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CRAYFISH NEUROMUSCULAR FACILITATION ACTIVATED BY CONSTANT PRESYNAPTIC ACTION POTENTIALS AND DEPOLARIZING PULSES

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SUMMARY

1. Experiments were conducted to test the hypothesis that facilitation of transmitter release in response to repetitive stimulation of the exciter motor axon to the crayfish claw opener muscle is due to an increase in the amplitude or duration of the action potential in presynaptic terminals. No consistent changes were found in the nerve terminal potential (n.t.p.) recorded extracellularly at synaptic sites on the surface of muscle fibres.

2. Apparent changes in n.t.p. are attributed to three causes.

(i) Some recordings are shown to be contaminated by non-specific muscle responses which grow during facilitation.

(ii) Some averaged n.t.p.s exhibit opposite changes in amplitude and duration which suggest a change in the synchrony of presynaptic nerve impulses at different frequencies.

(iii) Some changes in n.t.p. are blocked by γ-methyl glutamate, an antagonist of the post-synaptic receptor, which suggests that these changes are caused by small muscle movements.

3. The only change in n.t.p. believed to represent an actual change in the intracellular signal is a reduction in n.t.p. amplitude to the second of two stimuli separated by a brief interval.

4. Tetra-ethyl ammonium ions increase synaptic transmission about 20 % and prolong the n.t.p. about 15 %. This result suggests that an increase in n.t.p. large enough to increase transmission by the several hundred per cent occurring during facilitation would be detected.

5. The nerve terminals are electrically excitable, and most synaptic sites have a diphasic or triphasic n.t.p., which suggests that the motor neurone terminals are actively invaded by nerve impulses.

6. When nerve impulses are blocked in tetrodotoxin, depolarization of

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nerve terminals increases the frequency of miniature excitatory junctional potentials (e.j.p.s), and a phasic e.j.p. can be evoked by large, brief depolarizing pulses. Responses to repetitive or paired depolarizations of constant amplitude and duration exhibit a facilitation similar to that of e.j.p.s evoked by nerve impulses.

7. It is concluded that facilitation in the crayfish claw opener is not due to a change in the presynaptic action potential, but is due to some change at a later step in the depolarization–secretion process.

INTRODUCTION

Facilitation is the process by which successive presynaptic nerve action potentials evoke responses of increasing magnitude in a post-synaptic cell. The larger responses are due to increased amounts of chemical transmitter released by the presynaptic element (del Castillo & Katz, 1954*b*; Liley, 1956; Dudel & Kuffler, 1961*b*; Kuno, 1964; Martin & Pilar, 1964; Kuno & Weakley, 1972*a*, *b*). Beyond this, little is known of the mechanism responsible for increased transmitter release, although this problem has been a subject of investigation for three decades (Eccles, 1964).

Early investigations concentrated on the possibility that growth in the size or duration of the action potential in the presynaptic terminals was the primary cause of facilitation and the related phenomenon of post-tetanic potentiation (Lloyd, 1949; Wall & Johnson, 1958; Eccles & Krnjević, 1959; Takeuchi & Takeuchi, 1962; Hubbard & Schmidt, 1963; Dudel, 1965*a*). Although this has been eliminated as the mechanism in most other systems (Hubbard, 1963; Martin & Pilar, 1964; Katz & Miledi, 1965*b*; Braun & Schmidt, 1966; Miledi & Slater, 1966; but see Curtis & Eccles, 1960), it still seems to be regarded as the most likely mechanism of facilitation at crustacean neuromuscular junctions (Atwood, 1967; Bittner, 1968; Sherman & Atwood, 1972; Atwood & Lang, 1973; Lang & Atwood, 1973).

In this study, the experiments which provide evidence for this 'pre-spike hypothesis' of facilitation are repeated, and the results reinterpreted as providing no substantial support for the hypothesis. In essence, the nerve terminal potential is ordinarily not seen to grow during facilitation, and the terminals are shown to be excitable, and probably fully invaded by all-or-none action potentials. In addition, presynaptic spikes can be abolished by tetrodotoxin, and the nerve terminals stimulated repetitively by brief depolarizing pulses. These stimuli evoke transmitter release, and the post-synaptic responses show facilitation similar to that caused by presynaptic action potentials. It is concluded that crayfish neuromuscular facilitation is not caused by an increase in the presynaptic action potential.

METHODS

Procambarus clarkii were used in these studies. Experiments on changes in nerve terminal potentials during facilitation were performed on the claw opener muscle (dactyl abductor) of the pinching leg (cheliped). The dorsal surface of the muscle was exposed and the axon of the single exciter motor neurone to this muscle was dissected from the meropodite (Zucker, 1973). The procedures for intracellular recording from muscle fibres, extracellular recording from synaptic sites, recording from or stimulating the motor axon, and controlling temperature were standard, and have already been described (Zucker, 1973).

In most experiments, the amplified intracellular and extracellular responses to repetitive stimulation were averaged automatically with a signal-averaging computer (Biomac 500). A calibration pulse was introduced in series with the reference lead and averaged along with the muscle responses. The computer-averaged signal could be displayed and photographed, or it could be recorded by a pen writer at low speed to produce permanent records.

Stimuli from a square-pulse stimulator plus isolation unit could be fed into the extracellular micro-electrode for stimulating nerve terminals. For this purpose, micro-electrodes filled with 2 M sodium chloride, with outside diameters of about 10 μm (internal tip diameters of about 3 μm) and resistances of 1–3 MΩ were most suitable. The stimulation current was monitored across a resistor (10 kΩ or 100 kΩ) in series with the reference lead. Simultaneous recordings from the intracellular, extracellular, and axon electrodes and the current monitor could be displayed, photographed, and averaged.

In experiments on the effects of nerve polarization, two methods of polarization were employed. The focal technique involved the placement of an extracellular micro-electrode directly over a nerve terminal and synaptic site. Cathodal current through this electrode depolarized a small local area of the nerve and muscle membranes. This method has been discussed in detail by Katz & Miledi (1967*a*, *b*).

A second method of nerve depolarization used was the electrotonic method. In this technique, a motor neurone branch about 10 μm in diameter is sucked into a close-fitting suction electrode. Nerve spikes recorded from this electrode were usually monophasic positive, about 1–3 mV in amplitude. Positive stimulating pulses delivered to this electrode will depolarize the nerve membrane outside the electrode. Responses to nerve polarization were recorded intracellularly from a nearby muscle fibre or extracellularly from a synaptic site on a small nerve branch about 100–400 μm from the suction electrode. A detailed discussion of this technique may be found in Dudel (1971).

The dorsal surface of the opener muscle could be dimly illuminated by transmitted light, which must pass through the underlying thick adductor muscle, or by very bright oblique incident illumination from a fibre optics light guide and a 150 W focused light source. This surface of the muscle was nearly devoid of visible nerve branches and so was unsuitable for experiments using the electrotonic method of depolarization. For these experiments, the richly innervated ventral surface of the claw opener muscle of the first walking leg was used. The ventral exoskeleton of the propodite was cut away and the closer (dactyl adductor) removed. The underlying sensory nerves and blood vessel were carefully cut away, the membrane covering the opener removed, and the ventral surface of the opener thus exposed. The walking leg was mounted, and the exciter axon dissected from the meropodite, in the same manner as for the pinching leg (Zucker, 1973).

The composition of the bathing medium was (mM): NaCl 195, KCl 5·4, $CaCl_2$ 13·5, $MgCl_2$ 2·6, Tris maleate buffer 10, adjusted with NaOH 10 to pH 7·3. A few drops of

tetrodotoxin (10^{-5} g/ml.) could be added to the bath to block nerve conduction. When γ-methyl glutamate (γ-MG) (Lowagie & Gerschenfeld, 1973) was used to block synaptic transmission, an appropriate amount of 200 mM-γ-MG was added to the bath to obtain the desired final concentration. This procedure left the osmotic pressure of the solution virtually unchanged.

RESULTS

Changes in nerve terminal potentials during facilitation

Identification of nerve terminal potentials. When a micro-electrode is positioned on the dorsal surface of the claw opener muscle and the exciter axon stimulated in the meropodite, it is usually possible to record a very small potential, perhaps 50 μV in amplitude. If a careful search is made, and the electrode moved from point to point on the muscle, a spot will eventually be found from which much larger potentials can be recorded. Typically, each response now consists of a rapid diphasic (positive–negative) potential, about 1–2 msec in duration, which appears relatively constant from trial to trial. This is the nerve terminal potential (n.t.p.) (Dudel & Kuffler, 1961*a*). At some recording sites, it is monophasic positive; at others it is triphasic (positive–negative–positive).

The n.t.p. is followed, after about 1·5 msec at room temperature, or 3–5 msec at lower temperatures (5° C), by a second potential. This consists of a complex negative wave containing a variable number of unitary or 'quantal' responses, each about 10 msec in duration (Dudel & Kuffler, 1961*a*). This represents current flowing into the muscle, since it is abolished by post-synaptic blocking agents (see Fig. 4), and is called the extracellularly recorded junctional potential (e.r.j.p.). On occasion, a nerve spike evokes no transmitter release (a 'failure') and the e.r.j.p. is absent.

At some recording sites a small, regular, monophasic positive or diphasic positive–negative deflexion lasting many msec is seen underlying the e.r.j.p. and slightly preceding it. When a failure occurs and the e.r.j.p. is absent, this potential can be viewed in isolation (Fig. 1*A*, traces 5 and 7). This potential resembles those seen when the electrode is placed randomly on the muscle surface, not in the immediate vicinity of a nerve branch or a synaptic site. It is thus termed the non-specific muscle response (n.s.m.r.) (del Castillo & Katz, 1956; Katz & Miledi, 1965*a*). The first positive component represents current flowing locally out of the muscle to distant synapses which act as current sinks. If a number of synapses are fairly near the micro-electrode, a negative phase may follow as current tends to flow towards the electrode from earth.

When a signal-averaging computer is used to compute the average potential response recorded at a site following repeated stimulation, the

n.s.m.r. often appears to be a late positive phase of the n.t.p. (Fig. 1 *B*). There are three ways to distinguish an n.s.m.r. from an n.t.p. The first is by careful scrutiny of single responses, where at low temperature the n.t.p. is frequently complete, and the trace returns to zero, before the n.s.m.r. begins (Fig. 1 *A*). The second is by use of neuromuscular blocking agents, which selectively abolish the n.s.m.r. and the e.r.j.p. (Fig. 4). The third and most useful way is to move the electrode 10–20 μm away from the synaptic site. Then the n.s.m.r. can be recorded (Fig. 1 *D*) or averaged (Fig. 1 *C*) in isolation, and compared to the responses at the site which include the n.t.p. and the e.r.j.p.

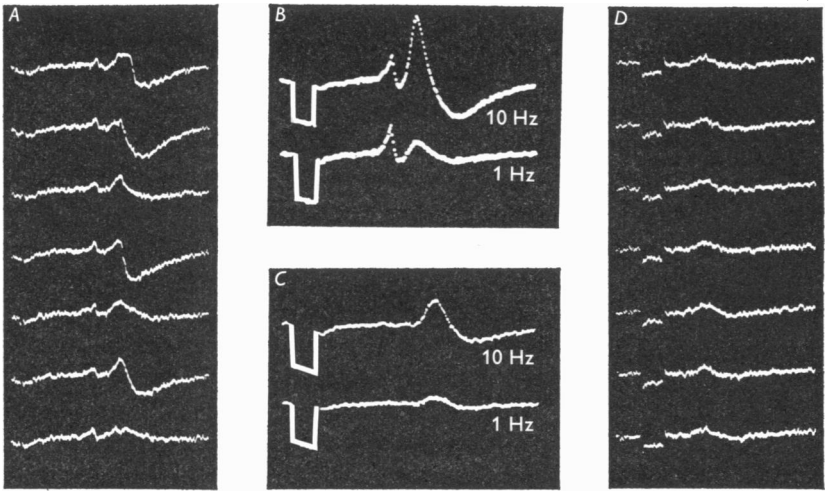

Fig. 1. Non-specific muscle responses (n.s.m.r.s) and nerve terminal potentials (n.t.p.s). *A*, a series of records from a synaptic site with a diphasic n.t.p., followed by a mainly positive n.s.m.r. and, finally, by negative extracellularly recorded junctional potentials (e.r.j.p.s). *B* is the computer display of the average of sixty-four responses. The electrode was lifted 20 μm above the muscle surface, and the records in *D* were taken showing only an n.s.m.r. *C* is the average of 64 of these responses. Stimulation frequency: 10 Hz in *A* and *D*, and 1 or 10 Hz in *B* and *C* as indicated. Calibration pulses: 100 μV, 2 msec. Temperature: 15° C.

It is crucially important to distinguish the n.t.p. from any n.s.m.r. because we expect the n.s.m.r. to change during facilitation. If the amount of transmitter released by spikes is increased by tetanic stimulation (Figs. 1*C*, 4*C*) or paired stimulation (Fig. 4*E*), then both the n.s.m.r. and e.r.j.p. should increase by about the same proportion, since both the local and distant synapses facilitate.

Effects of facilitation on n.t.p.s. The changes in n.t.p.s have been studied at thirty-one synaptic sites. The n.t.p. amplitude was measured from the

first positive peak to the negative peak. The duration was usually measured as the interval from the foot of the first positive phase to the peak of the second positive phase. If the foot was indistinct, the peak of the first phase was used to start the duration measurement. If the last phase was obscured by an n.s.m.r., or simply not present, the negative peak was used to end the duration measurement. In every case where duration changed during facilitation, the same type of change was observed, regardless of how the duration was estimated.

Facilitation was induced by increasing the stimulus frequency, or by using paired stimuli separated by 8–30 msec. The interval between pairs was usually 2 sec. Facilitation (F) is measured as the increase of the facilitated response (R) compared to the control response (R_0), divided by the control response, $F = (R-R_0)/R_0 = R/R_0-1$. Thus $F = 1$ means the facilitated response is twice the control; $F = 0$ indicates that the response is the same amplitude as the control.

The main result of these experiments is that there is no consistent change in the n.t.p. during facilitation. At eight sites the n.t.p. grew, at eleven it was diminished, at six the amplitude remained constant, while at the remaining six sites the effects were mixed, the n.t.p. sometimes increasing and sometimes decreasing during facilitation. For all thirty-one sites, the average amplitude of the unfacilitated e.r.j.p. was 56 ± 62 μV (mean $\pm$ S.D.), and the average facilitation caused by 10 Hz stimulation was $3{\cdot}0 \pm 2{\cdot}2$. There were no significant differences in these values among the four groups with different changes in n.t.p. during facilitation.

Changes in n.t.p. duration were also sometimes observed, but again bore no clear relation to facilitation. The duration increased at nine sites, decreased at five, and remained constant at seventeen. These experiments were performed at temperatures between 2 and 21° C; the results were unaffected by temperature.

The absence of a change in n.t.p. which was correlated in a consistent fashion with facilitation was true for all shapes of n.t.p. Fig. 2*A* illustrates a diphasic n.t.p. that remained constant at two frequencies of stimulation, and Fig. 2*B* shows a similar result for a monophasic positive n.t.p. Fig. 2*D* shows a positive n.t.p. that grew slightly during facilitation. These were the only two monophasic n.t.p.s encountered. Fig. 2*C* shows one of the sites with variable changes in the n.t.p., where an increase occurred between 3·3 and 10 Hz, and a decrease at 20 Hz. Other sites showing various n.t.p. changes during facilitation are illustrated in Fig. 3.

Causes of n.t.p. changes. Although the results demonstrate a lack of consistent relation between n.t.p. changes and facilitation of transmitter release, changes in n.t.p. do occur at some sites. One possible cause for a change in the computer-averaged n.t.p. is a change in the synchrony of the

nerve spikes arriving at the motor neurone terminals at different frequencies. Suppose that at one frequency of stimulation the nerve spikes are more synchronous (that is, are more precisely locked to the stimulus in time) than at another frequency. Then the time-average of the first group of n.t.p.s will be larger and briefer than the time-average of the second

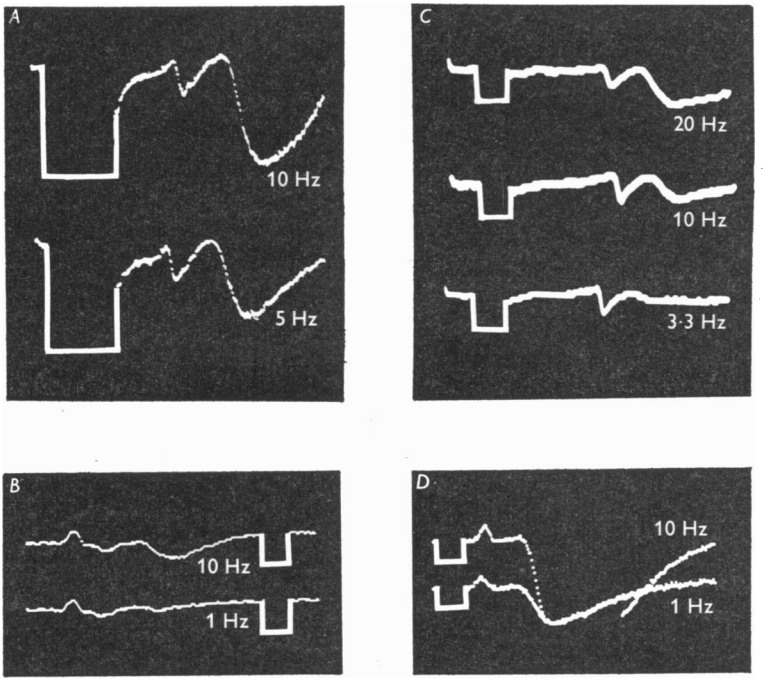

Fig. 2. Examples of n.t.p.s at the frequencies of stimulation indicated next to each trace. In these examples the n.t.p.s show no change (*A* and *B*), or slight (*D*) or variable (*C*) changes during facilitation. In *B*, the small signal between the monophasic n.t.p. and the e.r.j.p. is due to a sensory fibre excited antidromically in the leg nerve. It had a different threshold from the exciter axon. Calibration pulses: 100 μV in *A–C*, 250 μV in *D*; 5 msec in *A*, 1 msec in *B* and *C*, 2 msec in *D*. Temperatures: 23° C in *A–C*, 10° C in *D*. Each trace is the average of sixty-four responses.

group (since the computer is triggered by the stimulator), even though the individual n.t.p.s are identical at both frequencies. Such a situation would be suggested by opposite changes in n.t.p. amplitude and duration. Such a negative correlation was observed at eight of the twenty-five sites which showed a change in n.t.p. amplitude. Two examples are illustrated in Fig. 3*A* and *B*. Five of these eight sites showed a decrease in n.t.p. with increased frequency; the other three showed an increase or mixed effects. It seems quite possible that a change in synchrony of nerve responses to

different frequencies of stimulation accounts for some of the changes in n.t.p. during facilitation. However, since seventeen of the twenty-five n.t.p.s whose amplitude changed showed no change or a positively correlated change in duration, it is unlikely that variable synchrony can be the only factor contributing to n.t.p. changes.

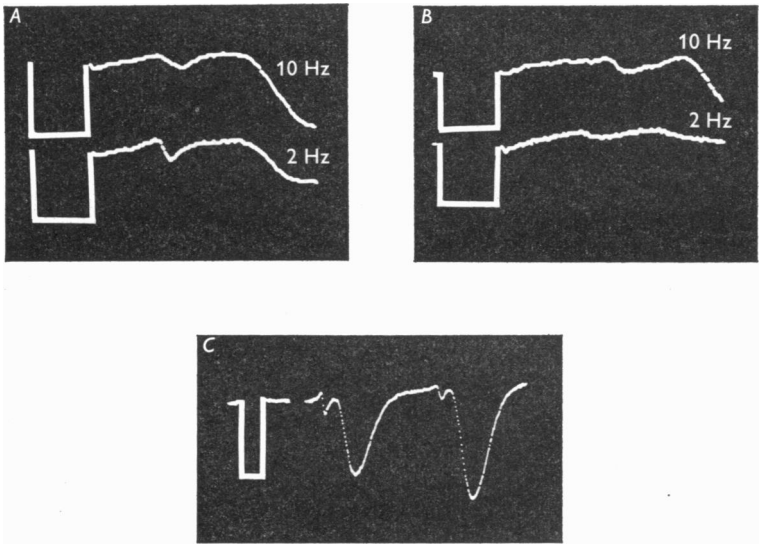

Fig. 3. Examples of n.t.p.s which change during facilitation. The n.t.p. became smaller and broader in *A*, and larger and briefer in *B* as the stimulus frequency was increased as indicated. In *C* the n.t.p. was reduced to the second of two stimuli separated by 30 msec. Calibration pulses: 100 μV in *A–C*; 1 msec in *A* and *B*, 5 msec in *C*. Temperatures: 13° C in *A* and *B*, 2° C in *C*. Number of responses averaged: sixty-four in *A* and *B*, 256 in *C*.

Another factor which might be involved is muscle movement. In every experiment, the muscle was observed during stimulation at each frequency and a landmark was located along an optical micrometre in one eyepiece of the dissecting microscope. Movements of 20 μm could be detected, and responses from such sites were rejected. Visible movements were infrequent because the claw opener is a slow muscle, whose fibres rarely develop action potentials and do not twitch. The muscle contracts slowly, and only to a fairly high-frequency tetanus (Bittner, 1968). The claw was fixed in a closed position. However, it may be noted that an intentional movement of the extracellular micro-electrode of 5 μm or less can greatly change the size of the n.t.p. (Katz & Miledi, 1965*a*). Such a muscle movement would have remained undetected in the microscope.

There are several clues that undetected muscle movements interfered

with the recording of the n.t.p. and were responsible for some of the changes during facilitation. One would expect these movements to cause some n.t.p.s to increase and others to decrease when the frequency of stimulation is changed. As already noted, this was observed (Figs. 2 and 3). Furthermore, sites were observed at which the n.t.p. grew at moderate frequencies but shrank at higher frequencies (Fig. 2*C*), as if the electrode moved first closer, then past the optimal recording position. At other sites, increasing the frequency of stimulation first changed the n.t.p. in one direction. A later repetition of the same experiment led to a change in the opposite direction (Fig. 4*A* and *B*).

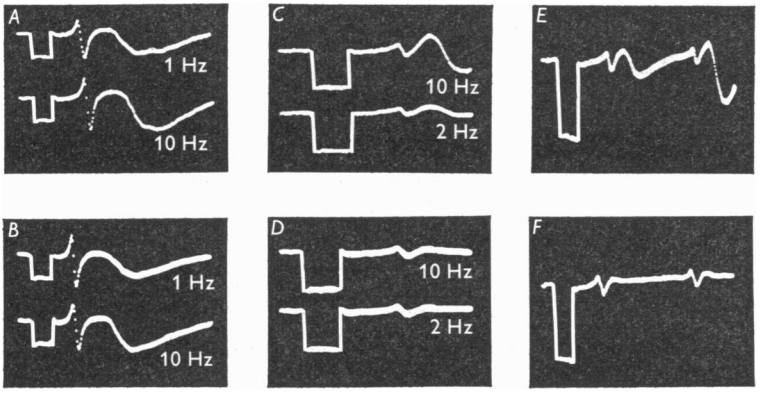

Fig. 4. Examples of n.t.p.s which may be influenced by muscle movements. In *A* and *B*, a site is illustrated whose n.t.p. first increased, and later decreased when the frequency was changed from 1 to 10 Hz. *C* shows a site whose n.t.p. changed with frequency. In *D*, this change was abolished in 10 mM-γ-methyl glutamate (γ-MG). *E* shows a site whose n.t.p. changed during paired stimulation with a separation of 10 msec. *F* shows that this change is unaffected by 10 mM-γ-MG. Calibration pulses: 100 μV and 1 msec in *A* and *B*; 200 μV and 2 msec in *C*–*F*. Temperatures: 15° C in *A* and *B*, 13° C in *C*–*F*. Each record is the average of sixty-four responses.

In order to further test the possibility of movement confounding the results, muscle movement was reduced by adding 10–25 mM-γ-methyl glutamate (γ-MG) to the medium. This is an antagonist for the postsynaptic receptor responses to glutamate and to the natural transmitter (Lowagie & Gerschenfeld, 1973). γ-MG immediately blocked excitatory junctional potentials (e.j.p.s) recorded intracellularly from surface fibres, as well as e.r.j.p.s and n.s.m.r.s (Fig. 4*D*, *F*). However, e.j.p.s could still be recorded in deep fibres, and very slight movement occurred at very high frequencies (over 30 Hz). Apparently, γ-MG fails to penetrate below the muscle surface fibres (see Zucker, 1974*a*).

γ-MG was added to the bath in three experiments while recording from sites showing n.t.p. changes during facilitation. In all three sites the changes observed during paired stimulation persisted in γ-MG (Fig. 4*E*, *F*). However, at the two sites where n.t.p. changed with frequency, the change was blocked by γ-MG. This suggests that some of the n.t.p. changes during facilitation, particularly those occurring at different frequencies, are caused by minute muscle movements. However, other changes, particularly those occurring during paired stimulation, are probably genuine.

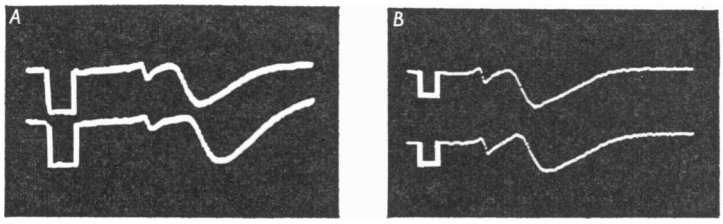

Fig. 5. Effects of tetraethylammonium chloride (TEA) on n.t.p. and synaptic transmission. *A* and *B* are two different sites. The upper trace shows responses in normal medium. The lower trace shows responses after adding 10 mM (*A*) or 5 mM (*B*) TEA to the medium. Stimulus frequency: 10 Hz. Calibration pulses: 200 μV and 2 msec in *A*; 100 μV and 1 msec in *B*. Temperatures: 8° C in *A*, 12° C in *B*.

Effects of tetraethylammonium (TEA). It seemed possible that extracellular recording of n.t.p.s might be too insensitive to detect small changes in membrane potential that might actually contribute to facilitation. The n.t.p. registers the local nerve membrane current, which is proportional to the second derivative (curvature) of the intracellular wave form of a conducted action potential. To test the sensitivity of this index of the presynaptic impulse, the effect of 5–10 mM-TEA on the n.t.p. and transmission was measured. TEA in the external medium is known to increase transmitter release and to prolong the n.t.p. in the frog (Benoit & Mambrini, 1970), presumably by interfering with delayed rectification and spike repolarization. The effects in crayfish (Fig. 5) are similar to those seen in the frog. The negative phase of the n.t.p. is prolonged about 50 %, the synaptic delay is increased about 50 %, the duration of the e.r.j.p. is prolonged about 15 %, and its amplitude is increased about 10–20 %. Thus we can detect a change in n.t.p. small enough to increase transmission by only 20 %. We certainly should be able to detect n.t.p. changes if they were responsible for a facilitation that often reaches several hundred per cent.

Excitability of nerve terminals

It has often been suggested that crayfish motor neurone terminals are not fully invaded by spikes, and that facilitation is due to an increased invasion of terminals (Atwood, 1967; Bittner, 1968; Sherman & Atwood, 1972; Atwood & Lang, 1973; Lang & Atwood, 1973). In the present experiments, it was possible to stimulate the exciter motor neurone antidromically at about half the sites from which an n.t.p. and e.r.j.p. were recorded. The failure to stimulate all sites may be related to the difficulty in placing the electrode close enough to the nerve terminal (Lang, Atwood & Morin, 1972). At most sites, however, an antidromic response could be recorded from the axon in the meropodite or as an e.j.p. in an adjacent muscle fibre. The orthodromic and antidromic axon spikes always had the same conduction time and similar wave forms. Simultaneous antidromic and orthodromic stimulation resulted in axon spike

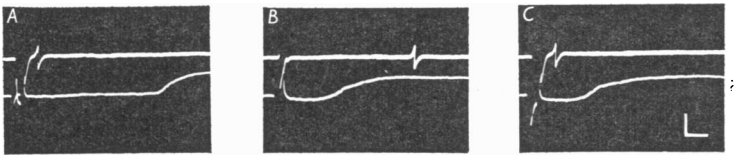

Fig. 6. Antidromic stimulation of motor neurone terminals. Upper trace records from a suction electrode on the exciter axon in the meropodite. Lower trace is an intracellular recording from an opener muscle fibre. In *A* the axon was excited orthodromically by a second suction electrode proximal to the first. In *B* the motor neurone was excited antidromically from a micro-electrode on a presynaptic terminal. In *C* both stimuli were delivered simultaneously, and collision occurs, blocking the orthodromic e.j.p. and the antidromic axon spike. Calibration marks: 0·5 mV for upper trace, 2 mV for lower trace; and 2 msec. Temperature: 21° C.

collision (Fig. 6). The threshold for antidromic stimulation varied critically with electrode location, just as did recordings from synaptic spots. Typical thresholds were 5–20 μA for 1 msec pulses, although sites with thresholds lower than 1 μA were encountered. Cathodal current was always more effective. Sometimes large pulses, several times threshold, were ineffective in eliciting spikes. This is probably the effect of 'anode surround' reported in frog (Katz & Miledi, 1965*a*), where the local outward depolarizing nerve current completes the circuit as inward hyperpolarizing current surrounding the depolarized region.

It was never possible to elicit antidromic motor neurone spikes from any point on the muscle from which an n.t.p. and e.r.j.p. could not be recorded, unless the electrode were placed against a visible large (10 μm diameter) motor neurone branch. Thus these results cannot be attributed to current

spread. Rather they suggest that the same general regions of the motor neurone that form synapses with the muscle are electrically excitable, and that they are fully invaded by all-or-none action potentials.

Facilitated responses to depolarizing nerve pulses in nerves made inexcitable by tetrodotoxin

A different approach to the question whether a changed presynaptic electrical signal contributes to facilitation was pursued. Instead of using action potentials to trigger transmitter release, spikes were blocked with tetrodotoxin 10^{-8}–2×10^{-7} g/ml., and nerve terminals were depolarized with electrical pulses applied to a nerve branch. The best results were obtained when the electrotonic method of depolarization was used (see Methods).

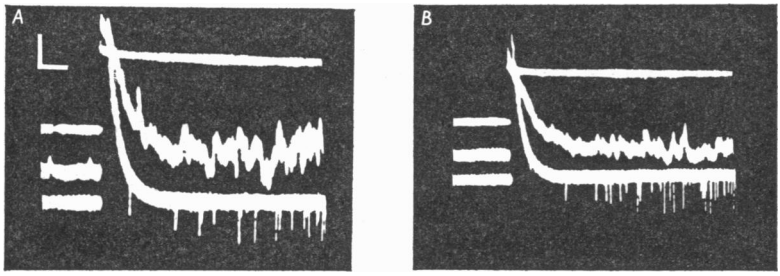

Fig. 7. Effect of long electrotonic depolarizing pulses applied to a motor neurone branch. The current intensity is monitored in the upper trace. The middle trace is from an intracellular micro-electrode, while the lower trace records extracellular synaptic currents. Records in *A* were obtained in normal medium; tetrodotoxin 2×10^{-7} g/ml. has been added in *B*. Calibration marks: 1 μA for upper trace, 200 μV for remaining traces; and 1 sec. Temperature: 10° C.

Responses to electrotonic depolarization of nerve. A suction electrode was placed on a suitable motor neurone branch on the innervated (ventral) muscle surface and muscle responses recorded intra- or extracellularly from a nearby muscle fibre. When depolarizing (anodal) current was passed through the suction electrode, the frequency of spontaneous miniature e.j.p.s and e.r.j.p.s was increased (Fig. 7*A*). If tetrodotoxin was then added to the bath, this response to nerve depolarization persisted (Fig. 7*B*). Apparently, as in the frog (Katz & Miledi, 1967*a*), tetrodotoxin does not interfere with the depolarization–secretion mechanism in crustacea.

Phasic transmitter release, consisting of many quanta released nearly simultaneously, and thus resembling the normal e.j.p. or e.r.j.p., could be evoked with brief, strong depolarizing pulses. The responses to such

pulses were graded, in that larger or longer pulses evoked more transmitter release, and hence larger post-synaptic responses. These pulses could be delivered at different frequencies, or in pairs, and the responses compared to those evoked by a similar sequence of action potentials.

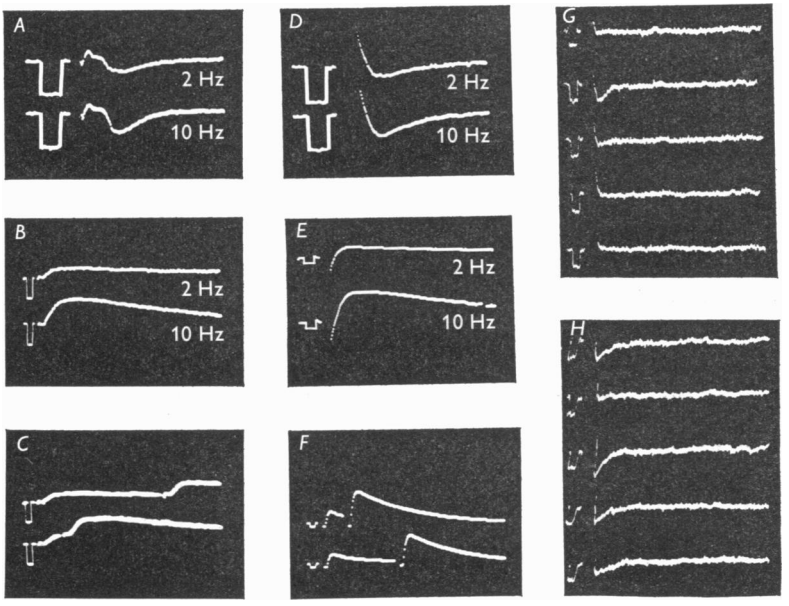

Fig. 8. Facilitated transmitter release evoked by action potentials and depolarizing pulses. In *A–C* transmission is triggered by presynaptic action potentials. In *D–H* records were obtained after adding tetrodotoxin 2×10^{-7} g/ml. to the medium, and transmission was induced by electrotonic depolarizing pulses. *A* and *D* show extracellular recordings from a site in response to stimulation at 2 and 10 Hz. *B* and *E* show intracellular responses to these frequencies. *C* and *F* show e.j.p. facilitation to paired stimuli. *G* shows a segment from the 1 Hz series of extracellular responses to depolarizing pulses making up the averaged response in the upper trace of *D*. *H* is a segment from the 10 Hz series averaged in the lower trace of *D*. The traces were obliquely slanted due to the rapid movement of the film; they have been remounted so that they run horizontally, hence the vertical deflexions are slanted. Depolarizing pulse parameters: 7 μA and 2 msec in *D*, *G* and *H*; 30 μA and 1 msec in *E* and *F*. Calibration pulses: 200 μV, 5 msec. Temperature: 12° C. Thirty-two responses were averaged in *A–F*.

One experiment is illustrated in Fig. 8. Synaptic potentials were recorded both intra- and extracellularly. On the left are shown responses to nerve action potentials at two different frequencies, or to pairs of action potentials. Then tetrodotoxin was added to the medium and the responses to repetitive or paired pulsed stimulation were recorded (middle column).

It is apparent that transmitter release evoked by depolarizing pulses facilitates in a manner similar to that seen with normal e.j.p.s and e.r.j.p.s. When responses were evoked by nerve spikes, the e.r.j.p. increased 57 % (F = 0·57) (Fig. 8*A*) when the frequency was increased from 2 to 10 Hz. The transmitter release by depolarizing pulses facilitated by 100 % when the frequency was changed from 2 to 10 Hz (Fig. 8*D*). The intracellular e.j.p.s showed a facilitation of 2·25 for these frequencies (Fig. 8*B*), and a facilitation of 2·64 using depolarizing pulses (Fig. 8*E*). Paired action potential stimulation led to an e.j.p. facilitation of 0·50 at 50 msec separation and 1·0 at 10 msec separation (Fig. 8*C*). Paired depolarizing pulses in tetrodotoxin evoked responses where the second showed facilitation of 1·20 at 30 msec separation and 1·25 at 10 msec (Fig. 8*F*). The minor differences between action potential and pulse-evoked facilitation are due in part to the fact that facilitation depends to some extent on pulse parameters, and also that the potential profile in the nerve terminals evoked by electrotonic depolarization is highly non-uniform, while the action potential is all-or-none.

It is difficult to distinguish the e.r.j.p. from the stimulus artifact in computer-averaged records (Fig. 8*D*). Actually, the artifact is quite brief, lasting less than 0·5 msec after the end of the pulse. This is clearly shown by records of single responses at 2 Hz (Fig. 8*G*) and 10 Hz (Fig. 8*H*). The e.r.j.p. shows large fluctuations due to the statistical nature of transmitter release (del Castillo & Katz, 1956; Dudel & Kuffler, 1961*a*). By assuming that Poisson statistics govern transmitter release, the average number of quanta released can be estimated from the proportion of failures (del Castillo & Katz, 1954*a*). Since facilitated transmitter release does not follow Poisson statistics precisely (Bittner & Harrison, 1970; Johnson & Wernig, 1971; Zucker, 1973), this estimate of m, the average number of quanta composing a response, is only an approximation. In the 2 Hz sequence illustrated in Fig. 8*G*, there were six failures in twenty responses (m = 1·20). In the 10 Hz sequence (Fig. 8*H*), there were three failures in thirty-nine responses (m = 2·33). Short runs were necessitated by the instability of these preparations. Facilitation at 10 Hz is calculated as 0·94, which compares well with the value (F = 1·00) obtained from the computer-averaged e.r.j.p. amplitudes.

Results similar to those in Fig. 8 were obtained in three other experiments. No controls of spike-evoked responses were collected, since the electrodes had to be repositioned after adding tetrodotoxin. The values of facilitation to depolarizing pulses at 10 Hz, compared to 2 Hz, were 5·7, 2·6 and 3·0. These values, plus those discussed above, fall well within the range of values of facilitation elicited by similar trains of action potentials.

Responses to focal nerve depolarization. In order to avoid the uncertainties inherent in the electrotonic method of depolarization (see Discussion and Katz & Miledi, 1967*a*), extensive attempts were made to evoke transmitter release by the focal method of stimulation (see Methods). With this technique, the area of nerve depolarized by an extracellular micro-electrode is restricted to the region immediately under the electrode, and the time course of depolarization more faithfully follows that of the current pulse (Katz & Miledi, 1967*b*). It is thus possible to study systematically the effects of pulse parameters on transmission and facilitation.

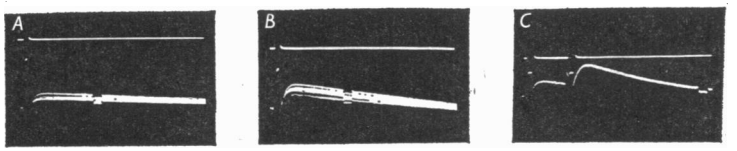

Fig. 9. Facilitated transmitter release evoked by focal nerve stimulation in a tetrodotoxin-blocked preparation. The top trace is a current monitor. The bottom trace is an intracellular record from the muscle fibre on which the focally depolarized synapse was located. Pulse frequency was 1 Hz in *A*, 10 Hz in *B*. Several successive responses are photographically superimposed. *C* shows an example of responses to paired stimulation with a separation of 20 msec. Calibration pulses: 200 μV, 5 msec. Temperature: 10° C.

Unfortunately, synaptic transmission was successfully evoked and observed in only one experiment using focal depolarization. Responses to repetitive stimulation at 1 and 10 Hz are shown in Fig. 9*A* and *B*. Superimposed traces at each frequency reveal a fluctuating response. The smallest deflexions are failures, revealing the stimulus artifact. Larger responses fall into a few amplitude classes, and can be classified as singlets, doublets or occasionally triplets. These fluctuating responses were reversibly abolished by magnesium-substituted calcium-free medium, and by the addition of 5 mM cobalt chloride.

The responses to about 100 stimuli at each frequency were recorded and classified as failures, singlets, etc. The total number of quanta released, divided by the number of trials, provides an estimate of m, the average number of quanta released. At 1 Hz, m was 0·55, and at 10 Hz it was 1·51. Thus facilitation at 10 Hz was 1·76. Facilitation also occurred using paired stimuli. With a 20 msec separation, and using longer pulses, m was 1·80 for the first pulse and 2·65 for the second, and facilitation was 0·48. An example of a response to paired pulses is shown in Fig. 9*C*.

An attempt was made to characterize the statistics of transmitter release evoked by constant nerve-depolarizing pulses for data obtained with

either depolarizing technique. Details of the statistical methods are presented elsewhere (Zucker, 1973). Unfortunately, trends appeared in the data, which invalidates the assumption of stationarity required by the statistical analysis. Furthermore, the estimates of the number of quanta in a response were subject to considerable error. Nevertheless, the data could be fairly well described by binomial statistics. An example is given in Table 1.

TABLE 1. Statistics of transmitter release evoked by focal nerve depolarizing pulses in a tetrodotoxin-treated preparation. Frequency: 10 Hz. Stimulus parameters: 18 μA, 2·5 msec. N, number of trials. n_x, number of responses containing x quanta. m, average number of quanta released per stimulus. p and n are the parameters of the binomial distribution which best describes the data. This distribution, and the predictions of the Poisson distribution ($p = 0$), are shown below the observed distribution

Source of n_x	N	n_0	n_1	n_2	n_3	$n_{\geqslant 4}$		
Observed	133	21	43	49	20	0	$m = 1{\cdot}51$	
Binomial prediction	133	19	48	46	18	2	$p = 0{\cdot}42$	$n = 3{\cdot}6$
Poisson prediction	133	29	44	34	17	9	$p = 0$	

DISCUSSION

A major result of these experiments is that the nerve terminal potential exhibits no consistent change during facilitation. There is thus no evidence for the hypothesis that facilitation is due to a growth in the presynaptic action potential. This conclusion contradicts the common view (Dudel, 1965*a*; Atwood, 1967; Bittner, 1968; Sherman & Atwood, 1972; Atwood & Lang, 1973; Lang & Atwood, 1973) that crustacean neuromuscular facilitation is due to a larger n.t.p. This view is largely based on earlier results in which monophasic positive n.t.p.s appeared to increase during facilitation. A comparison of the original figures (e.g. Dudel, 1965*a*) with Fig. 1 in this paper indicates that many, if not all, the computer-averaged positive n.t.p.s were heavily contaminated by non-specific muscle responses. This interpretation is consistent with the linear relation between the amplitudes of this positive potential and the e.r.j.p., when both change during facilitation (Dudel, 1965*a*). The present results show that when care is taken to distinguish n.t.p.s from n.s.m.r.s, even monophasic positive n.t.p.s do not necessarily grow during facilitation. Similar results were obtained in early experiments by Dudel (1963, Table 2) in which single records of n.t.p.s and e.r.j.p.s were photographically superimposed, rather than computer-averaged. Recently Ortiz (1972) and Lang & Atwood (1973) have also found facilitating synapses in the crayfish claw opener

with monophasic positive n.t.p.s which do not increase during facilitation. Linder (1973) has also reported such synapses in crab.

Dudel (1963, 1965*a*, *b*) believed that only sites with monophasic n.t.p.s were located at the very ends of nerve terminals. Although this interpretation is probably correct, there is no reason to concentrate exclusively on recordings from extreme tips of nerve terminals. Transmitter is released from points scattered all along fine nerve branches (Atwood & Morin, 1970). These sites were found to facilitate just as much as the relatively rare terminal sites.

One objection to the interpretation of recordings from sites with multiphasic n.t.p.s is that the prominent negative phase may represent current through parts of the motor nerve terminal distant from the electrode, or from nearby large nerve branches (Dudel, 1965*b*). In older experiments using the ventral muscle surface, this explanation is possible, because the motor neurone branches densely over this surface (Mangold, 1905; Lang *et al.* 1972). In contrast, very few neurone branches are present on the dorsal surface, whose fibres are innervated mainly by nerve branches running upward through the muscle. This was the main reason for working with this surface, where it is difficult to find synaptic sites. The paucity of nerve branches makes contamination of the n.t.p. by non-terminal nerve currents unlikely. This conclusion is supported by the fact that n.t.p.s were not observed at random electrode placements.

The present results indicate that most nerve terminals are excitable near points where transmitter is released. The diphasic or triphasic wave forms of most n.t.p.s imply an active invasion of the terminals by spikes. Even the monophasic positive n.t.p. recorded from nerve terminal ends is consistent with active spike invasion (Katz & Miledi, 1965*a*). These results and the absence of a consistent correlation between n.t.p. and e.r.j.p. amplitudes during facilitation suggest that most, if not all, nerve terminals are fully invaded by all-or-none action potentials which do not increase during facilitation. Only during presynaptic inhibition does the action potential appear to be blocked (Dudel, 1965*b*).

The variable changes in n.t.p. which sometimes accompany changes in stimulating frequency are often attributable to undetected muscle movements or to changes in synchrony of spikes arriving at a recording site. The reduction in n.t.p. frequently seen to the second of closely spaced paired stimuli may well represent a real change in action potential. It is possible that the n.t.p. reduction is due to a depolarizing afterpotential in the terminals. Such a phenomenon was earlier suggested by Dudel (1965*a*). In a following paper (Zucker, 1974*b*) evidence is presented in support of the existence of such an afterpotential. It is also shown to be unrelated to facilitation.

Recently, results of experiments with caesium on crab neuromuscular junctions (Atwood & Lang, 1973) have been adduced in support of the partial spike invasion hypothesis of facilitation (Lang & Atwood, 1973). Caesium, like TEA, prolongs the n.t.p. and enhances transmission. In crab, as in crayfish (Bittner, 1968), some muscle fibres display more facilitation than others. Atwood & Lang (1973) found that caesium had a larger effect on transmission in highly facilitating fibres, where the unfacilitated response is normally quite small. They concluded that this result supported the idea that highly facilitating terminals are not fully invaded by spikes, while non-facilitating terminals are. In view of the present results, a more likely explanation is that, whatever the mechanism for facilitation, non-facilitating terminals normally more nearly saturate the release mechanism. Hence their initial responses are large, and less subject to increase in response to a longer presynaptic spike in the presence of caesium than the facilitating synapses where the release mechanism is quite unsaturated.

Bittner & Harrison (1970) suggested that a correlation between variations in extracellularly recorded junctional potential (e.r.j.p.) and intracellular e.j.p. amplitudes from the same fibre might be due to variable terminal invasion. However, they found such correlations in only a very small proportion of the sites they studied, and much of the correlation may be due to the contribution to the e.j.p. of the individual synapse generating the e.r.j.p. Thus the effects of caesium ions, and the e.j.p.–e.r.j.p. correlations, are not inconsistent with the conclusion of this paper that the nerve terminals are invaded by action potentials.

The situation in crayfish can be compared to data on other facilitating synapses. Facilitation in the squid stellate ganglion giant synapse (Miledi & Slater, 1966), the chick ciliary ganglion (Martin & Pilar, 1964), rat neuromuscular junction (Hubbard & Schmidt, 1963), frog neuromuscular junction (Katz & Miledi, 1965*b*; Braun & Schmidt, 1966) and crab neuromuscular junction (Linder, 1973) was unaccompanied by increases in presynaptic spike amplitude or duration. At the vertebrate neuromuscular junctions, paired stimulation led to increased transmitter release, but a reduced n.t.p., to the second stimulus. Furthermore, motor nerve terminals in both frog and rat are excitable and actively invaded. These junctions are thus very similar to those in crayfish. The only known place where facilitation might be caused by changes in the presynaptic spike is the primary afferent synapse on to mammalian spinal motor neurones (Lloyd, 1949; Wall & Johnson, 1958; Eccles & Krnjević, 1959). The mechanism of facilitation and post-tetanic potentiation at this synapse is in dispute (Eccles & Rall, 1951; Curtis & Eccles, 1960).

The experiments with pulsed nerve stimulation in tetrodotoxin further demonstrate that facilitation does not depend on action potentials. These

results are similar to those obtained at the squid giant stellate synapse (Bloedel, Gage, Llinás & Quastel, 1966; Katz & Miledi, 1967*c*) and the frog neuromuscular junction (Katz & Miledi, 1967*a*), where facilitation was demonstrated to paired nerve depolarizations in tetrodotoxin. Thus facilitation, and transmitter release in general, do not require the sodium influx that accompanies nerve spikes, and cannot be caused by the accumulation of sodium intraneuronally, as proposed by Birks & Cohen (1968). (This may well be the mechanism responsible for long-term facilitation, however; see Sherman & Atwood, 1971.)

One problem with the experiments using the electrotonic technique of polarization is that constant current pulses may not generate constant potential changes at the synaptic release sites some distance from the stimulating electrode. However, the most likely effect is an increase in potassium conductance (delayed rectification) during a train of pulses. This will reduce the terminal length constant and hence the potentials developed at the synapses. Since a smaller depolarizing pulse evokes a smaller transmitter release, the likely effect of delayed rectification is to reduce transmission to repeated pulses, not to increase it.

It was hoped that the use of focal nerve depolarization would circumvent these difficulties. For this reason the one successful experiment with this technique is significant. Unfortunately, many properties of this preparation work against the use of this technique: the low density and inaccessibility of synaptic release sites (Atwood & Morin, 1970; Lang *et al.* 1972), the fact that this technique only depolarizes a very restricted area of nerve membrane (Katz & Miledi, 1967*b*), the necessity of recording intracellularly and the low amplitude of single quantum responses (Bittner & Kennedy, 1970), and the high susceptibility of the muscle membrane to 'breakdown' (Katz & Miledi, 1967*b*). Because of these limitations, attempts at focal polarization of synapses were usually unsuccessful.

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