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AN ELECTROPHYSIOLOGICAL INVESTIGATION OF MAMMALIAN MOTOR NERVE TERMINALS

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The small size of motor nerve terminals and their intimate relationship with the muscle membrane has made it difficult to use conventional electrophysiological techniques for exploring their properties. It is not surprising, therefore, that several fundamental questions have remained unsettled: namely, do impulses actually invade the terminals? What are the effects upon the terminals of repetitive nerve impulses? Have the terminals unique pharmacological properties? It has been postulated that nerve action potentials actually invade the terminals and that the liberation of acetylcholine (ACh) is in logarithmic proportion to the magnitude of the depolarization produced by the action potential (for reviews, see e.g. Eccles & Liley, 1959; Katz, 1962). However, it has been suggested recently that the nerve terminals cannot sustain an action potential but are invaded electrotonically (Riker, Roberts, Standaert & Fujimori, 1957; Riker, Werner, Roberts & Kuperman, 1959*a*, *b*; Riker, 1960; see also Dudel & Kuffler, 1961*b*; Dudel, 1962*b*). There is also considerable divergence of opinion with respect to the nerve terminal changes which are responsible for the potentiation of transmitter release after repetitive stimulation that gives post-activation or post-tetanic potentiation (Hutter, 1952; Liley & North, 1953; Liley, 1956*b*). Thus the process has been attributed to the changes in the amplitude of the presynaptic spike potential (Lloyd, 1949), in the volume of the presynaptic terminals (Eccles, 1953) and in the availability of transmitter for release (Eccles, 1957; Hubbard, 1959; Curtis & Eccles, 1960).

Our investigation of these problems has been made possible by the development of a reliable method of placing a micro-electrode close to motor nerve terminals in the rat diaphragm *in vitro*. With this technique it has been possible to study nerve terminal properties as revealed by extracellular recording and stimulating. A brief report of some of the present experiments has been published (Hubbard & Schmidt, 1961, 1962).

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METHODS

All experiments were performed *in vitro* using rat hemidiaphragm-phrenic nerve preparations, obtained from young albino rats of the Wistar strain. The method of dissection, the divided recording chamber, the mounting of the preparation and the aeration and composition of the bathing fluids have been previously described (Liley, 1956*a*; Hubbard, 1961). The temperature of the fluid over the preparation was monitored with a thermistor, while the temperature of the water-bath, in which the recording chamber was set, could be kept by a thermistor-controlled feed-back circuit at any desired temperature between 22–24° C (room temperature) and 40° C. Neuromuscular transmission was blocked by raising the concentration of $MgCl_2$ in the bathing solution to 11–12 mM.

In all experiments 4 M-NaCl-filled micro-electrodes with 1·5–3 μ tips and 1–3 MΩ resistance were used for both recording and stimulating. Conventional techniques (Hubbard, 1961) were employed for the extracellular recording of nerve spikes, miniature end-plate potentials (m.e.p.p.s) and end-plate potentials (e.p.p.s). When stimulating nerve terminals the micro-electrode was connected to the output of the isolation units of two Grass stimulators arranged in series and triggered, generally at 2 sec intervals, from the oscilloscope time base. The stimulating pulses were rectangular in form and from 0·06 to 0·1 msec in duration. They were recorded across a 50 Ω resistance in the stimulating circuit and monitored on the oscilloscope screen. The antidromic nerve impulses set up by stimulation through the micro-electrode were recorded from the phrenic nerve with a pair of platinum electrodes. The same electrodes could also be used for stimulation of the phrenic nerve. In some experiments separate pairs of electrodes on the phrenic nerve allowed simultaneous stimulation and recording.

In experiments in which nerve terminals were polarized the technique described by Hubbard & Willis (1962*a*) was used. An Agar-Ringer-filled polarizing electrode was moved to the vicinity of the recording micro-electrode, successful location being signalled by the progressive increase of extracellular e.p.p.s evoked during the passage of hyperpolarizing currents.

RESULTS

Location of micro-electrode

Throughout the present investigation a micro-electrode was judged to be located close to an end-plate only when extracellular m.e.p.p.s. were recorded from it. This criterion was selected first, because previous investigators had found that m.e.p.p.s. can be recorded extracellularly only in very close proximity to motor end-plates (del Castillo & Katz, 1956; Liley, 1956*a*; Dudel & Kuffler, 1961*a*). Secondly, as explained in later sections of these results, it became plain as the investigation proceeded that only when the electrode was in such a position was it invariably possible to record from or to stimulate nerve fibres in the vicinity of their endings. Finally, a number of observations could only be explained if, when recording m.e.p.p.s., the electrode was very close to the end-plate region of muscle fibres. Thus movement of a few microns upwards or sideways caused all trace of the m.e.p.p.s to be lost. A small downward movement caused impalement of the underlying muscle fibre. Further, certain features of the frequency and amplitude distribution of extracellular m.e.p.p.s suggested that the electrode could directly influence the

nerve endings and was thus in close proximity to them. For instance, an increase in extracellular m.e.p.p. frequency could almost always be provoked by deliberate small movements of the electrode. The amplitude distribution of such m.e.p.p.s often showed that there was an excess of the largest potentials, which presumably were those generated in response to quantal emission from presynaptic terminals that were in close proximity to the micro-electrode and hence most susceptible to damage by it. Indeed, when recording began at many junctions, there was often a high frequency of the largest m.e.p.p.s As it was transient, this local increase in frequency may have been due to leakage of hypertonic NaCl from the electrode.

Extracellular m.e.p.p.s were only found in the vicinity of very superficial nerve branches, which suggests that junctions on the surface of the preparation were selected for recording. Once located the electrode tip appeared to be held in position by the strong connective-tissue sheath of the muscle and this stability of recording enabled more extensive observations of extracellular m.e.p.p.s to be made than had previously been possible on a mammalian preparation (Liley, 1956*a*). Compared with intracellular m.e.p.p.s at the same junction (Fig. 1*B*) the extracellular m.e.p.p.s (Fig. 1*D*) showed a reversed polarity, faster time course, lower frequency and different amplitude distribution, as has previously been described for amphibian (Fatt & Katz, 1952) and crustacean (Dudel & Kuffler, 1961*a*) extracellular m.e.p.p.s. In the experiment illustrated (Fig. 1) the rise time was $0{\cdot}42 \pm 0{\cdot}06$ msec (mean ± I.S.D.) for 25 consecutive extracellular m.e.p.p.s and $0{\cdot}70 \pm 0{\cdot}07$ msec for the same number of intracellular m.e.p.p.s. The respective half-decay times were $0{\cdot}71 \pm 0{\cdot}04$ msec and $0{\cdot}81 \pm 0{\cdot}05$ msec. These times are much shorter than those previously published (Liley, 1956*a*, Table 2) for mammalian m.e.p.p.s. Sometimes the m.e.p.p.s were slow to rise and flat-topped, possibly indicating local saturation of ACh receptors (del Castillo & Katz, 1956). The higher frequency of intracellular m.e.p.p.s at this junction is illustrated by the fact that only 186 m.e.p.p.s were recorded extracellularly in 33·35 sec while 317 m.e.p.p.s were recorded intracellularly in 32·5 sec. Figure 1 shows further that the typical Gaussian distribution of intracellular m.e.p.p. amplitudes (Fig. 1*A*) changes to a skew distribution (Fig. 1*C*) when the recording electrode is placed outside the muscle fibre. The largest class of m.e.p.p.s is then just above the noise level (cross-hatched column). For the amphibian neuromuscular junction del Castillo & Katz (1956) concluded that the smaller frequency and the skew distribution of extracellularly recorded m.e.p.p.s would be explained if the electrode tip recorded potentials arising in an area which was smaller than the total area of the motor end-plate. They calculated that m.e.p.p.s generated more than

15–20 μ from the recording electrode would be lost in the amplifier noise. As similar differences between intra- and extracellular amplitude distribution and frequency occur in the rat, it seems that, despite the more compact nature of the rat end-plate (Cole, 1957), an extracellular electrode can in the rat, as in the frog, record only from some fraction of the end-plate area.

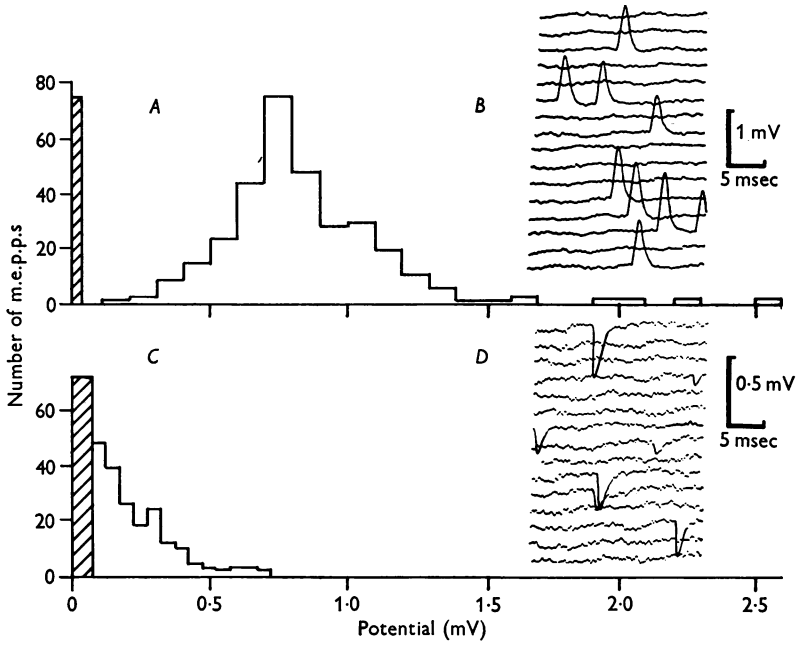

Fig. 1. A comparison of intra- and extracellular m.e.p.p.s recorded at the same junction. *A*, *C*. Amplitude histogram of intra- and extracellular m.e.p.p.s, respectively. The width of the cells in the histogram was 100 μV in *A* and 50 μV in *C*. The cross-hatched column represents the noise level. *B*, *D*. Sample records of intra- and extracellular m.e.p.p.s, respectively. The records are composite and do not give an accurate representation of m.e.p.p. frequency. The temperature was 25° C. The records have been retouched.

Recording at the neuromuscular junction

Spike potential. While the micro-electrode was recording extracellular m.e.p.p.s, the phrenic nerve was stimulated to evoke focal extracellular e.p.p.s. These e.p.p.s were distinguished in that they were invariably preceded by small diphasic (positive-negative) spike potentials (Fig. 2). When the stimulus strength was reduced, both spike and end-plate potential failed together, the spike potential being single and all-or-nothing (Fig. 2*A*, *B*, *C*). It can therefore safely be assumed that the spike potential was recorded from the nerve terminating at the junction from which both the

e.p.p. and the m.e.p.p.s were recorded. If, as occasionally happened, the spike was compound, it was not further investigated. Furthermore, on phrenic nerve stimulation composite spike potentials could be recorded from sites where there were no m.e.p.p.s and which presumably were close to nerve branches. At any given junction the terminal nerve spike potential was largest when m.e.p.p.s were best recorded. A small background component of extracellular e.p.p.s from neighbouring end-plates was usually found (Fig. 2*A*) except when the $MgCl_2$ content of the bathing fluid was so high that the e.p.p.s were extremely small. If the $MgCl_2$ content of the bathing solution was raised to a level at which intermittent

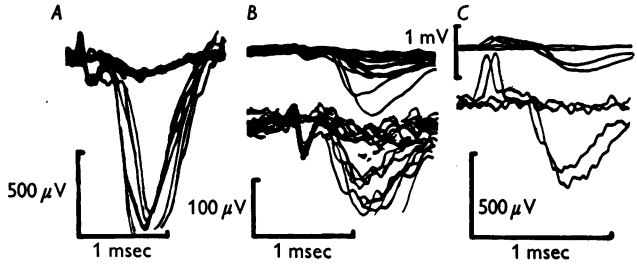

Fig. 2. Sample records from three junctions of the presynaptic spike potential and accompanying e.p.p. in response to threshold stimulation of the phrenic nerve. In *B* and *C* the records were taken simultaneously at two different gains. Note the separate voltage scale for the upper record in *C*. The voltage scale of *A* also applies to the upper record in *B*. For further description see text.

failure of e.p.p. response occurred, there was no failure of the accompanying spike potential. The failure of an e.p.p. response under such conditions was thus not due to a presynaptic blockage of nerve impulses (cf. del Castillo & Katz, 1954*a*). There was also no correlation between the variations in e.p.p. amplitude found in solutions with high levels of magnesium (del Castillo & Katz, 1954*b*) and the small individual variations in spike potential amplitude (see e.g. Fig. 6, CON). The variations in spike size appeared to be due to random fluctuations in the base-line noise level, for the range of variation was considerably reduced either by using a high-frequency cut-off in the recording circuit or by selecting spike potentials larger than the average (see below) for study.

The diphasic positive-negative form of the spike potential (Fig. 2*A*, *B*) is that expected for unipolar recording from the terminal of a nerve fibre in volume (Lorente de Nó, 1947). Similar spike potentials have been observed when recording from the terminal region of crayfish neuromuscular junctions (Dudel & Kuffler, 1961*a*; Dudel, 1962*a*, *b*). The relative size of the positive and negative components (Fig. 2*A*, *B*, *C*)

varied widely from ending to ending, which possibly is indicative of variations in the position of the micro-electrode tip relative to the presynaptic terminal. Most frequently, however, the initial positive component was smaller than the following negative one, as is shown in the typical records of Figs. 2*A*, *B*, 3, 6. Occasionally a purely positive spike was seen (Fig. 2*C*), suggesting blockage of impulse conduction and electrotonic spread of the action potential to the recording site. In these cases the passive behaviour of the membrane may have resulted from damage due to mechanical pressure of the micro-electrode (Freygang & Frank, 1959; Murakami, Watanabe & Tomita, 1961; Terzuolo & Araki, 1961).

The amplitude of the spike potential was always measured between the positive and negative peaks and it was assumed that this amplitude was directly proportional to the size of the action potential which would be recorded with an intracellular electrode, though it is realized that this is justified only as a first approximation. Very slight movements of the recording electrode caused the extracellular potential of the nerve terminal to vary in form between the characteristic time courses of the first or second derivative of the intracellular potential (Lorente de Nó, 1947). If conduction in the terminals was purely electrotonic the extracellular spike would be purely positive and would be directly proportional to the intracellular potential (Dudel, 1962*b*). Thus the above assumption would perhaps lead to an over-estimation of the actual variations of the intracellular spike under various experimental conditions (see below), but a more reliable calculation of the intracellular potential changes from our extracellular records is not possible. The amplitude of the presynaptic spike potentials, thus measured, was usually of the order of 80–300 μV (average of 53 junctions 140 μV) but occasionally potentials up to 820 μV were recorded. The neuromuscular delay was measured as the time interval between the peak of the positive potential and the onset of the e.p.p. The positive peak most probably signals the maximum rate of rise of the intracellular spike potentials (or its first derivative), and thus the beginning of transmitter release, according to the views of Liley (1956*c*). This interval was always measured in 30–60 superimposed records taken at a frequency of 1/sec (e.g. Fig. 3*B*). The average of such observations at 53 junctions was $0{\cdot}217 \pm 0{\cdot}004$ msec (mean $\pm$ S.E.), the temperature being 34–36° C.

Repetitive stimulation. During repetitive stimulation at frequencies of 10/sec or greater it was observed that, parallel with the expected increase in e.p.p. amplitude (Liley, 1956*b*), there was an increase in the amplitude of the accompanying spike potential. A systematic study of this effect of repetitive stimulation was made at a large number of junctions by taking advantage of the fact that after 10–20 impulses at a suitable frequency the e.p.p. amplitude reaches a new level and increases only slowly with

further stimulation (Liley, 1956*b*). The effects of frequencies of stimulation between 1 and 320/sec were then quickly assessed by superimposing photographically serial traces of e.p.p.s and spike potentials, usually recorded simultaneously at two different amplifications—a high gain to show the spike potential and a lower gain for the e.p.p. The expected increase in e.p.p. amplitude with increasing frequency of stimulation can clearly be seen (Fig. 3*A*). There is also an associated increase in spike size (Fig. 3*B*), as may be observed in more detail in Fig. 3*C* where the amplitudes of e.p.p. and spike potential at frequencies between 1 and 320/sec are plotted as percentages of their respective amplitudes at 1/sec. This frequency was selected for control observations, as frequencies of stimulation below 5/sec appeared in numerous experiments to have no consistent

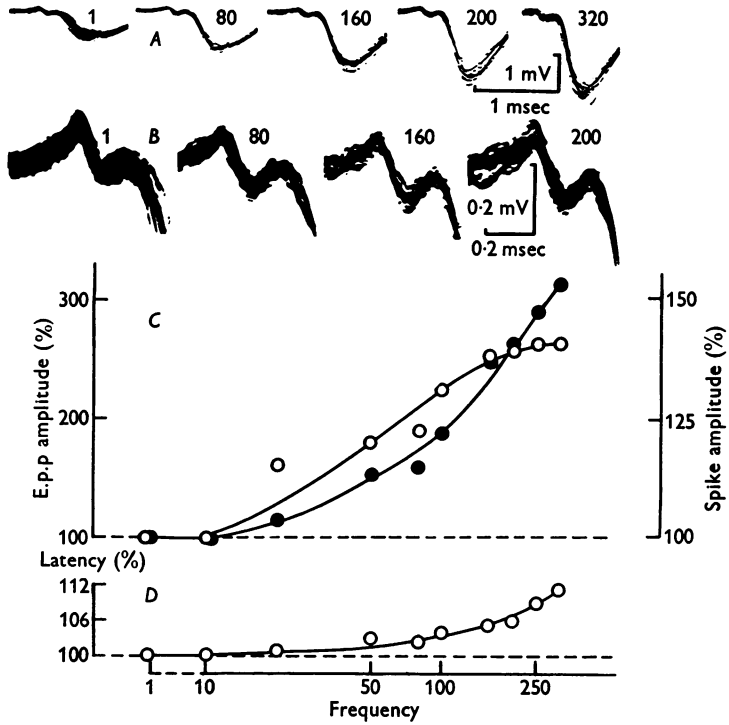

Fig. 3. Extracellular e.p.p.s and presynaptic spike potentials during repetitive stimulation at frequencies from 1 to 320/sec. *A*, records of extracellular e.p.p.s made by superimposing 30–60 faint traces during stimulation at the stated frequencies. *B*, as *A* but taken simultaneously at a much higher gain to show detail of the spike potential. *C*, spike amplitudes (○) and e.p.p. amplitudes (●) plotted as a function of frequency of stimulation (semi-log. scale). The amplitudes of spike and e.p.p. during stimulation at 1/sec were taken as 100 %. Note different ordinates for spike and e.p.p. amplitudes. *D*, latency of presynaptic spike potential as a percentage of the latency during stimulation at 1/sec. Temp. 35° C.

effect on e.p.p. and on spike potential amplitude. An increase in the spike potential latency (measured between stimulus artifact and peak of the positive component of the spike) was also consistently found during repetitive stimulation at frequencies of 10/sec or above (Fig. 3*D*).

It was initially puzzling that the spike potential amplitude did not appear to increase so much at frequencies above 100/sec as at lower frequencies. This discrepancy, together with the proportionately greater increase of e.p.p. amplitude, some 200–300 % compared with spike potential increases of 30–40 %, suggested to us that there might be a logarithmic relationship between spike and e.p.p. amplitudes during repetitive stimulation. Figure 4 illustrates one of many experiments in

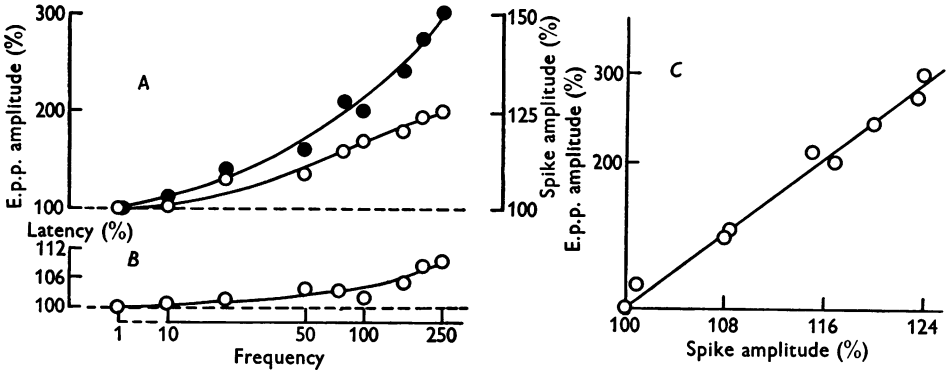

Fig. 4. The relationship between amplitudes of e.p.p. and spike potential during repetitive stimulation. *A*, *B*, spike potential amplitudes and latency, and e.p.p. amplitude plotted as in Fig. 3 (semi-log. scale). *C*, e.p.p. amplitudes replotted on a logarithmic scale against spike amplitudes on a linear scale. Temp. 35·5° C.

which this possibility was investigated. Figure 4*A* and *B* shows, as in Fig. 3, the increases in e.p.p. amplitude and in spike potential amplitude and latency which occurred during repetitive stimulation. In Fig. 4*C* the increased e.p.p. amplitude is plotted on a logarithmic scale against the increased amplitude of the spike potential (abscissa). The straight-line relation found in this experiment was obtained consistently in many similar experiments. If the spike potential amplitude is an index of the amount of depolarization applied to nerve terminals, the relationship would support Liley's (1956*c*) hypothesis that transmitter release is logarithmically proportional to the amount of this applied depolarization.

Post-tetanic potentiation. After repetitive stimulation the increased e.p.p. and spike amplitudes and the increased spike latency returned to the control values over very similar time courses. In the experiment illustrated (Fig. 5) serial records of e.p.p. and spike potential were taken at 0·5 sec intervals before and after a tetanus of 2000 impulses at 200/sec. As the

sample records (Fig. 5, CON) show, individual traces were superimposed for 10 sec periods before the tetanus and 3 sec periods during the first minute afterwards (Fig. 5, PTP). During the second and later minutes records were again superimposed for 10 sec periods (e.g. Fig. 5, PTP 60–70, 130–140). In the graph e.p.p. and spike amplitudes and spike latency are represented by points placed in the middle of the periods over which they were pooled. The periods increase in duration as they fall at later intervals after the tetanus. Clearly the e.p.p. and spike amplitudes run a parallel course after (Fig. 5, PTP) as well as during repetitive stimulation (Fig. 5, TET).

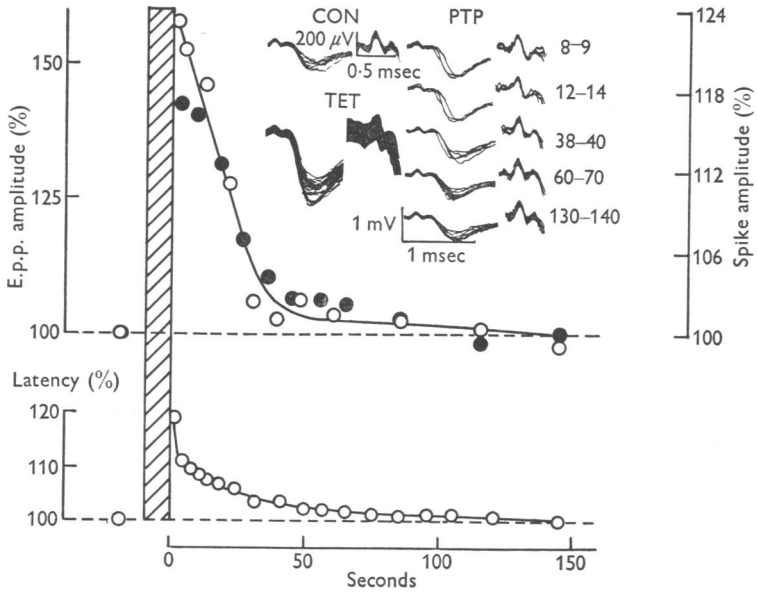

Fig. 5. The decline of extracellular e.p.p. and spike potential amplitudes after tetanic stimulation. The sample records show e.p.p.s and spike potentials simultaneously recorded at different gains, before (CON) during (TET) and after (PTP) a tetanus of 2000 impulses at 200/sec. The figures beside the PTP series refer to the time (sec) after the tetanus over which the sample records shown were superimposed. The same voltage calibration (200 μV) applies to all the spike potentials. Similarly the mV calibration is common to all the e.p.p.s. The graph based on this and other records shows e.p.p. (●) and spike (○) amplitudes and spike latency as percentages of the control values. For further description see text. Temp. 35° C.

In some experiments (Fig. 6) the spike potential was large enough to permit much more extensive observations of the effects of frequency and durations of stimulation upon the post-tetanic potentiation of spike potential amplitude.

Figure 6 shows that, as the number of stimulating volleys was increased from 500 to 1500, there was an increase in the maximum size and in the

duration of the potentiation of spike amplitude. After 300 volleys in the same experiment (not illustrated), the spike increase was smaller and shorter than after 500 (Fig. 6, 500 volleys). Representative sample records show the control size of the spike potentials (CON) and the first seven spike potentials after stimulation (PTP), in serial order from left to right. In the graph spike sizes for the first 5 sec after the tetanus are plotted directly, while at later intervals the points represent averages of the spike amplitudes over 5 sec, stimulation being at 1/sec before and after the period of tetanic stimulation.

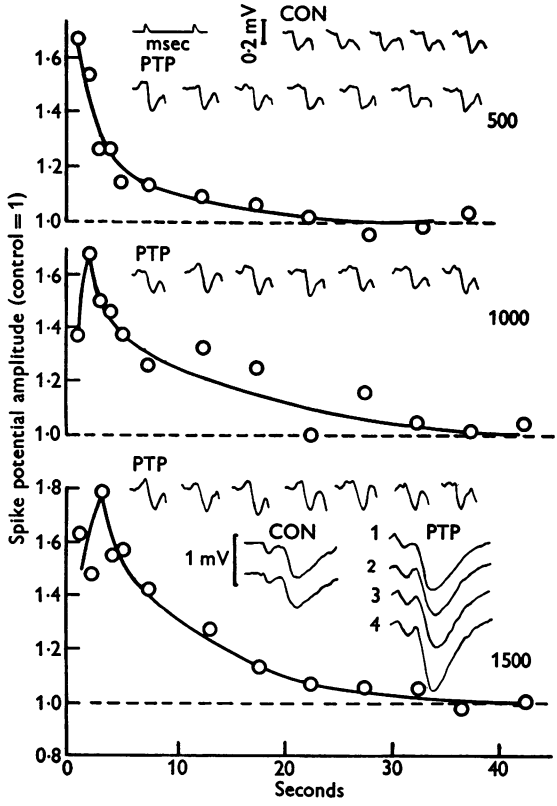

Fig. 6. Post-tetanic potentiation of spike potential amplitude after 500, 1000 and 1500 stimuli at a frequency of 100/sec. In CON sample records of the control pre-tetanic spike amplitude are shown. The control amplitude did not vary throughout the experiment. PTP indicates in each case the first seven post-tetanic spike potentials arranged in serial order from left to right. Stimulation before, and after the tetanus was always at 1/sec. The sample records of e.p.p.s were recorded simultaneously with the spike potentials but at a lower gain. CON indicates records of sample pre-tetanic e.p.p.s, and PTP denotes the first four post-tetanic e.p.p.s after a tetanus of 1500 stimuli at 100/sec. In the graph spike amplitudes are plotted individually for the first five post-tetanic records, thereafter the points represent the mean of five serial records. Temp. 32·5° C.

As the sample records show (Fig. 6, 1500 volleys), e.p.p.s were recorded at the same time as the spike potentials. The e.p.p.s showed comparable changes to the spike potentials in magnitude and duration of potentiation. An interesting point was that, as previously described (Hubbard, 1959), after a tetanus of about 1000 volleys the e.p.p. potentiation took a few seconds to reach its peak. Thus after 1500 volleys (Fig. 5) the 3rd and 4th e.p.p.s are bigger than the 1st and 2nd e.p.p.s. Paralleling this short delay there was a similar delay in reaching the peak of spike amplitude. The sample records and graphs show (Fig. 5) that after 500 volleys the first spike recorded was the largest. After 1000 volleys however the second was the largest and after 1500 volleys the third was largest. This parallel delay of e.p.p. and spike potential amplitude should not of course be confused with the delayed development of potentiation found in a curarized preparation. This late delay is much longer after comparable stimulation than that described here.

In Fig. 7, taken from the same experiment as Fig. 6, the effect of 1000 stimuli at frequencies of 100, 50 and 25/sec is displayed. Here again there was a close parallel with post-tetanic potentiation of e.p.p.s, the magnitude of the potentiation increasing with the increase in frequency, but the duration being longest with the lower frequency. This latter feature was only true when the number of stimulating volleys was of the order of 1000 or more. After 500 volleys at 50/sec for instance in the same experiment the potentiation was much shorter than after 500 volleys at100/sec,whereas after 1500 volleys the effects of stimulation at 50/sec also outlasted the effects at 100/sec. This somewhat paradoxical finding has been described for the potentiation of e.p.p.s in similar circumstances (Hubbard, 1960).

Potentiation after one impulse. The similarity in time course between the post-tetanic potentiation of the amplitude of e.p.p.s and spike potentials (Figs. 6, 7) prompted us to investigate whether this relationship held for the e.p.p. potentiation which occurs when a second stimulus follows a first at a close interval in a preparation paralysed by excess of magnesium (del Castillo & Katz, 1954*b*). This was done in six experiments in which two stimuli, 4 msec apart, were repeated every second for 150–250 trials. The spike potential and associated e.p.p. were recorded simultaneously at two different gains (cf. Figs. 3, 5) the e.p.p. amplitudes of the paired responses being measured from the records taken at the lower gain and the corresponding spike potentials from the records taken at the higher gain. As expected, the second e.p.p. was usually larger than the first, the mean increase in individual experiments ranging between 26 and 35%. The amplitude of the second spike potential was, however, not increased but rather decreased, the mean decrease in individual experiments ranging from 2·5 to 14% (mean of six experiments, 9%).

Effects of hyperpolarization. The increases in spike potential amplitude found during and after repetitive stimulation (Figs. 5, 6, 7) could well be explained by a hyperpolarization of nerve fibres brought about by the stimulation (Lloyd, 1949; Eccles & Krnjević, 1959). It was therefore of interest to try to mimic these changes by applied hyperpolarizing currents.

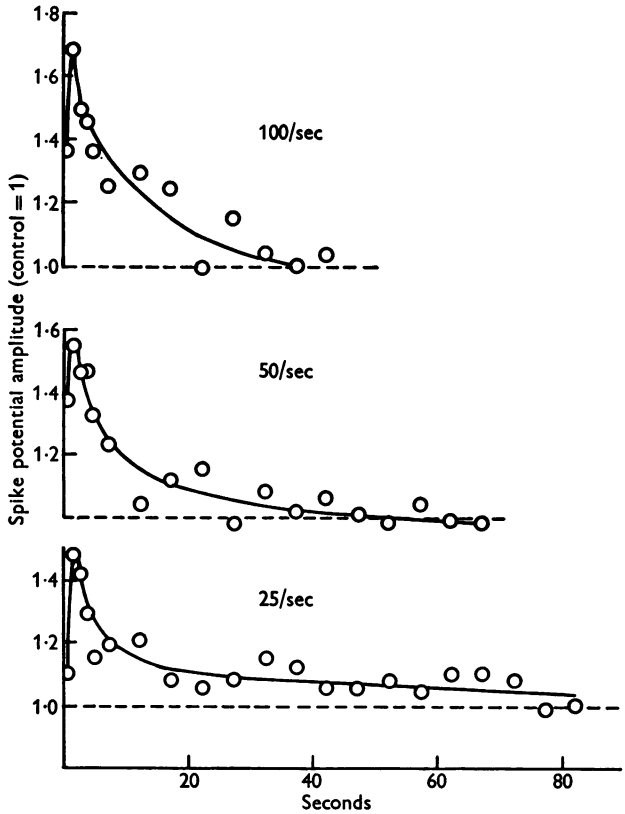

Fig. 7. The post-tetanic potentiation of spike potential amplitudes after 1000 stimuli at 100/sec, 50/sec and 25/sec, respectively. The results are drawn from the same junction as that illustrated in Fig. 6. The points in the graph represent individual spike amplitudes for the first five post-tetanic seconds and thereafter represent the mean of five consecutive spike potentials. These latter points are placed in the middle of the 5 sec period over which the spike amplitudes were pooled. Before and after the tetanus stimulation was at 1/sec.

As the sample records (Fig. 8*A*) and graph (Fig. 8*D*) show, when a current of 8·5 μA was applied the spike potential increased in amplitude within the 2 sec period between observations. The new amplitude was thereafter maintained until the current ceased, when it reverted to the control size, again within the 2 sec period between observations. The latency of the

presynaptic spike was increased during the application of current and reverted to the control value when the current ceased to flow. As expected, the amplitudes of the accompanying e.p.p.s. (Fig. 8*A*, *D*) were increased progressively during the period of current flow (Hubbard & Willis, 1962*a*) despite the static spike potential amplitude. When the effects of a wide range of currents were compared (Fig. 8*B*, *C*) the increases in spike potential amplitude appeared directly proportional to the applied current for the smaller values of current (μA) but approached a maximum of 170–180 % of the control value as the current was increased. Individual spike amplitudes were occasionally increased to a greater extent (Fig. 8*B*, max.) The increases in spike amplitude produced by hyperpolarizing currents were not affected by previously applying a depolarizing current.

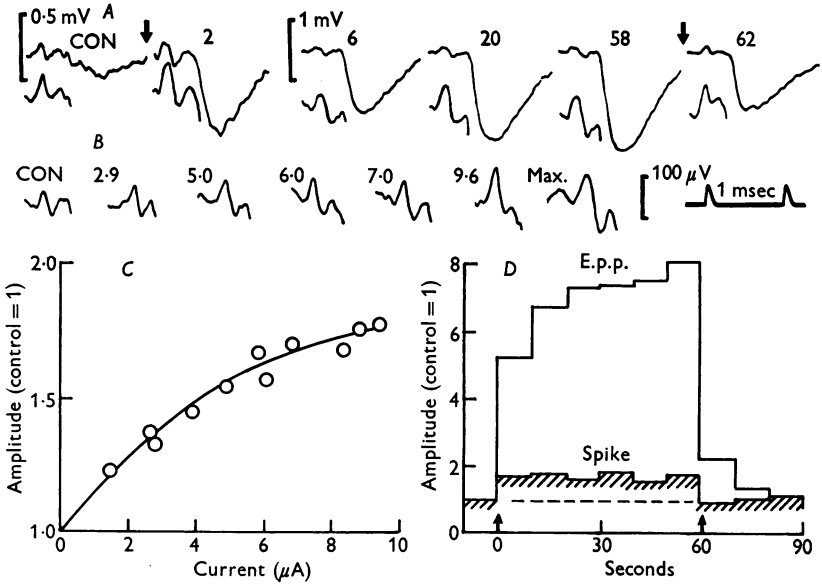

Fig. 8. The effect of hyperpolarizing currents upon spike and e.p.p. amplitudes. *A*, sample records from an experiment in which a current of 8·5 μA was applied for 60 sec. Stimulation was at 2 sec intervals before, during and after the period of current flow. The numbers indicate the time in seconds after the onset of current. In the graph *D* the measurements of e.p.p. and spike potential amplitude from this experiment are averaged over 10 sec periods. The arrows in *A* and *D* mark the turning on and off of the current. *B*, *C*, the relationship between spike amplitude and the strength of hyperpolarizing current. Each current step was applied as shown in *A* and *D*, and there was a minimum interval of 2 min between each current application. *B* shows sample records of spike potential selected because their amplitude was close to the average amplitude during the application of the indicated current (μA). In *C* these averages, based on 10–30 records taken at 2 sec intervals during current flow, are plotted against current strengths in μA. All records from the same junction. Temp. 35° C.

This procedure has been found to reduce the magnitude of the increases in e.p.p. amplitude brought about by a standard hyperpolarizing current (Hubbard & Willis, unpublished observations).

Antidromic stimulation

When the micro-electrode was placed in a position for recording extracellular m.e.p.p.s and e.p.p.s with preceding nerve spike potential, a stimulating pulse through the micro-electrode invariably produced an impulse antidromically conducted up the phrenic nerve. This response was recorded from the nerve in oil in the second compartment of the chamber. In most cases this response was a small all-or-nothing spike potential of the same size as the spontaneous spike potentials sometimes recorded from the nerve (Fig. 9*A*). Occasionally two such potentials were evoked and more rarely compound action potentials were recorded. By using the single antidromic responses as indicators of threshold it was possible to determine, under a variety of conditions, both absolute changes in threshold and the sequence of changes following conditioning stimuli.

The usual form of the excitability cycle after one suprathreshold conditioning stimulus is shown in Figs. 9*D*, *E*; 10; 11*A* (open circles). The relative refractory period lasted from 1·5 to 4 msec and was followed by a supernormal period which, 10–20 msec after the conditioning stimulus, passed into a period of subnormal excitability. This became undetectable 50–90 msec after the conditioning stimulus. Identical changes in excitability after one conditioning stimulus were described by Gasser & Grundfest (1936) and Lehmann (1937) for the A fibres of the cat phrenic nerve *in vitro*. After subthreshold conditioning pulses the threshold of a second testing pulse was reduced. When the conditioning pulse was less than 50–60 % of threshold these changes lasted less than 0·5 msec. After larger but still subthreshold pulses, however, small threshold decreases could be detected for up to 3 msec, suggesting that the stimulated structure was capable of developing local responses (Erlanger & Blair, 1931; Katz, 1937). As the duration of the *in vitro* observation increased, the period of supernormal excitability lengthened, while the period of subnormal excitability shortened (Fig. 9*E*) and eventually became undetectable, as was observed by Gasser (1935) on whole nerve trunks.

In three experiments the effect of cooling the preparation was explored. When the temperature was reduced from 35 to 22° C (Fig. 9*D*) or from 36 to 27° C (two experiments), there was a small increase in the refractory period and a marked increase in the supernormal period but no significant change in the duration of the subnormal period. These changes were completely reversible upon warming to the original temperature. In

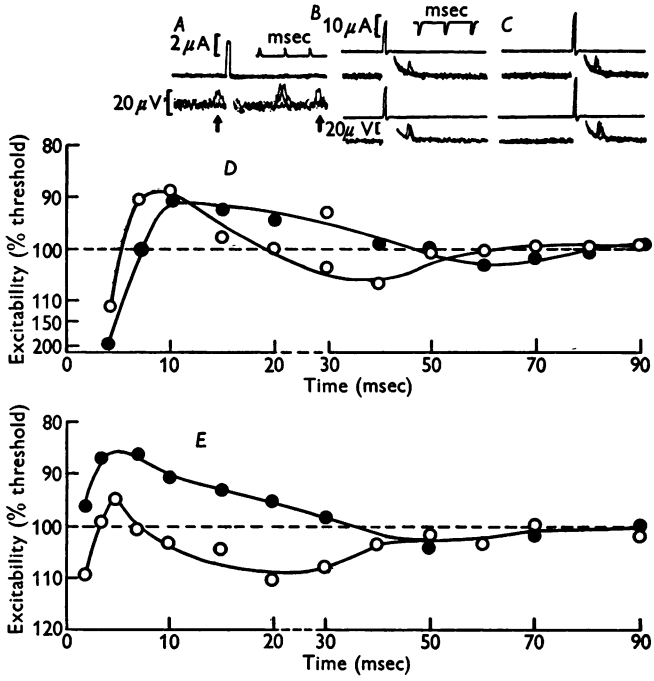

Fig. 9. The antidromic potentials and the excitability changes found on stimulation at the neuromuscular junction. *A*, sample records of antidromic and spontaneous (arrows) potentials recorded from the phrenic nerve in oil (lower trace). The upper trace shows the stimulus current. *B*, *C*, sample records as in *A* of antidromic potentials in the phrenic nerve, on stimulation at the junction from which the records shown in Fig. 3 were obtained. The upper pair of records in *B* and *C* show the antidromic potentials (lower traces) recorded after threshold stimulation. In the lower pairs these potentials were preceded by a conditioning stimulus, the interval being 5 msec in *B* and 50 msec in *C*. Note the unchanged stimulating current required in *B* (lower pair of traces) and the increased current required in *C* (lower pair of traces). In *D* the excitability changes of the terminals from which the record in *A* was obtained are plotted after a single conditioning stimulus, at intervals from 4 to 90 msec. The excitability changes were investigated at 35° C (○) and after the preparation had been cooled to 22° C (●). The changes were completely reversible upon rewarming and the open circles represent the mean values of the excitability at 35° C before and after cooling. Excitability at each interval was determined as the ratio of the conditioned to the unconditioned threshold current (μA). *E*, changes in the excitability cycle of another nerve terminal after one conditioning impulse in the course of a prolonged investigation. ○ results shortly after the beginning of the experiment, ● the excitability cycle 2 hr later. The temperature in *E* was 34° C.

contrast, Lundberg (1948) reported that cooling A fibres of cat phrenic nerve preparations below 37° C markedly reduced the amplitude and duration of negative after-potentials. In non-myelinated fibres, on the other hand, negative after-potentials (Lundberg, 1948; Greengard & Straub, 1958) and thus presumably the supernormal period (Gasser & Erlanger, 1930) increased in duration upon cooling to the temperatures investigated in our experiments.

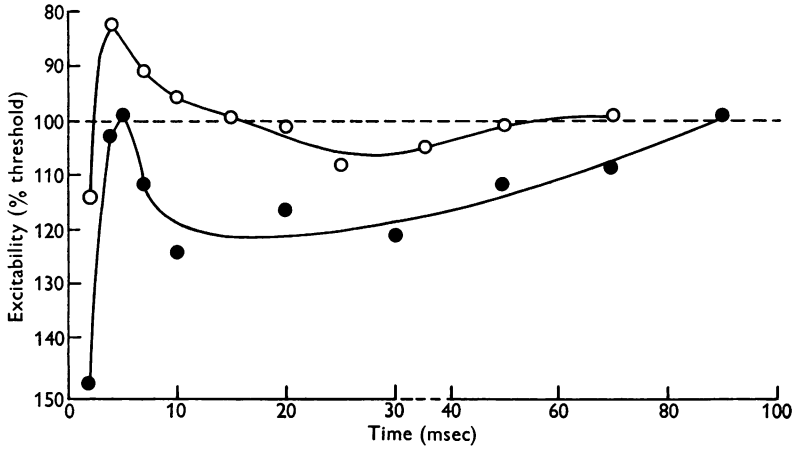

Fig. 10. The excitability changes of the motor nerve terminals after one (○) and five conditioning impulses (●) at 200/sec recorded and plotted as in Fig. 9. Temp. 34·5° C.

The changes in the excitability cycle brought about by increasing the number of conditioning impulses from one to five at 200/sec are illustrated in Fig. 10. There was an increase in the duration of the refractory period and a curtailment of the supernormal period, which appeared only as a notch in a prolonged and increased subnormality. The effects of two impulses in the same experiment were intermediate between those of one and five conditioning impulses. After nine impulses at 200/sec (Fig. 11*B*) the magnitude and duration of the subnormal excitability was still greater. The maximum subnormality was increasingly delayed as the number of conditioning impulses was increased. It seems possible therefore that the post-tetanic delay in reaching the maximum potentiation of e.p.p. and spike potential amplitudes (Fig. 6) could well be explained by a similar delay in reaching the maximum hyperpolarization of the nerve terminals. Figure 11 also illustrates the effect of varying the frequency of stimulation. Ten impulses at 100/sec (Fig. 11*A*, filled circles) had less effect on the duration and magnitude of the subnormal period than nine impulses at 200/sec (Fig. 11*B*). In the same experiment the effect of ten impulses at

10/sec was also explored, but the excitability changes did not differ from those found after a single impulse.

Attempts were also made to determine the duration of the subnormal excitability after large numbers of conditioning stimuli. In one procedure the testing pulse was set at threshold for the antidromic response, and, when stimulating, every second the time was noted at which the responses reappeared after tetanic stimulation. Small random variations in threshold made this procedure difficult, but in our most successful experiment the duration of the subnormal excitability appeared to increase approximately exponentially as the number of conditioning volleys (at 100/sec)

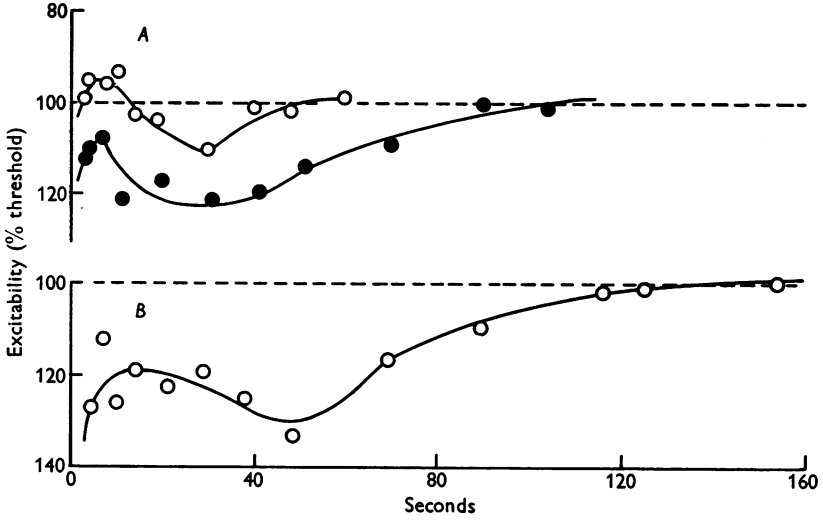

Fig. 11. The effect of frequency of stimulation upon post-tetanic hyperpolarization of the motor nerve terminals. *A*, the excitability changes after one impulse (○) and 10 impulses at 100/sec (●). *B*, the excitability changes after 9 impulses at 200/sec at the same junction. Recording and plotting as in Fig. 9. Temp. 34° C.

was increased. Thus after 200 volleys the threshold returned to normal in 9·6 sec (mean of 10 trials), while after 300 volleys it took 32·4 sec (5 trials), after 400 volleys 54 sec (5 trials), and after 500 volleys 185 sec (2 trials). After longer tetanic stimulation it was possible to measure the threshold at various intervals after the tetanus and thus to plot the return to normal excitability. Measured in this way, for example, it took 4–5 min for the threshold to return to the control value after 10 sec of stimulation at 100/sec. The actual threshold change for the greater part of this prolonged subnormal period was generally small, being often within 5 % of the control threshold. The duration of subnormal excitability was comparable with the duration of the increases in spike amplitude and in e.p.p. potentiation

to be expected after similar stimulation. The subnormality found in the present investigation appeared to last longer than that described by Gasser & Grundfest (1936) following comparable stimulation of the cat phrenic nerve.

DISCUSSION

The results of this investigation will be considered in relation to two problems mentioned in the introduction; namely, does the nerve impulse invade the nerve terminals actively or electrotonically? and what changes are brought about in the terminals by repetitive nerve impulses?

In the present investigation a soundly based criterion, the recording of the extracellular m.e.p.p.s. (del Castillo & Katz, 1956; Dudel & Kuffler, 1961*a*), has been employed in locating the micro-electrode close to nerve terminals. In this position a diphasic positive-negative potential was recorded preceding the appearance of an e.p.p. Moreover, at the sites where there was this characteristic response of a propagated impulse, electrical stimulation through the micro-electrode invariably evoked nerve impulses that were antidromically propagated up the phrenic nerve. In our preparation it thus seems probable that the presynaptic nerve terminals are electrically excitable and are actively invaded by a nerve impulse. In contrast, in crayfish muscle an electrode similarly located often recorded only a positive nerve potential (Dudel, 1962*b*) and it was not possible to obtain an antidromic response on stimulation (Dudel & Kuffler, 1961*b*). Conduction in these terminals is thus thought to be electrotonic (Dudel, 1962*b*).

It must be admitted that an alternative explanation of our results would be that the terminals conduct only electrotonically, and that the most peripheral excitable structure is the first node of Ranvier beyond the terminals. This possibility seems unlikely for the reasons already given, and in any case some of our observations can be explained only if the nerve terminal membrane or the membrane of the first node has properties (besides the ability of the terminals to liberate ACh) not shared by the parent fibres. It has been suggested (e.g. Holmes, 1962) that because of their non-myelinated nature nerve terminals would resemble C fibres. In fact, the time course of the excitability cycle after one impulse (Figs. 9, 10, 11) was that of the parent A fibres and gave no indication of the presence of a presynaptic generator potential as postulated by Riker *et al.* (1957). However, the response to temperature changes was similar to that found in C fibres (Fig. 9) and the subnormal excitability after tetanic conditioning appeared longer lasting than that reported for the parent A fibres. Further, the ability to modify the after-potential cycle with prostigmine and other drugs (Hubbard & Schmidt, 1961; and unpublished experiments) argues for unique pharmacological properties in this site.

Our observations of the changes in the presynaptic spike potentials (Figs. 3, 4) after tetanic stimulation are in full agreement with Lloyd's (1949) hypothesis that, after repetitive stimulation, the spike potential in the terminal region of nerve fibres is larger in amplitude and thus more effective in transmission. The finding that the increased e.p.p. amplitudes during repetitive stimulation (Figs. 3, 4) were logarithmically proportional to the increased spike amplitudes (Fig. 4) effectively answers the criticism that spike increases hitherto observed were too small to account for the increased transmitter release (Ritchie & Straub, 1956; Bishop, Burke & Hayhow, 1959). The spike potential increases found in our experiments (Figs. 3–7) were larger than those previously reported in peripheral nerves (Ritchie & Straub, 1956; Gasser, 1958) and the optic nerve (Bishop *et al.* 1959). Spike potential increases of comparable size were recorded by Eccles & Krnjević (1959) in primary afferent fibres close to their termination in the spinal cord. It seems possible, as these workers suggest, that the ability to produce an increased spike potential after repetitive stimulation is most highly developed in the terminal region of nerve fibres.

Lloyd's (1949) hypothesis cannot, however, explain the results of our experiments with two stimuli separated by an interval of only 4 msec. Under these conditions, while the second e.p.p. response was larger than the first, the second spike potential was smaller than the first. Similar observations were made by Eccles & Rall (1951) in the spinal cord. They found that after a small number of conditioning impulses at a high frequency, the post-synaptic response was increased while the presynaptic spike potential was depressed. There are at least two other grounds for postulating that the e.p.p. potentiation after a single impulse or a few high frequency impulses is caused by a different mechanism from that following longer stimulation. First, this type of potentiation may be increased if it is evoked when e.p.p. amplitudes have been increased by the application of a hyperpolarizing current to nerve terminals (Hubbard & Willis, 1962*a*, *b*). In contrast, after tetanic stimulation in similar circumstances, the time course and relative magnitude of the post-tetanic potentiation of e.p.p.s was unchanged (Hubbard & Willis, 1962*b*). Secondly, after one impulse e.p.p. potentiation can be detected at a time when the nerve terminals exhibit supernormal excitability (Figs. 9, 10, 11). Post-tetanic potentiation, on the other hand, is accompanied by a prolonged subnormal excitability of nerve terminals, which presumably is due to a long-lasting hyperpolarization (Gasser, 1935; Graham & Gasser, 1935). These differences suggest that the potentiation which follows one or a few impulses must be of a different type from that attributable to an increased size of the impulse. This primary potentiation, as it may be called, was also observed at motoneuronal synapses by Curtis & Eccles (1960), who

suggested that it is caused by a temporary increase in the amount of the transmitter substance available for release. The intensification of primary potentiation by hyperpolarizing current supports this hypothesis, because there is evidence that such currents when applied to nerve terminals also increase e.p.p. amplitudes by increasing the amounts of transmitter available for release by nerve impulses (Hubbard & Willis, 1962*a*, *b*).

During and after repetitive stimulation it seems probable that a secondary mechanism for increasing transmitter release is superimposed on or replaces the primary potentiation. This secondary mechanism is presumably induced (Lloyd, 1949) by the long-lasting hyperpolarization of nerve terminals which has been detected after repetitive stimulation both in motor nerve terminals (the present investigation) and in central afferent fibre terminals (Wall & Johnson, 1958). During this hyperpolarization there was an increase in the amplitude of presynaptic action potentials accompanied by a simultaneous increase in e.p.p. amplitudes (Figs. 3–6). It was found that the amplitude of the terminal nerve spike could be increased by applied hyperpolarizing current and that this increase was accompanied by an increase in transmitter output (Fig. 8). Similar observations have been made at nerve terminals in the squid (Hagiwara & Tasaki, 1958; Takeuchi & Takeuchi, 1962). These observations together with the findings of an association between hyperpolarization and increase in spike potential amplitude in single afferent nerve fibres (Eccles & Krnjević, 1959) and in motor nerve fibres (Eccles, Kostyuk & Schmidt, 1962) support the hypothesis that the hyperpolarization is responsible for the increase in spike amplitude. The analogy between the effect of hyperpolarizing current and the effects of post-tetanic hyperpolarization is, however, not complete. For instance, it is now known that hyperpolarizing currents produce progressive increases in transmitter release as measured by e.p.p. amplitudes (Hubbard & Willis, 1962*a*). During the application of current, presynaptic spike potentials are immediately increased in amplitude, but show no progressive changes (Fig. 8*A*, *D*). Further, Holmes (1962) has found that mammalian nerve fibres are not invariably hyperpolarized after repetitive stimulation. The recent development of methods of diminishing or increasing post-tetanic hyperpolarization in nerve fibres (Greengard & Straub, 1958, 1962) should be of value in attempting to determine whether hyperpolarization is causally or only coincidentally related to the post-tetanic potentiation of e.p.p.s.

SUMMARY

1. Nerve terminals were located in the $MgCl_2$ paralysed rat diaphragm-phrenic nerve preparation, *in vitro*, by the recording of extracellular m.e.p.p.s.

2. E.p.p.s recorded in the region of nerve terminals were preceded by a diphasic (positive-negative) nerve action potential, which was all-or-nothing in character.

3. During and after repetitive stimulation the amplitude of the spike potential was increased, this increase having a similar time course to the simultaneously recorded increase of e.p.p. amplitude.

4. During repetitive stimulation the increased e.p.p. amplitudes were logarithmically proportional to the simultaneously increased spike potential amplitudes.

5. After a single conditioning impulse the e.p.p. response to a second impulse at a close interval was potentiated, but the amplitude of the accompanying spike potential was decreased.

6. Hyperpolarizing currents caused an immediate increase in spike potential amplitude maintained for the duration of current flow, and reverting to the control value immediately the current ceased. E.p.p.s simultaneously recorded increased progressively in amplitude during the duration of current flow.

7. Stimulation in the region of nerve terminals, as revealed by the diphasic spike potentials, invariably resulted in the appearance of antidromic spike potentials.

8. The excitability cycle was investigated following both single and repetitive stimulating pulses.

9. It was concluded that: (i) the nerve impulses actively invaded nerve terminals: (ii) after repetitive stimulation the potentiation of e.p.p. amplitudes was due to an increase in presynaptic spike amplitudes induced by hyperpolarization of nerve terminals; and (iii) after single impulses the potentiation of e.p.p.s, designated primary potentiation, was brought about by another mechanism, possibly an increased availability of ACh.

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