted to nerve terminals where the extracellular space is most constricted, is also consistent with the present interpretation. Finally, since the relative excitability is proportional to the difference between internal threshold and membrane potential, the exponential decay of the supernormal excitability indicates an exponential time course for the d.a.p. This is expected if the potassium ions around nerve terminals are confined to a limited extracellular space by a diffusion barrier (Frankenhaeuser & Hodgkin, 1956).

The small reduction in supernormal excitability sometimes observed in calcium-free solution may be related to effects of calcium on the potassium flux during and between nerve impulses. In low calcium the spike amplitude

is reduced (Frankenhaeuser, 1957) while the resting potassium conductance is increased (Frankenhaeuser & Hodgkin, 1957). The latter effect may lead to an increased potassium concentration just outside the nerve membrane, while the former effect could reduce the potassium efflux during the spike. These effects can reduce the potassium-dependent d.a.p. in low calcium (Graham & Blair, 1947; Narahashi & Yamasaki, 1960). A similar situation in crayfish would reduce the supernormal excitability.

The effects of cobalt are less easily explained. Perhaps cobalt acts as a general calcium antagonist (Miledi, 1966; Hagiwara & Takahashi, 1967; Geduldig & Junge, 1968; Ito *et al.* 1970; Hagiwara & Kidokoro, 1971) and is equivalent to reducing the external calcium concentration. In this respect, the lack of effect of manganese ions on the post-activation excitability cycle is puzzling.

One result which appears to be not wholly consistent with an increased external $[K^+]$ as the basis for the supernormal excitability is the high temperature-dependence of the decay rate ($Q_{10}$ about 2·5). A $Q_{10}$ of about 1·3 would be expected if the external potassium is removed from the terminals by diffusion (Frankenhaeuser & Hodgkin, 1956). Three explanations are possible. (i) Prolonged refractoriness probably contributes to the late part of the post-activation excitability cycle at low temperature. The time course of relative refractoriness is known to have a high $Q_{10}$, about 2·9 (Adrian, 1914). (ii) Some of the excess potassium may be actively reabsorbed by nerve terminals. This process has a high temperature-dependence, and it appears to be the cause of a high $Q_{10}$ (2·0) for d.a.p. decay rate in mammalian nerve (Greengard & Straub, 1958). (iii) The extracellular space around nerve terminals might expand slightly at low temperature.

The d.a.p. which is implicated at crayfish motor nerve terminals may be compared to d.a.p. in other neural structures. Depolarizing afterpotentials and related phenomena have been analysed in squid giant axons (Shanes, 1949*a*, Frankenhaeuser & Hodgkin, 1956), crab nerve (Shanes, 1949*b*), mammalian nerve (Greengard & Straub, 1958), cockroach giant axons (Yamasaki & Narahashi, 1959; Narahashi & Yamasaki, 1960), sympathetic ganglion cells (Blackman, Ginsborg & Ray, 1963), hippocampal neurones (Kandel & Spencer, 1961), leech central neurones (Baylor & Nicholls, 1969) and glial cells (Orkand, Nicholls & Kuffler, 1966), and all appear to be caused by an increased external potassium concentration following nerve impulses. A post-activation period of increased excitability has also been observed in frog (Braun & Schmidt, 1966) and rat (Hubbard & Schmidt, 1963) motor nerve terminals. Whether these excitability changes are also due to changes in external potassium remains to be shown.

The present results have some bearing on the hypothesis that neuromuscular facilitation is caused by afterpotentials. Although a hyperpolarizing afterpotential could conceivably facilitate transmitter release, there is no evidence for such an afterpotential in crayfish nerve terminals. On the contrary, as is usually true in crayfish sensory neurones (Eyzaguirre & Kuffler, 1955) and central interneurones (Kennedy & Mellon, 1964), only a depolarizing afterpotential appears to follow single impulses. Furthermore, in very recent experiments using a sucrose-gap technique, Dudel (1973) has recorded a d.a.p. in very fine motor nerve branches in the crayfish claw opener muscle. This finding is in full agreement with the present results. Dudel (1971) has also shown that depolarization of crayfish motor nerve terminals can only reduce transmission, so the d.a.p. itself cannot be responsible for facilitation. Similar effects of presynaptic depolarization have been reported in squid (Takeuchi & Takeuchi, 1962) and rat (Hubbard & Willis, 1968).

It might be supposed that the transient increase in extracellular potassium is the cause of facilitation, especially since a direct effect of external potassium on transmitter release has been reported in rat (Cooke & Quastel, 1973). However, synaptic transmission in crayfish is affected little by the probable changes in potassium concentration following a nerve spike. In the present experiments with altered potassium concentration, it was observed that adding 2 mM potassium chloride to the medium had no effect at all on synaptic transmission. A slight reduction in nerve terminal threshold occurred, however, so the potassium was reaching the extracellular space around terminals. Further increase in $[K^+]$, up to 16 mM, caused only a slight (20 %) *reduction* in transmission. The experiments with cobalt also serve to dissociate facilitation from the d.a.p. Apparently, in crayfish at least, facilitation and the d.a.p. indicated by the supernormal excitability in nerve terminals are quite unrelated.

I wish to express my appreciation to Professors B. Katz and R. Miledi for providing the opportunity to work in their laboratory. I am particularly grateful to Professor Katz and Dr John Heuser for their many helpful discussions. The author was supported by the Helen Hay Whitney Foundation.

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