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HYPERPOLARIZATION OF MAMMALIAN MOTOR NERVE TERMINALS

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The present investigation was prompted by the discrepancy which has been observed between the effect of hyperpolarizing currents upon the release of transmitter spontaneously and by nerve impulses. Thus at neuromuscular junctions in the rat (Liley, 1956*c*) and the frog (Kraatz & Trautwein, 1957) hyperpolarization of nerve terminals reduced the rate of spontaneous transmitter release as measured by the frequency of miniature end-plate potentials. In contrast, the release of transmitter by nerve impulses was increased by hyperpolarizing currents applied to the presynaptic element of frog neuromuscular junctions (del Castillo & Katz, 1954*b*); cat motoneuronal synapses (Eccles, Kostyuk & Schmidt, 1962); and synapses on squid giant axons (Hagiwara & Tasaki, 1958; Takeuchi & Takeuchi, 1962). This discrepancy is difficult to understand in view of the parallel potentiation of both types of transmitter release (Liley, 1956*b*; Hubbard, 1959) during the hyperpolarization of nerve terminals induced by repetitive stimulation (Hubbard & Schmidt, 1961).

The experimental method chosen for hyperpolarizing the motor nerve terminals in this investigation differed from methods previously employed in two respects: it allowed the passage of a large range of hyperpolarizing current strengths without nerve block; and these currents were applied for long periods of time. This latter procedure led to the discovery that, whereas the effect of hyperpolarizing current upon spontaneous transmitter release is practically instantaneous (Liley, 1956*c*), the effect upon the release of transmitter by nerve impulses is delayed in onset and increases greatly with the passage of time. Our investigations have been extended to the elucidation of this new phenomenon which appears to be due to the action of hyperpolarizing current in causing a mobilization of transmitter in nerve terminals. Hyperpolarization of nerve terminals thus very effectively produces controlled changes in the mobilization and release of transmitter in the nerve terminals, which hitherto could be

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effected only indirectly by such a procedure as repetitive nerve stimulation (Liley & North, 1953; Hubbard, 1959).

The effects of depolarizing currents are described in another paper (Hubbard & Willis, 1962*b*). A preliminary account of part of this work has been published (Hubbard & Willis, 1962*a*).

METHODS

The experiments were performed on isolated rat hemidiaphragm-phrenic-nerve preparations (Bülbring, 1946) obtained from young albino rats of the Wistar strain. The dissection technique, the recording chamber, the methods of aeration and of temperature control of the solutions, and the intracellular recording techniques have been described previously (Hubbard, 1961). The temperature of the bathing solution over the preparation was measured by a thermistor. Muscle fibre membrane potentials were monitored continuously by a pen recorder (Texas Instruments Inc.), in addition to being displayed on a d.c. voltmeter.

The recording, stimulating and polarizing circuits are represented diagrammatically in Fig. 1. A glass micro-electrode (tip diameter less than 0·5μ; resistance 10–15 MΩ), filled with 3 M-KCl, was used to record intracellular potentials from muscle fibres (Fig. 1*A*). This was

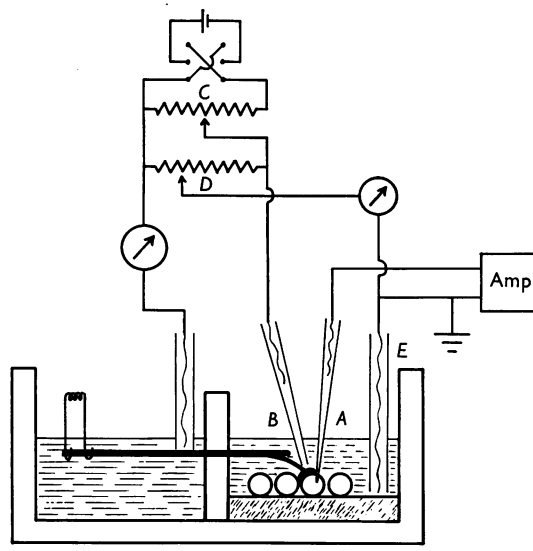

Fig. 1. Diagram of the method employed for polarizing the presynaptic terminals of the rat neuromuscular junction. The muscle (represented by circles) was placed in the right-hand side of the divided recording chamber. The nerve passed beneath the partition into the left compartment where it was mounted upon stimulating electrodes. A fine micro-electrode (*A*) was used to record intracellular potentials from muscle fibres. Polarizing currents were passed between a micro-pipette (*B*) placed near an end-plate region and an electrode in contact with the nerve in the paraffin pool in the left compartment. The source of current was a battery connected to a variable resistance through a reversible switch (*C*). A bridge circuit was established by shunting the polarizing circuit with a potentiometer (*D*) with the sliding contact connected to earth (*E*). Currents were monitored by micro-ammeters as shown. See text for further details.

mounted in a micro-manipulator (Eccles, Fatt, Landgren & Winsbury, 1954) and was inserted under visual control (Zeiss binocular dissecting microscope with magnifications up to 40×) into fibres near the tip of the polarizing electrode (Fig. 1*B*) until an end-plate potential (e.p.p.) was found which could be altered by polarizing currents. The light source for this procedure was a bulb with a focused beam directed down on the preparation from near the microscope. When recording e.p.p.s, neuromuscular transmission was blocked by one of three procedures: by adding curare (tubocurarine chloride, B.P. Burroughs Wellcome and Co., $1{\cdot}5$–$2{\cdot}5 \times 10^{-6}$ w/v) to a modified Krebs–Ringer solution (Liley, 1956*a*); by increasing the magnesium concentration to 11 mM; or by increasing the magnesium and decreasing the calcium concentrations to 8 mM-Mg and 1 mM-Ca, respectively. E.p.p.s were elicited by stimulation of the phrenic nerve in a paraffin pool with supramaximal condenser discharges. Miniature end-plate potentials (m.e.p.p.s) were recorded in preparations immersed either in modified Krebs–Ringer solution or in a similar solution in which the magnesium concentration was 11 mM.

Polarizing currents were obtained from a 45 V battery; the amount of current was controlled by a variable resistance and the direction by a reversing switch (Fig. 1*C*). In most experiments, the current was switched on, the variable resistance having been pre-set; in some experiments, the current was increased more slowly by varying the resistance manually. The currents were passed between an electrode on the phrenic nerve and an electrode located near the presynaptic terminals (Fig. 1*B*). One electrode was a silver–silver-chloride wire which made contact with the nerve through an agar-Ringer column. The other electrode was a silver–silver-chloride lead inserted into a glass micro-pipette filled with agar-Ringer solution. The micro-pipette was prepared by breaking the tip of a finely drawn micro-electrode until the outside diameter was 30–100 μ; the best results were obtained when the tip was squarely broken rather than obliquely and when the resistance was about 2 MΩ. The range of resistances used was 0·5–4MΩ. The micro-pipette was mounted in a second micro-manipulator and placed near a junctional region by using it to record foci of extracellular e.p.p.s. It was then removed from the recording circuit and put into the polarizing circuit. Current was passed from the micro-pipette along the nerve to the electrode in the paraffin pool, thus hyperpolarizing the nerve terminals, or in the reverse direction, depolarizing the terminals. The current in this circuit was monitored by a micro-ammeter; the values of current used ranged from 0·1 to 20 μA. When currents in excess of about 7–10 μA were used, the nerve impulse was often blocked, but m.e.p.p.s could still be recorded. There was no evidence that the current through the micro-pipette was affected by electrode polarization.

In preliminary experiments, it was found that large currents produced a potential drop in the bath which altered the post-synaptic membrane potential by as much as 10 mV. When the amplitudes of m.e.p.p.s were measured under these conditions, the mean amplitude varied linearly with the membrane potential and with the current; during a steady current, the amplitude did not vary with time. The changes in m.e.p.p. amplitude could be ascribed to passive alterations in the post-synaptic membrane potential. To avoid this complication the bridge circuit shown in Fig. 1*D* was employed. A potentiometer was inserted across the polarizing circuit, with the sliding contact connected to the same point as the bath earth (Fig. 1*E*). By an appropriate adjustment of the position of the sliding contact, a ratio between the two sides of the potentiometer resistance could be found at which no potential drop in the bath near the tip of the polarizing electrode was detected by the recording electrode. In effect, the tip of the polarizing electrode was at the same potential as the bath earth (Fig. 1*E*). With this procedure, the amount of current which crossed the muscle membrane was minimized, as shown by the lack of significant changes in muscle resting potential and in m.e.p.p. amplitude during the flow of large polarizing currents. Current flowing between the sliding contact of the potentiometer and the bath earth was monitored in a few experiments by a second micro-ammeter, as shown in Fig. 1. The current in this

arm of the circuit never exceeded 1 μA when the bridge was balanced to eliminate changes in the post-synaptic membrane potential. In any case, this current had no effect on the changes produced in the e.p.p. by currents in the polarizing circuit; for example, it was possible to reverse the direction of this current without influencing the e.p.p. changes.

In a few experiments, a Grass stimulator (Model S4), isolated from earth, was employed to pass rectangular pulses of current with durations up to 1 sec and also direct current. The bridge circuit was balanced during the passage of the direct current, and the value of current was read on the micro-ammeter. Then pulses of current (200–1000 msec) were passed at the same settings of the potentiometer and of the Grass stimulator. Finally, the direct current was again passed, and the balance point and current value were checked.

RESULTS

Effect of hyperpolarizing currents on e.p.p.s

Amplitude and time course of e.p.p. The amplitude of an intracellularly recorded e.p.p. was progressively increased (Fig. 2*A–F* and superimposed tracings *A* to *D*) when a constant current was passed for several seconds from an extracellular electrode placed within 100μ of the end-plate. No effects were seen when a greater distance separated the two electrodes. Current flow in the bath other than the localized current applied from the extracellular electrode could not therefore be responsible for the increase in e.p.p. amplitude. This progressive increase in amplitude was accompanied by an increasing rate of rise of the e.p.p. and by an increasing time between the onset and peak of the e.p.p. The falling phase of the e.p.p. had an approximately exponential time course and the time constant was not appreciably changed by the polarization. When the current was turned off (Fig. 2*G-L* and superimposed tracing *H* to *K*) the amplitude, the rate of rise and the time to peak gradually reverted to the control values. These progressive effects could be obtained without any change in the latency of the e.p.p., but usually at the onset of the current there was an immediate small increase in latency (Fig. 2, compare *A* and *B*). The magnitude of this increase varied with the magnitude of the applied current and from junction to junction. It was never progressive unless nerve block was imminent and always reverted to the control value as soon as the current ceased. For example, in Fig. 2, the latency was 2·16 msec before and after the current and 2·30 msec during the current.

These changes were similar in preparations in which neuromuscular transmission was blocked by curare (Figs. 2–6, 8, 9 and 11) and in those in which the block was due either to a high magnesium (Figs. 7 and 10), or to a high magnesium and low calcium concentration in the bathing medium. There was no qualitative difference in the results at room temperature (20–23 °C) and at 37 °C.

Varying durations of current. The height of the e.p.p. was found to depend upon the length of time a given current flowed and upon the

strength of current employed. Figure 3 shows the changes in e.p.p. amplitude during and after the passage of a fixed value of current (5·2 μA) for several successive periods of time. In each case after the current was turned off, the decay in height of the e.p.p. was followed until the control value was approximately reached. For each successive series, the current was left on for a longer time, so that the effects of the same current on 5, 10, 15 and 20 consecutive e.p.p.s at 3·5 sec intervals were recorded in that

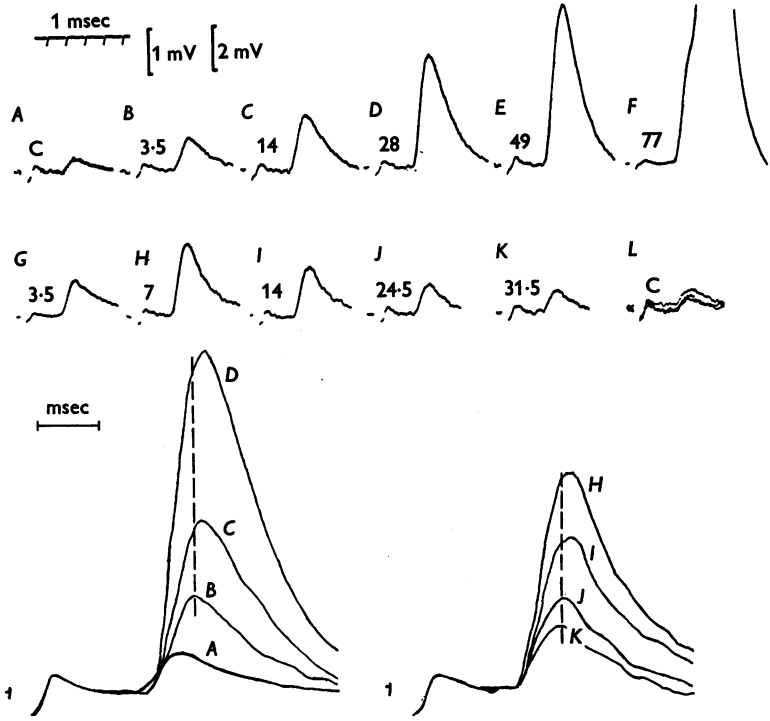

Fig. 2. Changes in e.p.p. amplitude during and after hyperpolarizing current. *A* shows four superimposed records of an e.p.p. in a curarized preparation at room temperature. The e.p.p. was recorded at 3·5 sec intervals throughout the experiment. As soon as the last e.p.p. shown at *A* was recorded, a hyperpolarizing current of 4 μA was begun; *B–F* are sample records taken during the period of current flow at the indicated intervals (in sec). After *F*, the current was stopped, and *G–K* were taken at the indicated intervals (sec); *L* shows four superimposed records of the e.p.p. when it had regained the control size after 52·5 sec. The 1 mV potential scale applies to *A–E* and *H–L*, while the 2 mV scale applies to *F* and *G*. Upstrokes of e.p.p.s retouched. Enlarged tracings of *A–D* and of *H–K* are shown at the bottom of the figure. In each case, the shock artifacts are superimposed. Note that *A* has a shorter latency than *B–D*. A broken vertical line has been drawn through the peaks of *B* and of *K*, showing the delay in time to peak which occurs when the e.p.p. is increased in amplitude. Note the separate time scales (msec) for the records *A–L* at the top of the figure and the superimposed tracings at the bottom of the figure.

serial order. The rising phases of the curves are superimposed in Fig. 3 showing that there was no appreciable change with repeated applications of the current. As the duration of the current flow was increased the height of the e.p.p. grew initially at a rapid rate and then progressively more slowly. Figure 4 illustrates the time course of the changes in e.p.p. amplitude when the current was left on for a long period of time. In this experiment the e.p.p. began to increase in height soon after the current was begun, then it grew in a nearly linear fashion for over 50 sec and finally reached a plateau after the current had been on for about 150 sec.

The decay of e.p.p. amplitude when the current was turned off is illustrated in Figs. 3, 4, 5*A* and *B*. There were invariably two phases of

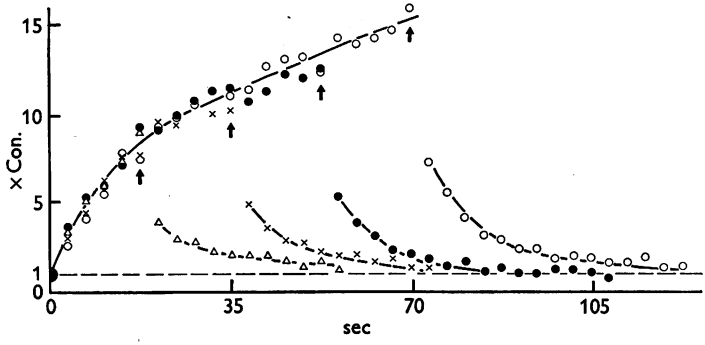

Fig. 3. Varying durations of hyperpolarizing current. E.p.p.s were sampled at 3·5 sec intervals during and after the passage of a fixed value of current (5·2 μA). The current was left on for 5 (Δ), 10 (×), 15 (●) and 20 (○) consecutive e.p.p.s in successive trials. The height of the e.p.p. relative to the control value is plotted against the time after the current was begun (sec). The times at which the current was stopped for the different series are indicated by arrows. Curarized preparation.

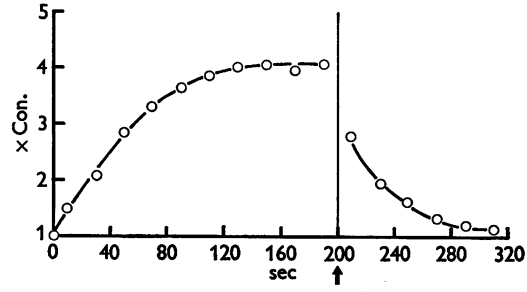

Fig. 4. Changes in e.p.p. amplitude during and after a prolonged period of current flow. E.p.p.s were sampled at 2 sec intervals. The mean amplitudes for successive 20 sec periods during and after the passage of a current of 4·2 μA are plotted relative to the control amplitude against the time (sec) after the current was begun. Each point is placed in the centre of the 20 sec period which it represents. The arrow and the vertical line show the time at which the current was stopped. The preparation was curarized.

decay, an initial rapid phase over 10–15 sec, followed by a slow phase having a duration that depended largely upon the strength of current previously applied. The duration of current flow also influenced the length of the subsequent decay in e.p.p. amplitude, but this could only be

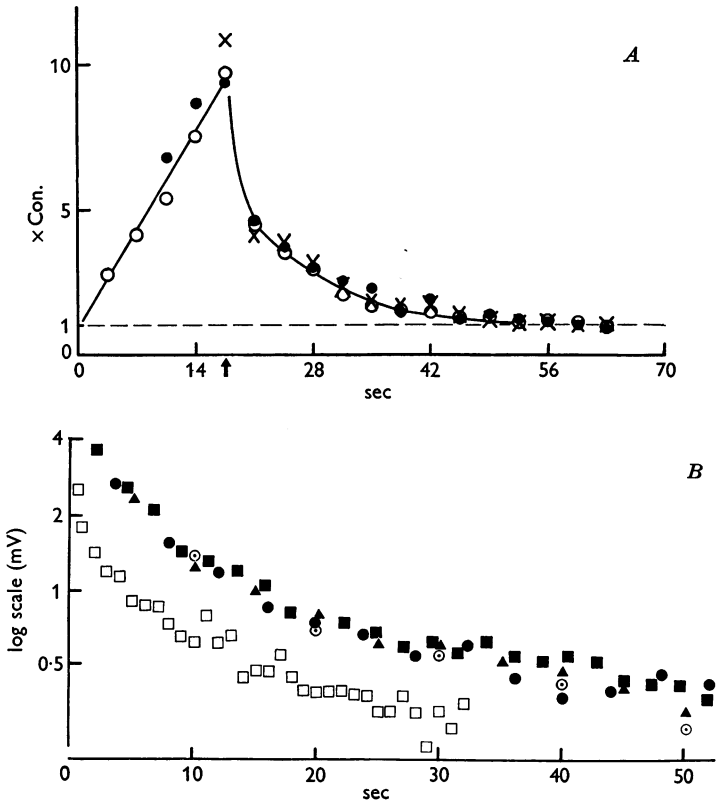

Fig. 5. *A*. Effect of sampling of e.p.p.s on degree of potentiation. E.p.p.s were recorded at 3·5 sec intervals during and after the passage of a hyperpolarizing current of 7·8 μA. The sampling of e.p.p.s began as soon as the current started in two series (the mean of the results of these two series was taken, giving the points shown by open circles), but in two other series the sampling was not begun for 10·5 sec (●) and 17·5 sec (×). The current was discontinued after 17·5 sec. The amplitudes of the e.p.p.s relative to the control amplitude are plotted against the time (sec) after the current was begun. Curarized preparation. *B*. Effect of sampling e.p.p.s at various rates on the decay in amplitude when current is discontinued. A hyperpolarizing current of 6 μA was passed for 40 sec before each series. No e.p.p.s were elicited during the period of current flow. When the current was stopped, e.p.p.s were elicited at constant rates for the different series (⊙ for every 10 sec; Δ every 5 sec; ● every 4 sec; ■ every 2·25 sec; and □ every 0·5 sec. For the 0·5 sec series only every alternative e.p.p. is plotted). The amplitude of the e.p.p. in mV is plotted on a log. scale against time in sec after the current was discontinued. Curarized preparation.

demonstrated when large differences in duration were compared. Thus in Fig. 3 the e.p.p. amplitude took almost the same time to return to the control amplitude after currents lasting 17·5, 35, 52·5 and 70 sec. In another experiment, however, after 200 sec of current flow (Fig. 4), although the potentiation of e.p.p. amplitude was less than that found in the experiment illustrated in Fig. 3, the decay took much longer, the control amplitude not being regained at 110 sec after the cessation of current flow. Both slow and rapid phases of decay appeared approximately linear when the e.p.p. amplitudes were plotted on semi-logarithmic co-ordinates (Fig. 5*B*). The separation of the two phases was then clear, the rapid phase in this figure (upper curve) lasting about 15 sec.

Neither the increase nor the decay of e.p.p. amplitude was affected by the sampling of e.p.p.s at the repetition rates (every 2–4 sec) normally used in our experiments. For example, in Fig. 5*A* a current of 7·8 μA was applied for 17·5 sec on several different occasions. On the first and last occasions the sizes of the e.p.p.s were tested at 3·5 sec intervals throughout this period (open circles). During the second repetition the first two stimuli were omitted (filled circles) and on the third repetition the first four stimuli in the 3·5 sec sequence were omitted (crosses). When the e.p.p. amplitudes are all plotted on the same graph (Fig. 5*A*), the increased e.p.p. amplitudes during the period of current flow lie almost on the same curve. A slight initial increase in amplitude when fewer stimuli were given would be expected because there was an increased interval between the control and test stimuli. After the current ceased with all four series the amplitudes of e.p.p.s observed at 3·5 sec intervals followed the same decay curve.

In other experiments of this type it was found that e.p.p. amplitudes reached a plateau during current flow independently of the sampling procedure. For instance, in one experiment e.p.p.s were elicited every 4 sec during the application of current until, after 40 sec, they were of constant size. The current was then turned off and the e.p.p.s decayed to the control size. The same current was now applied again but e.p.p.s were not elicited until 3 min later. Despite the much longer time of current flow and the absence of stimulation the e.p.p. amplitudes were almost the same as the plateau of amplitude reached in the preceding trial.

Figure 5*B* illustrates one of several experiments in which the decay of e.p.p. amplitude after a fixed period of hyperpolarizing current was followed at varying rates of stimulation. In this experiment the current was 6·0 μA applied for 40 sec. No stimuli were given during the period of current flow, but afterwards e.p.p.s were elicited at constant frequencies which ranged from 5/sec to one every 10 sec. When the e.p.p. amplitudes (ordinates) are plotted on the same time scale (Fig. 5*B*), the points representing

e.p.p.s elicited at intervals of 2 sec or longer all lie on the same curve. At these frequencies, therefore, the rate of decay of e.p.p. amplitudes was a function of time and was not increased by the loss of transmitter induced by stimulation. When the rate of stimulation was increased above 1/2 sec the decay of e.p.p. amplitude did occur in a shorter time, being more rapid the faster the stimulus frequency. The more rapid decay of e.p.p. amplitude found with stimulation at 2/sec is illustrated (Fig. 5*B* open squares). It was intermediate between the effects of 5/sec and 1/sec stimulation (not illustrated).

In several experiments, the time of onset of the progressive increase in e.p.p. amplitude was further studied by means of a modification of the technique employed for most of these experiments. Instead of using long current pulses from a battery polarizer, pulses of 200–1000 msec were passed from a Grass stimulator. The e.p.p. could be evoked at various times after the beginning of the pulses, so that a change in the height of an e.p.p. could be detected at any interval from a few milliseconds to 1 sec after the start of the hyperpolarizing current. In one unit investigated in this manner, there was no change in the e.p.p. height for over 700 msec, whereas by 800 msec a progressive increase in the height of the e.p.p. had commenced. In three other units there was no progressive increase in e.p.p. amplitude during a one second pulse although it had occurred by 4 sec after the onset of a long pulse of the same strength.

The effect of current pulses with durations of 1 sec was found to be cumulative when the pulses were repeated at 4 sec intervals. An e.p.p. evoked during the pulse might show no increase in amplitude when the pulse was applied only once or a few times, but, when the pulse was repeated every 4 sec for many seconds, the e.p.p. often grew progressively in height. When the pulses were discontinued the e.p.p. amplitude fell gradually to the control value in the same manner as after the application of a continuous current. When the e.p.p. was subjected to repeated brief pulses of current in this manner its rate of growth was less than when the same value of current was passed continuously.

Varying strengths of current. The effect of several values of current strength upon the time course of the increase in e.p.p. amplitude is demonstrated in Fig. 6. Small currents had little or no effect; and, when an increase in the height of the e.p.p. was produced, this was often not detected until the current had flowed for several seconds (Fig. 6*A*, curve produced by 0·5 μA). Intermediate strengths of current resulted in an increased e.p.p. height by the time the first test e.p.p. was elicited, which in most experiments was 2–4 sec after the current had begun. Thereafter the amplitude of the e.p.p. increased progressively toward a plateau, the level of which depended on the current strength. The largest values of current

strength caused the e.p.p. to become sufficiently large to generate an action potential in the muscle membrane (Fig. 2*F*).

Figure 6*A* shows that during the initial period of the increase in e.p.p. height there was an approximately linear relationship between e.p.p. amplitude and time. Figure 6*B* shows that during this period the e.p.p. height was approximately a logarithmic function of current strength. The e.p.p. amplitudes were measured at intervals of 12, 24 and 36 sec after the onset of current flow (i.e. at the vertical lines in Fig. 6*A*) and in Fig. 6*B*

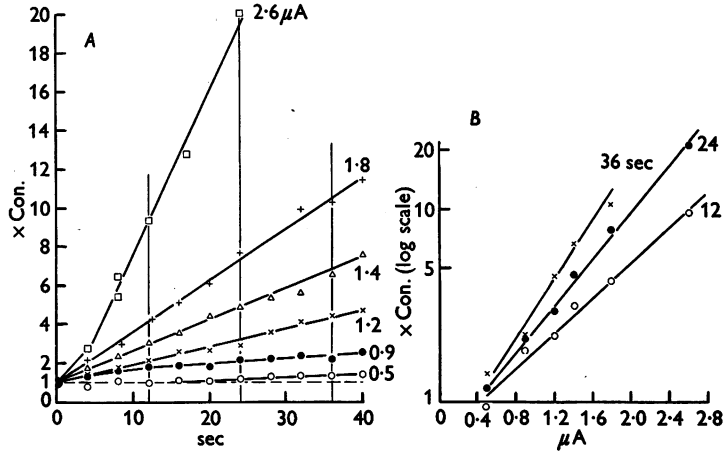

Fig. 6. Effect of varying strengths of current on e.p.p. amplitude. *A* shows the changes in e.p.p. amplitude relative to the control amplitude during the passage of various current strengths, as indicated, for periods of 40 sec. E.p.p.s were sampled at 4 sec intervals. In *B*, the amplitudes of e.p.p.s relative to the control size are plotted on a logarithmic scale against the various current strengths used (μA); e.p.p. amplitudes were compared at three intervals after the onset of current flow—12 (○), 24 (●) and 36 (×) sec, as indicated also by the vertical lines in *A*. Curarized preparation.

are plotted as open circles, filled circles, and crosses respectively on a logarithmic scale (ordinates) against the various current strengths used. As would be expected, the slopes of the resulting straight lines increase with the duration of the applied current.

Effect of hyperpolarizing currents on spontaneous m.e.p.p.s

M.e.p.p. amplitude. In magnesium-poisoned preparations both e.p.p.s and m.e.p.p.s could be recorded together. In this way it was possible to determine whether hyperpolarizing currents that produced progressive increases in e.p.p. amplitude also effected the mean amplitudes of m.e.p.p.s photographed on the same sweeps. For the accurate measuring of both e.p.p.s and m.e.p.p.s recorded in this way strengths of hyperpolarizing

currents were chosen which produced e.p.p. increases no greater than two to ten times the control size. Figure 7 illustrates results typical of the 20 units studied in this fashion. The mean amplitudes both of e.p.p.s and of m.e.p.p.s are plotted for successive 5 sec periods before, during and after the passage of current. At least 15 m.e.p.p.s were present in each 5 sec period. No change occurred in the mean amplitude of the m.e.p.p.s, whereas the e.p.p. was increased to nearly three times the control height. When the bridge circuit was balanced (see Methods) there was never any increase in m.e.p.p. amplitude during the passage of hyperpolarizing currents.

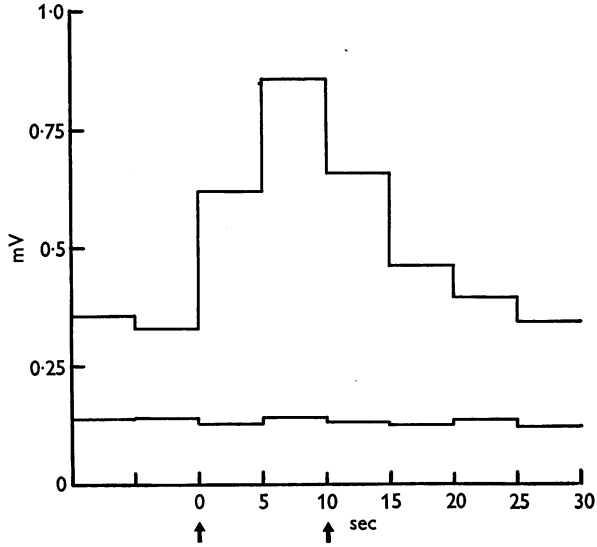

Fig. 7. Effect of hyperpolarizing current on m.e.p.p. amplitude. E.p.p.s and m.e.p.p.s were recorded on the same sweeps in magnesium-poisoned preparations. In this experiment, the amplitudes of both e.p.p.s and m.e.p.p.s were averaged over 5 sec periods, and the mean amplitudes are plotted in mV against time (sec). A current of 5·3 μA was begun at the time indicated by the first arrow and was stopped at the time shown by the second arrow.

M.e.p.p. frequency. The effect of polarizing currents upon the frequency of m.e.p.p.s was studied in preparations bathed either in a modified Krebs–Ringer solution (Liley, 1956*a*) or in a solution containing 11 mM magnesium. In preparations bathed with the Krebs–Ringer solution, the frequency of the m.e.p.p.s was found to be a logarithmic function of the current strength, being increased by depolarizing currents and decreased by hyperpolarizing currents. The frequency changed within milliseconds after the onset of current flow to a level which remained constant until the current ceased, at which time the frequency reverted immediately to the

original level. Thus, the results agree in every respect with those obtained by Liley (1956*c*), who used a similar preparation but a somewhat different technique for applying polarizing current. The strengths of current required to produce marked frequency changes in these experiments in Krebs–Ringer solution were generally much larger than the strengths of current effective in increasing the e.p.p. amplitude in curarized and magnesium-poisoned preparations.

Also in agreement with previous reports (del Castillo & Katz, 1954*b*; Liley, 1956*c*) when the preparation was bathed with solutions containing a high concentration of magnesium, the action of polarizing currents on the frequency of m.e.p.p.s was found to be greatly reduced or abolished. There was no change in m.e.p.p. frequency with junctions showing a progressive increase in e.p.p. amplitude (Fig. 7). When the frequency of m.e.p.p.s during hyperpolarizing current was compared with the frequency during intervals of the same duration before and after the application of current in a total of 30 units, the mean ratio of frequencies was $1{\cdot}04 \pm 0{\cdot}05$ (S.E.).

Effect of hyperpolarizing currents on neuromuscular depression

Following two successive impulses at the mammalian neuromuscular junction, the second e.p.p. is on the average smaller than the first at all intervals from 10–15 msec to 7–10 sec (Eccles, Katz & Kuffler, 1941; Lundberg & Quilisch, 1953; Hubbard, 1959). This depression has been attributed to a partial exhaustion of the acetylcholine which is immediately available for release in the nerve terminals (Liley & North, 1953). If this explanation is correct it provides a means of deciding whether the increase in e.p.p. amplitude found during the application of hyperpolarizing current is due to an increase in the available transmitter in the nerve terminals, or to an increased ability of the nerve impulse to liberate transmitter without change in the amount available. Thus if the ability of the nerve impulse to liberate transmitter was increased, the depression of a second e.p.p. at a suitable interval after the first would increase with the increased amplitude of the first e.p.p., as for example occurs with the increase in e.p.p. amplitude produced by the drug guanidine (Otsuka & Endo, 1960). Alternatively, if the e.p.p. increase is due to an increased amount of available transmitter from which the nerve impulse liberated a constant fraction, the ratio of the second e.p.p. to the first e.p.p. of the pair should be unaffected.

Figure 8 illustrates the results from one of 14 units investigated by this technique. E.p.p.s were evoked by pairs of stimuli separated by a 1 sec interval, and each set of paired stimuli was repeated every 8 sec. As shown in Fig. 8*B–E*, when a hyperpolarizing current was passed both members

of the pairs increased progressively in height. The time course of the increase of the first e.p.p. of each pair is plotted in Fig. 8*F*. After the current had been on for about 40 sec, the amplitudes of the e.p.p.s had reached a plateau. The time course of changes in the degree of neuromuscular depression is shown in Fig. 8*G*. Neuromuscular depression was plotted as the ratio of the amplitude of the second to the amplitude of the first e.p.p. of each pair. Soon after the onset of the hyperpolarizing current

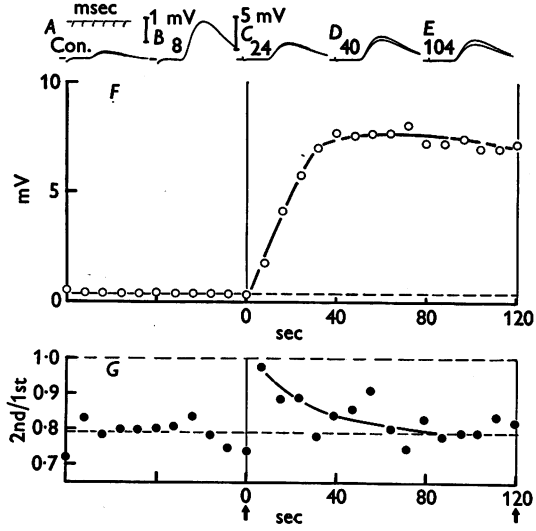

Fig. 8. Effect of hyperpolarizing current on neuromuscular depression. *A–E* are sample records of the pairs of e.p.p.s taken before (*A*) and during (*B–E*) the flow of a current of 1·7 μA. The interval between the members of each pair was 1 sec, while the paired stimuli were repeated every 8 sec. The 1 mV potential scale refers to *A* and *B*, while the 5 mV scale applies to *C–E*. Note 1 msec time scale. The first e.p.p. of each pair is plotted in mV against time in sec in *F*. After a series of control pairs, the current was begun and was kept on for 120 sec. The degree of neuromuscular depression is plotted in *G* as the ratio of the height of the second to that of the first e.p.p. of each pair. An interrupted horizontal line indicates the mean control level of depression. Curarized preparation.

the amplitude of the second e.p.p. became nearly equal to that of the first e.p.p. (Fig. 8*B*); there was a decrease of the neuromuscular depression. When the e.p.p. height had attained a plateau, the ratios of e.p.p. amplitudes for each pair was approximately the same as the mean control ratio. A transient reduction of depression was found in 11 of the 14 units investigated in this way. It appeared to be correlated with a rapid increase in e.p.p. size, for it did not occur in 3 units in which the e.p.p. increase was small and the plateau slowly attained. During the period when the e.p.p. height had attained a plateau, the ratio of e.p.p. amplitudes was approximately the same as the mean control ratio in all 14 units. Therefore, in the

terms of the initial argument of this section, the increase in e.p.p. amplitude was due to an increased amount of available transmitter. The transient reduction in depression which was associated with the phase of rapid increase in e.p.p. size (Fig. 8*B*) could then be explained by this increase in available transmitter occurring at such a rapid rate that it almost compensated for the loss of transmitter produced by nerve impulses.

Effect of hyperpolarizing currents on neuromuscular facilitation

In curarized preparations, the second e.p.p. of a brief tetanic train may be larger than the first if the frequency of stimulation is such that it is evoked within 15 msec after the first; however, the remaining members of the tetanic train are successively smaller until a plateau is reached. This plateau has been explained by Liley & North (1953) as due to the attainment of an equilibrium between transmitter depletion and replenishment. The typical effect of hyperpolarizing current on brief tetanic trains of e.p.p.s evoked in a curarized preparation is shown in Fig. 9*A–F*. During the current flow, each member of the tetanic train displayed a progressive increase in its height similar to that described previously for single e.p.p.s. In Fig. 9*G* the time course of the changes in amplitude during and after the current are shown for the first (open circles), the

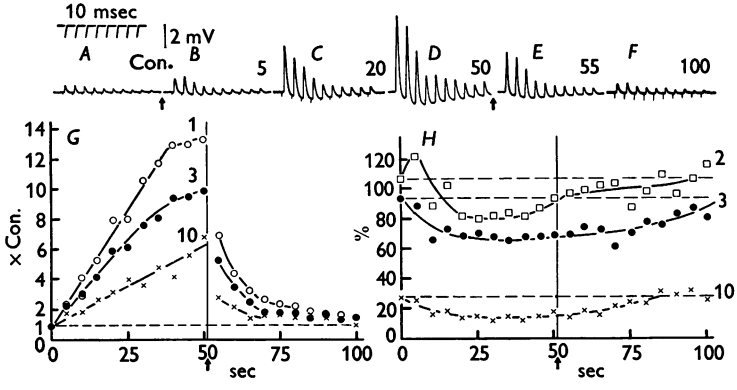

Fig. 9. Effect of hyperpolarizing current on brief tetanic trains of e.p.p.s in a curarized preparation. *A* shows a control record of a tetanic train produced by 10 stimuli at 100/sec. After *A*, a current of 4·5 μA was begun, as indicated by the first arrow. *B–D* are records taken during the current at the indicated times. After *D*, the current was turned off and records *E* and *F* were taken. *G* shows the time course of changes in the amplitudes of the first (○), the third (●) and the tenth (×) e.p.p.s; note that they are plotted relative to their respective control heights. In *H*, the ratios of the second (□), the third (■) and the tenth (×) e.p.p.s to the first e.p.p. are plotted against time. In *G* and *H*, the current was begun just after the control records were taken, and it was discontinued at the time indicated by the arrows and vertical lines.

third (filled circles) and the tenth (crosses) e.p.p.s of the train. With the second e.p.p. of the train there was an absolute potentiation in the initial control observation (Fig. 9*A*) and also 5 sec after the current was begun (Fig. 9*B*). At later intervals this potentiation was replaced by a small depression relative to the height of the first e.p.p. of the train (Fig. 9*H*, open squares). Toward the end of the period of current flow this depression became less. This recovery may be correlated with the tendency of the amplitude of the first e.p.p. to reach a plateau (Fig. 9*G*; open circles). A similar change occurred in the ratio of the heights of later e.p.p.s in the train to the height of the first e.p.p. In Fig. 9*H*, filled circles represent the ratio of the heights of the third to the first e.p.p., while crosses represent the ratio of the tenth to the first e.p.p. In each case, the ratios altered soon after the onset of the current to a new value which was maintained through the rest of the period of current flow. There was no tendency for the ratios to progress towards smaller values. Since there is evidence that neuromuscular depression is unchanged by hyperpolarizing currents (Fig. 8), the changes in the ratios of the later e.p.p.s to the first e.p.p. in the tetanic train during current flow probably reflect a relative decrease in the degree of neuromuscular facilitation.

This possibility was further investigated by testing the effect of hyperpolarizing currents on tetanic trains evoked in preparations in which neuromuscular transmission was blocked by magnesium. The successive members of such tetanic trains show increasing facilitation of the quantal release of transmitter (del Castillo & Katz, 1954*a*; Liley, 1956*b*). When hyperpolarizing current was passed, all the members of the tetanic train increased progressively in height (Fig. 10*B*, *C* and *G*, *H*). Just as in curarized preparations, the early members of the train increased in height more rapidly than did later members, so that, at intermediate current strengths, all the e.p.p.s of the train became nearly equal in height (Fig. 10*C*), and at high current strengths, the early e.p.p.s surpassed in height the later ones (Fig. 10*H*), so that the pattern resembled that in curarized preparations (Fig. 9). The very large e.p.p.s in Fig. 10*H* failed to evoke muscle action potentials because the threshold of the muscle membrane was elevated under the conditions of the experiment: the preparation was bathed in a high-magnesium-containing solution; and it was kept at room temperature.

DISCUSSION

Post-synaptic changes. The large and progressive increases in the amplitude of the e.p.p. produced by applied hyperpolarizing currents (Figs. 2–5*A*, 6–10) can be explained satisfactorily only if the site of action of the current is the presynaptic nerve ending. These changes are too

large to be accounted for by increases in the post-synaptic membrane potential (Fatt & Katz, 1951). The unchanged time course of decay of the e.p.p. (Fig. 2) indicates that there was no large change in the activity of acetylcholinesterase. Further, the increased response cannot be due to removal of curare from receptor sites since similar results were obtained whether neuromuscular transmission was blocked by curare or by alterations in the concentration of magnesium or calcium ions. An increase in the time to peak of the e.p.p. when its amplitude was increased (Fig. 2) was also found by Liley & North (1953, their figure 3) after post-activation

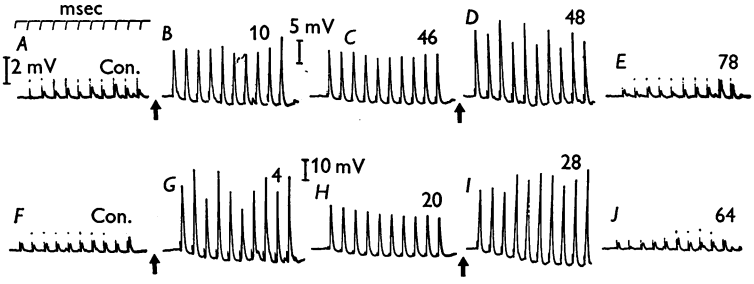

Fig. 10. Effect of hyperpolarizing current on tetanic trains of e.p.p.s in a magnesium poisoned preparation. *A* and *F* are control records of brief tetanic trains produced by ten stimuli at 100/second. After *A* was taken, a current of 4 μA was passed and *B* and *C* were recorded at the indicated times (sec) after the start of the current. *D* and *E* were taken after the current was stopped. The same sequence of events applies to *F*–*J*, except that the current strength was 9 μA. The 5 mV potential scale applies only to record *C*, and the 10 mV scale applies only to *H*; the 2 mV scale applies to all the other records. The 1 msec time scale refers to all the records. M.e.p.p.s can be seen interspersed among the e.p.p.s.

potentiation. This suggests that a greater output of acetylcholine (ACh) caused nearby receptors to be saturated. Receptors at a greater distance than usual from the recording electrode would then be contributing to the depolarization. Another possibility might be that the current flow decreases the rate of diffusion of the positively-charged ACh molecules in the synaptic cleft.

In deciding the cause of the increase in e.p.p. amplitude during hyperpolarizing current, a key observation is the concomitant unchanged m.e.p.p. amplitude (Fig. 7). This finding implies that the response of the muscle membrane to ACh is unchanged and that there is no inward current across the muscle membrane affecting our results (cf. Takeuchi & Takeuchi, 1961). Moreover, since the m.e.p.p. size is equivalent to the mean quantal size (del Castillo & Katz, 1954*a*) the increased e.p.p. amplitude must be due to an increase in the number of quanta released by nerve impulses.

Comparison with previous investigations. In previous investigations of

the effects of presynaptic hyperpolarization an increase in synaptic potential amplitude has also been detected and attributed to an increased transmitter output (del Castillo & Katz, 1954*b*; Hagiwara & Tasaki, 1958; Takeuchi & Takeuchi, 1962; Eccles *et al.* 1962). The results of the present investigation differ from those previously reported chiefly in the finding of a progressive increase in e.p.p. amplitude during the continued application of current (Figs. 2–5*A*, 6–10). Apparently this effect was not detected because the longest time for which current was applied in previous studies was only 3–4 sec (del Castillo & Katz, 1954*b*). Unfortunately nerve block prevented these workers from observing e.p.p. amplitudes during this period but an indication that a progressive change might have occurred in their experiments is the post-anodic increase of e.p.p. amplitude that they found after passage of hyperpolarizing currents (cf. Figs. 3–5).

As would be expected, it was possible to produce results similar to those of previous investigations by measuring e.p.p. amplitudes at a fixed time after the onset of varying strengths of current. For instance, in Fig. 11 the sizes of the e.p.p.s relative to the control size are plotted against currents of various strengths, both in the hyperpolarizing and in depolarizing directions, at two fixed intervals after the onset of current (4 sec, shown by open circles, and 8 sec, shown by filled circles). The forms of the two curves are similar but the size of the e.p.p. was greatly increased at the later interval by currents larger than 4 μA in the hyperpolarizing direction. There is a striking resemblance between the curves of Fig. 11 and those of previous investigators. If the amplitude of the presynaptic spike potential in the squid giant synapse was proportional to the amount of applied current, then current could be substituted for spike amplitude in the figures of Hagiwara & Tasaki (1958) and of Takeuchi & Takeuchi (1962). Their curves would then be of identical form with that of Fig. 11. The major difference between the curves of del Castillo & Katz (1954*b*) obtained at the frog neuromuscular junction and Fig. 11 lies in the effects of depolarizing currents; this problem will be considered in a later paper (Hubbard & Willis, 1962*b*). The curve produced by Eccles, *et al.* (1962, their Fig. 5) differs from Fig. 11 mainly in that it shows a linear relationship between excitatory post-synaptic potential height in motoneurones and current strength; however, with their method of polarization it is unlikely that the currents applied to the nerve terminals were as large as those applied in the present investigation. Their curve therefore probably only covers the range of currents below 4 μA in Fig. 11, over which the e.p.p. amplitude is approximately linearly related to the current strength.

Increased transmitter output. In previous investigations, the effects of hyperpolarizing current have been attributed to an increase in the amplitude of the presynaptic spike during current flow, which might cause it to liberate more transmitter (del Castillo & Katz, 1954*b*; Hagiwara & Tasaki, 1958; Takeuchi & Takeuchi, 1962; Eccles *et al.* 1962). An increase in spike amplitude has been observed during hyperpolarization of nerve terminals of squid giant axons (Hagiwara & Tasaki, 1958; Takeuchi & Takeuchi, 1962) and primary afferent fibres in the mammalian spinal cord (Eccles *et al.* 1962). It is doubtful, however, whether an increase in the total amplitude of a presynaptic spike potential would necessarily lead to the liberation of more transmitter.

The only evidence for this assumption comes from the experiments of Liley (1956*c*) who concluded that because of the logarithmic relationship between the membrane potential of the presynaptic terminals and the m.e.p.p. frequency, most of the quantal content of the e.p.p. would be released by that part of the presynaptic spike which exceeded a potential 60 mV more depolarized than the resting potential. Only an increase in this part of the spike would be expected to increase quantal output

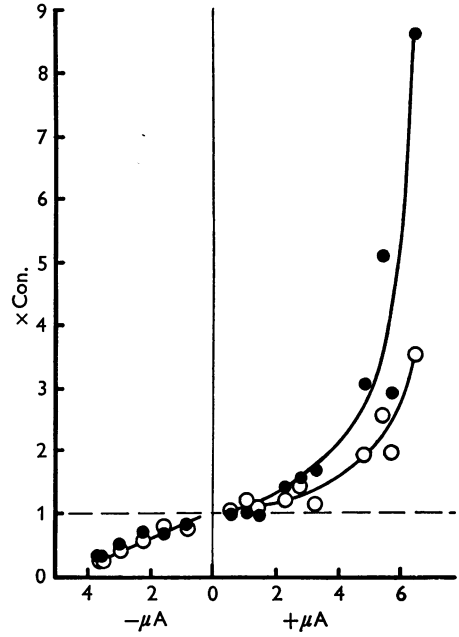

Fig. 11. E.p.p. amplitude relative to the control amplitude is plotted against various values of current strength which had been passed either for 4 sec (○) or for 8 sec (●). The effects of depolarizing currents are plotted in the left side of the graph ($-\mu$A), while the effects of hyperpolarizing currents are plotted to the right ($+\mu$A).

significantly. An increase in spike amplitude merely because of a hyperpolarized base line would have little effect. The previous studies on the effects of polarizing currents on synaptic transmission could not (del Castillo & Katz, 1954*b*) or did not (Hagiwara & Tasaki, 1958; Takeuchi & Takeuchi, 1962; Eccles *et al.* 1962) specify whether or not there was any change in the amount of overshoot of the spike. Increases in the overshoot of the spike of primary afferent fibres during applied hyperpolarizing currents have been recorded (Eccles & Krnjević, 1959), but these were very small and were not an invariable finding.

Certain features of the present results provide difficulties for this

explanation of the effects of hyperpolarizing current on transmitter release by nerve impulses. The most striking characteristic of these results is the progressive nature of the changes in e.p.p. amplitude (Figs. 2–5*A*, 6–10). The other is the long duration of the latency of onset (Fig. 6*A*). Neither the progressive increase in the e.p.p. amplitude nor the delay often observed in the onset of this change can be accounted for by changes in the current density at the presynaptic terminals. The current in the polarizing circuit was monitored continuously by a micro-ammeter (Fig. 1) and was constant over the whole time during which the e.p.p. was increasing in height. Constancy of the current affecting the nerve could also be presumed because the latency of onset of the e.p.p. was observed to change to a value which remained constant throughout the period of current flow and returned immediately to the control value when the current was turned off (Fig. 2). When m.e.p.p. frequencies were studied in preparations in normal solution (p. 125) the frequency changed immediately the current was begun, remained constant during the period of current flow, and reverted to the control level as soon as the current was turned off; this suggests that the effective current at the nerve terminals was constant throughout the period of current flow.

If, as these observations suggest, the membrane potential of the presynaptic terminals was altered immediately to a new maintained level by the passage of current, it would be difficult to postulate that there were progressive changes in the amplitude of the presynaptic action potential; nor does it seem likely that during the pasage of current the presynaptic action potential would remain unchanged for a period of seconds and then begin to increase progressively. Also it would seem unlikely that the presynaptic spike potential was responsible for increases in e.p.p. amplitude that persisted for many seconds after the cessation of current (Figs. 4, 5).

In view of these difficulties we have considered another explanation of these results. This is that the hyperpolarizing current increases the amount of transmitter available for release in the nerve terminals. If the presynaptic spike normally liberates a constant fraction of this available transmitter (Liley & North, 1953), a progressive increase in e.p.p. amplitude would occur in parallel with a progressive increase in the amount of available transmitter. That this occurs is suggested by investigation of the effect of hyperpolarizing current on the depression of neuromuscular transmission which follows activation (Fig. 8). As mentioned in the Results section (p. 126), an increase in the amount of available transmitter with no concomitant change in the ability of the presynaptic spike to release transmitter should result in larger e.p.p.s but there should be no change in the degree of neuromuscular depression. In most of the experiments the degree of neuromuscular depression underwent a transient decrease and

then remained constant at the control level (Fig. 8); in the remaining experiments the depression did not change at all. All the increase in e.p.p. amplitude was apparently due to an increase in the store of available transmitter rather than an increase in the ability of the presynaptic spike to liberate transmitter from an unchanged level of available transmitter.

The effect of hyperpolarizing currents on tetanic trains of e.p.p.s supported the conclusion that an increase of available transmitter had occurred. All the e.p.p.s in a tetanic train were increased progressively (Figs. 9, 10). On the alternative view that the increase in e.p.p. amplitude was due to an increased effectiveness of transmitter liberation, it might be expected that only the first two to three members of the train would be increased, the later members being compensatorily decreased, as happens after repetitive stimulation (Liley & North, 1953). If the plateau phase of the tetanic response in curarized preparations represents an equilibrium between transmitter output and transmitter replacement, as suggested by Liley & North (1953), then the observed increase in the plateau level must mean that the equilibrium has shifted to a higher level, and therefore the rate of replacement of the available transmitter must be increased.

Further, if the hyperpolarizing current brings about an increase in the amount of available transmitter it might be expected that the potentiation of a second e.p.p. at a short interval after the first would disappear (Fig. 9), for this potentiation has itself been ascribed to a transient increase in available transmitter, brought about by the first impulse (Curtis & Eccles, 1960).

The finding that e.p.p. amplitudes were increased by hyperpolarizing current while m.e.p.p. frequency was unchanged or decreased (p. 125) is, however, a serious obstacle to acceptance of the view that there is an increase in the amount of available transmitter. The model of transmitter release suggested by del Castillo & Katz (1956) and Katz (1958) implies that m.e.p.p. frequency would vary with the amount of available transmitter for spontaneous release of transmitter is assumed to occur following random collision of sterically-related surfaces of the presynaptic vesicles and the presynaptic membrane. An increase in vesicle number should then increase the number of successful collisions. On this model therefore a change in the amount of available transmitter could not occur without a change in m.e.p.p. frequency. However, the possibility that m.e.p.p. frequency is not closely linked to the amount of available transmitter warrants further study for it is known, for instance, that when the rat diaphragm *in vitro* is deprived of glucose the e.p.p. amplitude gradually declines (Jeffries, 1953) but the frequency of m.e.p.p.s is unchanged, falling only after prolonged repetitive stimulation is added to the effect of the glucose deprivation (Liley, 1956*d*).

A question of some interest is the means by which a hyperpolarizing current could bring about the movement of transmitter in the nerve terminals. The simplest hypothesis would be that the structural correlates of the quantal units of transmitter, presumably the presynaptic vesicles, are negatively charged and so would move toward the presynaptic membrane in the electrical field produced by applied current. The slow time course of the changes in the amount of available transmitter might reflect the resistance of the viscous axoplasm to the migration of large particles. On the basis of electron microscopic evidence (Birks, Huxley & Katz, 1960), Curtis & Eccles (1960) postulated that the available transmitter is 'those quanta of transmitter at any instant in immediate juxtaposition to the presynaptic membrane'. By means of hyperpolarizing currents it should thus be possible to produce a large increase in the number of vesicles in close proximity to the nerve membrane. For instance, an increase of twenty times in e.p.p. size (Fig. 6, 2·6 μA), would mean that at least twenty times the usual number of vesicles were in virtual contact with the presynaptic membrane. Such a large change in vesicle distribution might well be demonstrable by electron microscopy.

SUMMARY

1. A method for polarizing motor nerve terminals in the rat diaphragm *in vitro* is described. It allows the use of a large range of polarizing currents for long periods of time without nerve block.

2. E.p.p.s elicited at 2–4 sec intervals during the passage of hyperpolarizing currents increased progressively in amplitude. The magnitude of these increases varied with the strength and duration of the applied current. In both curarized and magnesium-poisoned preparations e.p.p.s became large enough to generate muscle action potentials.

3. After the current was turned on, there was a delay which could last several seconds, before any increase in e.p.p. amplitude was detected.

4. After the current was turned off, the e.p.p. amplitude fell, at first rapidly and then more slowly, often taking many seconds to return to control height.

5. The amplitude of m.e.p.p.s was unaltered during hyperpolarizing currents despite concomitant e.p.p. increases. M.e.p.p. frequency was decreased during current flow when preparations were bathed with a Krebs–Ringer solution, but there was no frequency change when the magnesium concentration of this solution was increased to 11 mM.

6. All the e.p.p.s of a tetanic train were progressively increased in amplitude during the passage of hyperpolarizing current.

7. Neuromuscular depression was either unaffected or transiently decreased by hyperpolarizing currents.

8. The hyperpolarizing current is thought to increase the amount of transmitter available for release by nerve impulses.

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