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THE ORIGIN OF THE POST-TETANIC HYPERPOLARIZATION OF MAMMALIAN MOTOR NERVE TERMINALS

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SUMMARY

1. Motor nerve terminals in magnesium-poisoned rat hemidiaphragm-phrenic nerve preparations *in vitro* were stimulated with short depolarizing pulses of approximately threshold strength and the evoked antidromic responses recorded from the phrenic nerve. The percentage of these 1/sec or 0·5/sec stimuli to which there was no antidromic response was used as a quantitative measure of the terminal excitability. After standard tetanic stimulation (1000 impulses at 100/sec) the excitability of the terminals was depressed for an average duration of 60–70 sec, during most of which time no antidromic responses to stimuli of pretetanic intensity were recorded. There was no significant interaction between stimuli to the terminals at rates of 1 or 0·5/sec.

2. Potassium-free solutions at first increased, then decreased, the post-tetanic depression of excitability. Raising $[K]_o$ threefold (15 mM) abolished the post-tetanic depression and often converted it to an exaltation of excitability.

3. Polarizing currents were applied to the terminals with a second electrode. Depolarizing currents increased, while hyperpolarizing currents decreased, the post-tetanic depression of excitability.

4. In solutions with 70% of the normal NaCl content replaced by sucrose, the post-tetanic depression of excitability was reversibly prolonged.

5. In the presence of $7{\cdot}7 \times 10^{-6}$ M digoxin or 0·42 mM ouabain there was a small reversible reduction of post-tetanic excitability.

6. After exposure to solutions containing no glucose or to solutions containing 3–5 mM sodium azide the excitability of the terminals was not altered by the tetanus. After washing with the control solution, post-tetanic depression of excitability returned. Antimycin-A ($1{\cdot}8 \times 10^{-6}$ M) had little or no effect upon post-tetanic excitability.

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7. It was concluded that the post-tetanic depression of excitability reflected hyperpolarization of the terminals and that this hyperpolarization was caused by a shift of the membrane potential towards the potassium equilibrium potential because of an increase in potassium permeability.

INTRODUCTION

It is well known that spike potentials in nerves are followed by positive and negative afterpotentials (Adrian, 1920; Amberson & Downing, 1930; Gasser, 1933). Positive afterpotentials have been shown to summate during and after repetitive (tetanic) stimulation (Gasser, 1935; Gasser & Grundfest, 1936; Grundfest & Gasser, 1938; Straub, 1961; Ito & Oshima, 1962) and this increase in membrane potential after the stimulation is proportional in magnitude and duration to the frequency and number of stimuli (Ritchie & Straub, 1957; Connelly, 1959; Meves, 1961; Hubbard & Schmidt, 1963).

Within the framework of the ionic theory two basic hypotheses have been advanced to explain this post-tetanic hyperpolarization (P.T.H.). First, it has been postulated that P.T.H. is generated directly by the increase in activity of the sodium pump that is caused by the uptake of sodium ions during repetitive activity (Hodgkin & Keynes, 1956; Caldwell, 1960). The hyperpolarization would then be produced either electrogenically by uncoupled extrusion of sodium ions from the interior of the nerve (Connelly, 1959; Straub, 1961; Holmes, 1962) or by a coupled transfer of sodium and potassium ions causing depletion of potassium ions in a restricted extracellular space adjacent to the membrane (Ritchie & Straub, 1957).

Alternatively, there is evidence (Hodgkin & Huxley, 1952) that the after-hyperpolarization which follows a spike occurs because the permeability to potassium ions increases during the spike and does not return at once to its resting value. Since in excitable tissues the resting potential is generally at least 10–20 mV less than the potassium equilibrium potential (V_K), this state of increased permeability raises the membrane potential above its resting value. This explanation was first proposed to account for the positive afterpotential following the single nerve spike (Hodgkin & Huxley, 1952; Coombs, Eccles & Fatt, 1955) and has been extended to the hyperpolarization detected after repetitive stimulation of nerve cells or their processes (Meves, 1961; Ito & Oshima, 1962).

The present investigation was initially undertaken while testing the postulated correlation between P.T.H. in nerve terminals and post-tetanic potentiation of synaptic potentials (Lloyd, 1949; Eccles & Krnjević, 1959; Hubbard & Schmidt, 1963; Hubbard & Gage, 1964). For this purpose it was necessary to determine whether drugs and ionic changes which had

been shown to abolish P.T.H. in other tissues were equally effective in our particular preparation (the rat hemidiaphragm-phrenic nerve *in vitro*). The hyperpolarization was assessed from the concomitant excitability change (Gasser & Grundfest, 1936; Grundfest & Gasser, 1938) in the terminals, since it was clearly impossible to measure the membrane potential directly in such fine structures. Our results support the hypothesis that P.T.H. is due to a prolonged increase in potassium permeability and provide some explanation for other experimental results which conflicted with this interpretation (Ritchie & Straub, 1957). A preliminary report of part of this investigation has been published (Gage & Hubbard, 1964).

METHODS

All experiments were done *in vitro* using rat hemidiaphragm-phrenic nerve preparations mounted in a divided recording-bath and bathed by a continuously flowing, aerated solution kept at a controlled temperature (for further details see Hubbard, 1961; Hubbard & Schmidt, 1963).

There were two types of solution: 'control' and 'test'. Control solutions had the composition described by Liley (1956), except that the bicarbonate concentration was 2 g/l. to give a pH of 7·3–7·4 at 37° C when bubbled with 5 % CO_2 and 95 % O_2. In several preliminary experiments in which Liley's solution containing 1 g/l. sodium bicarbonate was used, the pH was found to be 7·0–7·1. Neuromuscular transmission was blocked by raising the $MgCl_2$ concentration to 12–14 m-moles/l. or raising the $MgCl_2$ concentration to 6–8 m-moles/l. and reducing the $CaCl_2$ concentration to 1 m-mole/l. Test solutions differed from control solutions in that either certain constituents were omitted (e.g. glucose, sodium, potassium), or certain reagents were added (e.g. sodium azide (Drug Houses of Australia); digoxin (Burroughs Wellcome); Antimycin-A (C grade, Calbiochem.). Sucrose was added in amounts sufficient to compensate for the sodium withdrawal, measurements of freezing point depression being made with a Fiske osmometer. The digoxin was dissolved in alcohol, the final alcohol concentration in the test solution being 40 mM.

Control and test solutions passed to the recording chamber through independent systems of tubing set in the same heating-bath. The rates of flow in each system were matched so that there was no temperature change in the recording chamber when the test solution was substituted for control. At the usual flow rate of 3 ml./min, the time for a 95 % change of the fluid in the chamber was found, by a thermal dilution method, to be 4 min. This time coincides with the theoretical 95 % changeover time assuming an exponential change in contents. It was therefore probable that new solutions were equally distributed throughout the bath.

Excitability testing. As shown diagrammatically in Fig. 1, 4 M-NaCl-filled glass microelectrodes with 1–3 μ tips and 0·5–3 MΩ resistance were judged to be in the vicinity of motor nerve terminals when extracellular miniature end-plate potentials (Fig. 1*B*) could be recorded (del Castillo & Katz, 1956; Hubbard & Schmidt, 1963). The electrodes were then connected to a stimulating circuit (Hubbard & Schmidt, 1963) and a depolarizing square pulse of very short duration was applied at a rate of 0·5 or 1/sec. The single all-or-nothing antidromic responses set off by stimulation (Hubbard & Schmidt, 1963) were recorded from a pair of platinum electrodes on the phrenic nerve in oil (Fig. 1*A*). Before the tetanus the strength of the pulse was adjusted so that 10–80 % of the stimuli produced an antidromic response. All the antidromic responses were recorded sequentially on moving film. If the number of responses was satisfactory a tetanus of 1000 supramaximal stimuli at 100/sec was applied to the phrenic nerve through another pair of platinum electrodes. The stimulating

pulses applied to the terminals through the micro-electrode continued at a rate of 0·5 or 1/sec before, during and after the tetanus.

After the tetanus in our control solution there were initially no antidromic responses for some 60 sec (see Results). Thereafter antidromic responses appeared, at first infrequently and then at a frequency approximating to the pretetanic level. To produce a graphical record of the responses it was found most convenient to count in each selected period, not the percentage of responses to stimulation, but the percentage of failures. Thus the percentage

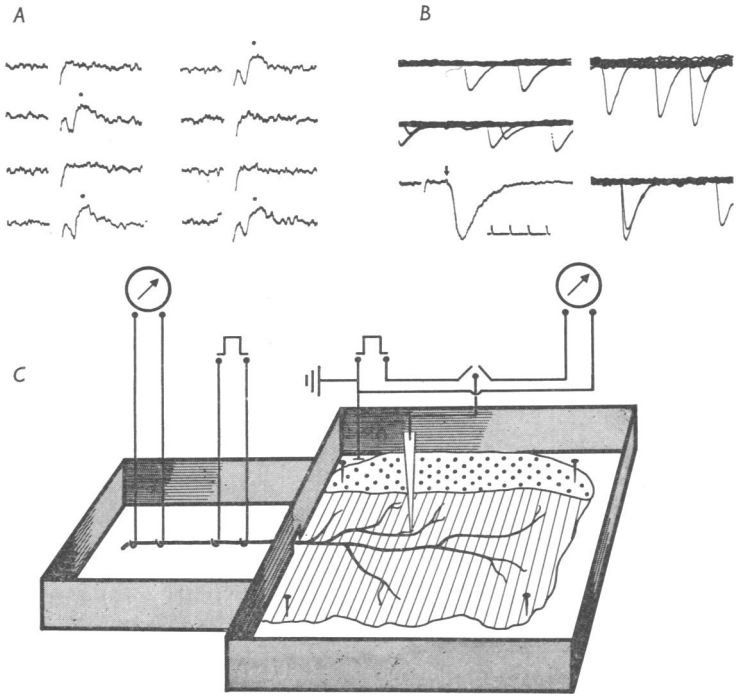

Fig. 1. The method of testing the post-tetanic excitability of nerve terminals.

A. Successive oscilloscope traces recorded during threshold terminal stimulation at 1 sec intervals. Antidromic responses (successes) recorded from the phrenic nerve in oil are marked with a dot. The break in the sweep is due to the stimulus artifact. The percentage of failures in this series was 50 %.

B. Records of extracellular miniature end-plate potentials and an end-plate potential (first column, third record) obtained in the vicinity of nerve terminals. Note the presynaptic spike potential (arrow) preceding the e.p.p. set up by phrenic nerve stimulation (1 msec timer).

C. Schematic diagram of recording and stimulating circuits (see Methods).

of failures in 20–40 sec periods before and in 20 sec periods after the tetanus was measured and the percentages obtained were used as a measure of the nerve threshold at that period and thus presumably of the membrane potential of the terminals (Gasser & Erlanger, 1930; Wall, 1958).

One of the test procedures was to pass currents through the terminals with a second electrode while assessing the post-tetanic excitability changes. The methods used have been fully described (Hubbard & Willis, 1962; Hubbard & Schmidt, 1963).

Solutions were changed in the order control–test–control, several trials of the experimental

procedure being made in each solution. The temperature of the bathing solution was constant to within 0·5° C in any one experiment but ranged between 34 and 37° C in different experiments.

RESULTS

The average duration of the post-tetanic reduction of excitability in the control solution in forty-one trials at fourteen different terminals following tetani of 1000 impulses at 100/sec was 66·2 ± 18·2 sec (mean ± S.D.). This is rather longer than the duration of the post-tetanic reduction of excitability in cat phrenic nerves following a comparable tetanus (Gasser & Grundfest, 1936). No significant correlation was found between the post-tetanic depression of excitability and the pH of the bathing solution in the range 7·0–7·4. It may be inferred that in this preparation the depression

TABLE 1. Results of a run test applied to continuous series of responses in three motor nerve terminals

Unit	Trial	N	M_1	M_2	U_0	μ_u	σ_u^2	T_0	$P_D(T_0)$
A	1	103	43	60	45	51·1	24·11	0·958	$0{\cdot}30 < P < 0{\cdot}40$
A	2	100	45	55	45	50·5	24·25	1·016	$0{\cdot}30 < P < 0{\cdot}40$
A	3	100	64	36	48	47·1	20·98	0·092	$0{\cdot}30 < P < 0{\cdot}40$
A	4	149	93	56	64	70·9	32·55	1·121	$0{\cdot}20 < P < 0{\cdot}30$
A	5	152	57	95	64	72·0	33·15	1·302	$0{\cdot}10 < P < 0{\cdot}20$
B	1	100	61	39	47	48·6	22·39	0·228	$0{\cdot}80 < P < 0{\cdot}90$
B	2	121	67	54	52	60·8	29·30	1·531	$0{\cdot}50 < P < 0{\cdot}60$
C	1	100	33	67	38	45·2	19·31	1·530	$0{\cdot}10 < P < 0{\cdot}20$
C	2	159	85	74	58	80·0	39·12	1·825	$0{\cdot}05 < P < 0{\cdot}10$

N = total number of trials = $M_1 + M_2$

M_1 = total number of positive reactions

M_2 = total number of negative reactions

U_0 = total number of runs

$\mu_u = \frac{2M_1M_2}{N} + 1$

$\alpha = 0{\cdot}05$

$T_0 = \frac{|U_0 - \mu_u| - \frac{1}{2}}{\sigma^u}$

$\sigma^2{}_u = \frac{2M_1M_2\,(2M_1M_2 - N)}{N^2\,(N-1)}$

Null hypothesis in no trial rejected since $P_D(T_0) > \alpha$

of excitability was not an artifact caused by acidic solutions (Holmes, 1962). Because of the difference between terminals, comparisons of the magnitude and duration of the post-tetanic reduction of excitability under test and control conditions were always made at the same presynaptic terminal.

Two problems arise in connexion with these results. First, it was possible that the excitability of the terminals was progressively changed by the continued direct stimulation. This was suggested to us by the observation that the post-tetanic depression of excitability in the terminals following tetanic stimulation of the nerve trunk was of shorter duration than the depression of excitability reported by Hubbard & Schmidt (1963) following tetanic stimulation of the same frequency and duration applied to the terminals themselves. This possibility of progressive change at frequencies

of 1/sec and 0·5/sec was excluded by analysing for independence the responses to stimulation in nine trials at three terminals (Table 1) using a statistical Run Test (Swed & Eisenhart, 1943; Verveen, 1960). The probability of a significant difference between the observed number of runs (Table 1, U_0), and the number expected in a random series (Table 1, μ_u) was estimated. In none of the nine trials shown in Table 1 could the hypothesis of randomness be rejected (Table 1, $P_D(T_0)$), since the probability that there was a significant difference between U_0 and μ_u never exceeded 0·05 (α). Therefore it was assumed that there was no interaction between stimuli or action potentials at these frequencies.

Secondly, it might be argued that a failure of the normal depression of excitability might occur because the tetanic stimulation had not reached the terminals. Nerve block was in fact a late consequence of exposure to the cardiac glycosides digoxin and ouabain, and to sodium azide. Furthermore, the nerve under these conditions failed to conduct all the action potentials set up by tetanic stimuli sometime before single action potentials failed to reach nerve terminals. To exclude the possibility of complete or partial nerve block whenever there was no depression of excitability after a tetanus, the micro-electrode was reconnected to the recording circuit (Fig. 1) and the tetanus repeated. Only experiments in which there was a complete correspondence of extracellular e.p.p.s and stimuli during and after the tetanic stimulation are reported here. The changes in the post-tetanic potentiation of e.p.p.s will be described in a following paper.

Effects of varying the extracellular potassium concentration. The rival theories of P.T.H. generation predict opposite effects following this procedure, so it provides a crucial test. For instance, the active extrusion of sodium ions is decreased by a reduction of the extracellular potassium ion concentration and is increased by an increase in the extracellular potassium ion concentration (Hodgkin & Keynes, 1955). Thus if P.T.H. is a direct result of activity of the sodium pump, whether by electrogenic, uncoupled sodium extrusion (Connelly, 1959; Straub, 1961; Greengard & Straub, 1962) or by a coupled transfer of potassium ions causing a depletion of immediately extracellular potassium ions (Ritchie & Straub, 1957), it should be reduced by decreasing the extracellular potassium concentration $[K]_o$, and increased by increased $[K]_o$. Conversely, if P.T.H. is due to an increase in potassium permeability (Meves, 1961) it should be changed in just the opposite manner, for the hyperpolarization should, on this theory, be proportional to the difference between V_k and the resting potential. Decreasing $[K]_o$ will increase V_k and increasing $[K]_o$ will have the opposite effect. These changes in V_k should be associated with changes in P.T.H. of similar kind.

The first detectable effect of decreasing the extracellular potassium con-

centration of the solution was an increase in the magnitude and duration of the post-tetanic subnormality (Fig. 2*B*). After an exposure of 30 min or more, the opposite effect was found, there being little or no change in excitability after the tetanus (Fig. 2*C*). At this terminal, excitability was depressed for about 70 sec in the control solution and the first antidromic spike appeared 58 sec after the tetanus (arrow in Fig. 2*A*), but after 20 min in a potassium-free solution the depression lasted more than 120 sec and although the first antidromic spike was at 58 sec (Fig. 2*B*, first arrow)

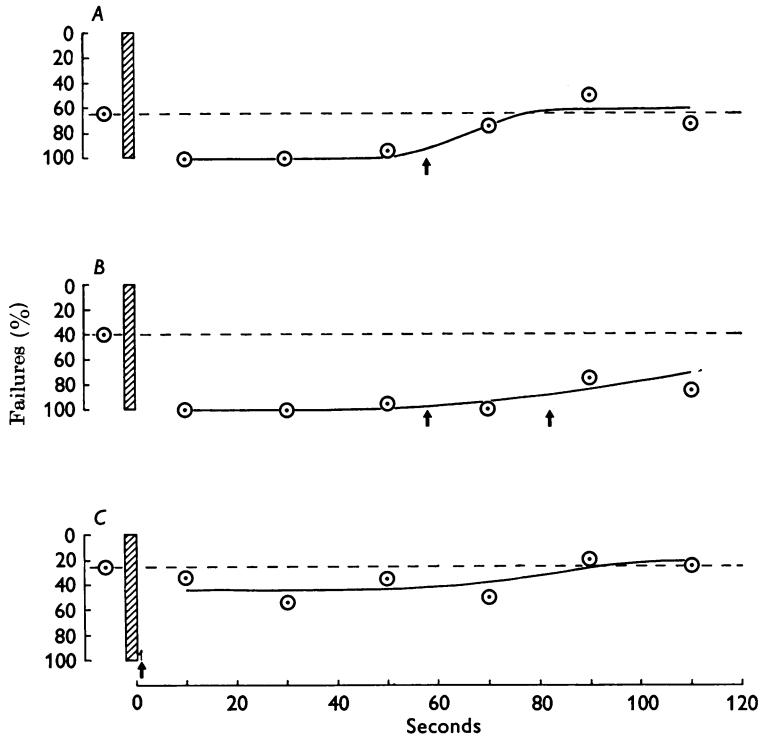

Fig. 2. The effect of a potassium-free solution upon post-tetanic excitability. *A*. Control. *B*. After 20 min in the test solution. *C*. After 35 min in the test solution. *Ordinates*: Percentage of threshold stimuli applied to the nerve terminals at a rate of 1/sec which did not evoke an antidromic action potential (% failures). Interrupted horizontal line represents the percentage of failures in the observation period (20–40 sec) before the tetanus. The vertical hatched block indicates a standard tetanus of 1000 stimuli at 100/sec applied to the phrenic nerve. In the post-tetanic period, the circles represent the percentage of failures in consecutive 20 sec periods after the tetanus, stimulation being at 1/sec and the same strength as before the tetanus. *Abscissae:* Time (sec) measured from the end of the tetanus. The circles are placed in the centre of the 20 sec periods over which the percentage of failures was calculated. The arrows mark the time at which the first post-tetanic antidromic response occurred. In *B* the second arrow denotes when the second antidromic response was seen.

the next response was not seen until 82 sec after the tetanus (Fig. 2*B*, second arrow). Fifteen minutes later, after a total time of 35 min in a potassium-free solution, there was no depression of excitability immediately after the tetanus (Fig. 2*C*), and the first antidromic spike was seen after 2 sec. Both effects were completely reversible upon soaking in a solution with the normal (5 mM) potassium content.

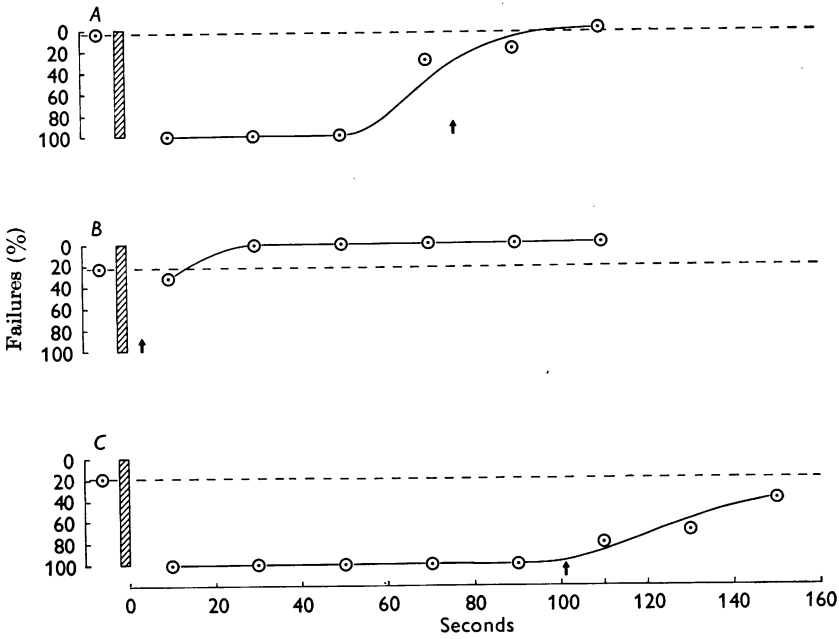

Fig. 3. Post-tetanic exaltation of excitability in 15 mM-K. *A*. Control. *B*. After 19 min in the solution containing 15 mM-K. *C*. 26 min after return to the control solution. The ordinates and abscissae and the significance of the hatched block, horizontal lines and arrows are the same as in Fig. 2.

Solutions containing 15 mM-KCl (three times normal) reversibly abolished the post-tetanic depression of excitability (Fig. 3). In this experiment in the control solution the post-tetanic reduction of excitability lasted about 100 sec (Fig. 3*A*). After 19 min in 15 mM-KCl, an antidromic spike was elicited 3 sec after a tetanus, and instead of a post-tetanic reduction of excitability there was a post-tetanic increase in excitability (Fig. 3*B*). This excitability change was fully reversed when the preparation was returned to the control solution (Fig. 3*C*).

It may be concluded that the results of varying the extracellular potassium concentration are those expected from the permeability hypothesis and are the opposite of those expected if the depression of excit-

ability was generated by uncoupled extrusion of sodium ions (Connelly, 1959) or the uptake of potassium ions from a confined space adjacent to the nerve terminal (Ritchie & Straub, 1957).

Effect of applied currents. The changing of the membrane potential of terminals by means of hyper- or depolarizing currents again afforded a discriminative test of the rival hypotheses for P.T.H. generation. The active extrusion of sodium ions is independent of membrane potential (Hodgkin & Keynes, 1954), but on the permeability theory alteration of membrane potentials nearer to or further from V_k should decrease or increase P.T.H. respectively.

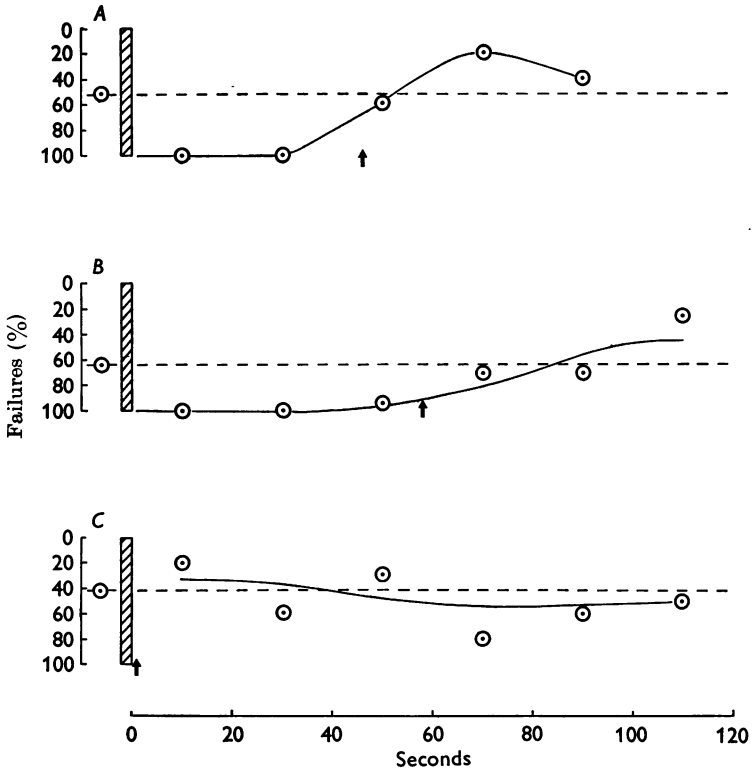

Fig. 4. The effect of polarizing currents upon post-tetanic excitability. *A*. Control. *B*. After application of a 1·56 μA depolarizing current to the terminal through a second micro-electrode. *C*. After application of a 0·88 μA hyperpolarizing current to the same terminal. The ordinates and abscissae and the significance of the hatched block, horizontal lines and arrows are the same as in Fig. 2.

As in the case of varying $[K]_o$, our results were those predicted by the permeability theory. The magnitude of the post-tetanic reduction of excitability could be graded by passing suitable hyperpolarizing and depolarizing currents. In the experiment illustrated (Fig. 4), after a post-

tetanic reduction of excitability of normal magnitude and duration had been observed (Fig. 4*A*), depolarizing currents of 1·56 μA were applied to the terminal through a second electrode. Following tetanic stimulation there was no antidromic response for 59 sec, and the excitability did not return to the control level for 80–90 sec (Fig. 4*B*). The current was now turned off, and after an interval a hyperpolarizing current of 0·88 μA was applied. There was now no significant post-tetanic excitability change, the first stimulus evoking a response (Fig. 4*C*).

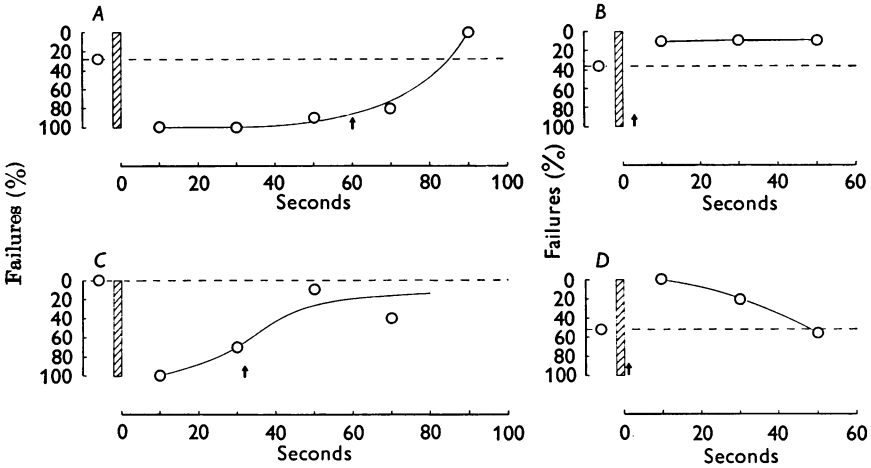

Fig. 5. The effect of hyperpolarizing currents upon post-tetanic excitability. *A*. Control. *B*. After the application of a 2·15 μA hyperpolarizing current. *C*. 3 min after the current was switched off. *D*. After application of a 1 μA hyperpolarizing current. (*D*) 3 min after (*C*). The ordinates and abscissae and the significance of the hatched block, horizontal lines and arrows are the same as in Fig. 2.

In other experiments the hyperpolarizing current was progressively increased, from levels producing a small diminution of the excitability change, up to current strengths at which the post-tetanic depression of excitability was replaced by a post-tetanic increase in excitability. In these cases, presumably, the membrane potential was changed to a level negative to the potassium equilibrium potential. For example, in the experiment illustrated in Fig. 5, before the application of current, the first antidromic spike was seen 60 sec after a tetanus and the depression of excitability lasted 80–90 sec (Fig. 5A). Immediately after the application of a hyperpolarizing current of 2·15 μA to the terminal, this depression was replaced by a post-tetanic increase of excitability (Fig. 5*B*). When the current was turned off, post-tetanic depression of excitability was again detected but was smaller in amplitude and duration than in the initial control, the first antidromic response occurring 32 sec after the tetanus (Fig. 5*C*). A second

application of a smaller hyperpolarizing current (Fig. 5*D*) again changed the post-tetanic depression of excitability to an increase of excitability which was of shorter duration than that found with the higher current (Fig. 5*B*).

The reduction in the depression of excitability seen in Fig. 5*C* illustrates another feature of hyperpolarizing currents. Apparently, some change in terminal properties outlasted the current application, for, after repeated application of hyperpolarizing currents, there was a post-tetanic increase in excitability after tetanic stimulation even in the absence of current. This was reversed to the normal depression either by the application of depolarizing current or by the passage of time (min).

Alternative mechanisms of hyperpolarization. Although the effects of varying $[K]_o$ and the use of depolarizing currents clearly indicate that the post-tetanic excitability change was generated by a change in potassium permeability, it was of interest to make three further tests which have been used by those experimenters who favour the generation of P.T.H. by sodium pump activity. These were: (1) reduction of the extracellular sodium concentration, (2) the exhibition of cardiac glycosides, and (3) the exhibition of metabolic inhibitors.

If P.T.H. is due to sodium extrusion following the increased uptake of sodium ions provoked by repetitive activity (Connelly, 1959), it should be reduced in magnitude by reducing the influx of sodium ions which is assumed to act as a stimulus to sodium pump activity (Hodgkin & Keynes, 1956). One means of doing this is to reduce the extracellular sodium ion concentration. When the phrenic nerve terminals were exposed to solutions in which 70% of the sodium had been osmotically replaced by sucrose, we found the exact opposite result to that predicted—the post-tetanic depression of excitability was reversibly prolonged. For instance, in one complete experiment the excitability change lasted 70–80 sec in control solution but after the terminal had been exposed for 43 min to a solution containing 70% less sodium the tetanus was followed by depression of excitability for 120–130 sec. The preparation was then returned to the control solution and a tetanus 44 min later was followed by excitability depression of a similar duration to that observed before lowering the sodium ion concentration.

The cardiac glycosides digoxin and ouabain have been shown to inhibit active sodium and potassium ion transfer in red blood cells, frog muscle, rat muscle and squid axons (Schatzmann, 1953; Greeff & Westermann, 1955; Johnson, 1956; Glynn, 1957; Caldwell & Keynes, 1959). These agents would thus be expected to reduce P.T.H. if this is dependent on increased sodium extrusion (Connelly, 1959) alone or on coupled increased potassium uptake (Ritchie & Straub, 1957). A reduced influx of potassium ions would

of course also lower V_k so that abolition of P.T.H. would be consistent with both types of hypothesis. In several experiments after 17–18 min exposure to solutions containing $7{\cdot}7 \times 10^{-6}$ M digoxin, some reversible reduction in the duration and magnitude of the post-tetanic depression of excitability was detected. Thus in one complete experiment in the control solution the depression of excitability lasted 50–60 sec and the first antidromic spike was recorded 45 sec after the tetanus. After 17·5 min in digoxin the depression of excitability lasted 40–50 sec and the first antidromic spike was noted 15 sec after the tetanus. Thirteen minutes after returning to the control solution the post-tetanic depression of excitability was of the control magnitude and duration. With ouabain (0·42 mM) lesser effects were detected. For example, in one experiment the depression of excitability lasted 70–80 sec in the control solution, the first antidromic spike being recorded 62 sec after the tetanus. After 12 min in ouabain the depression of excitability lasted 50–60 sec and the first antidromic spike was seen 42 sec after the tetanus. After 20 min exposure to this concentration nerve block occurred.

Metabolic inhibitors. This again is not a specific test for the origin of P.T.H. because, although active sodium extrusion and potassium uptake by nerve are dependent upon energy-yielding reactions, paralysis of this system will rapidly lead to a fall in the intracellular potassium ion concentration and thus reduce the equilibrium potential for potassium. Thus, all of the theories so far discussed would predict abolition of P.T.H. by metabolic inhibitors. The methods used to inhibit metabolic reactions were withdrawal of the only substrate (glucose) in the bathing medium, paralysis of aerobic respiration using a specific blocker of the cytochrome system, antimycin A (Slater, 1958), and inhibition of phosphorylation by the exhibition of sodium azide (Loomis & Lipmann, 1949).

When preparations were exposed to solutions containing no glucose, there was a very slow loss of the ability to generate a post-tetanic depression of excitability (Fig. 6). Thus after 9 min in a glucose-free solution, the depression was of normal duration (Fig. 6*A*). After 27 min in the glucose-free solution, however, the depression lasted only 20–30 sec and antidromic spikes returned 2 sec after the tetanus (Fig. 6*B*). Recovery in control solution was slow. Six minutes after restoration of glucose it could be seen that the magnitude of the depression was smaller than in the control (Fig. 6*C*). Indeed only after about 75 min had elapsed did the depression return to the control value (Fig. 6*D*).

Antimycin A ($1{\cdot}8 \times 10^{-6}$ M) which was found to abolish P.T.H. in C-fibres (Greengard & Straub, 1962) had little effect on post-tetanic depression of excitability at rat motor nerve terminals. In several experiments the depression was reduced in duration within 7 min of the exhibition of the

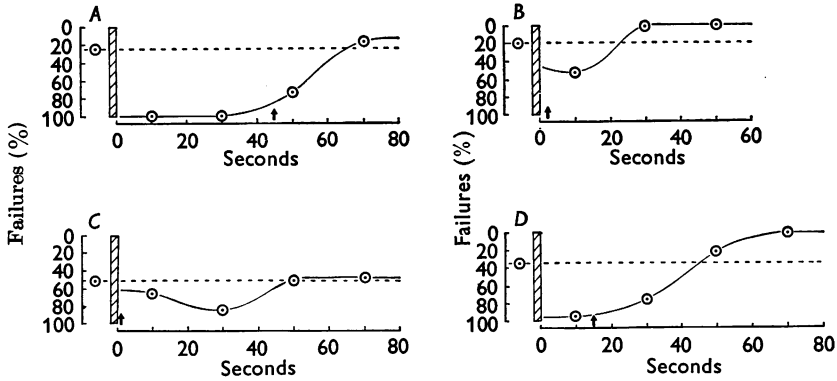

Fig. 6. The effect of omission of glucose from the bathing solution upon post-tetanic excitability. *A*. After 9 min in a glucose-free solution. *B*. After 27 min in a glucose-free solution. *C*. 6 min after return to the control solution (glucose concentration 1 mM). *D*. 75 min after returning to the control solution. The ordinates and abscissae and the significance of the hatched block, horizontal lines and arrows are the same as in Fig. 2.

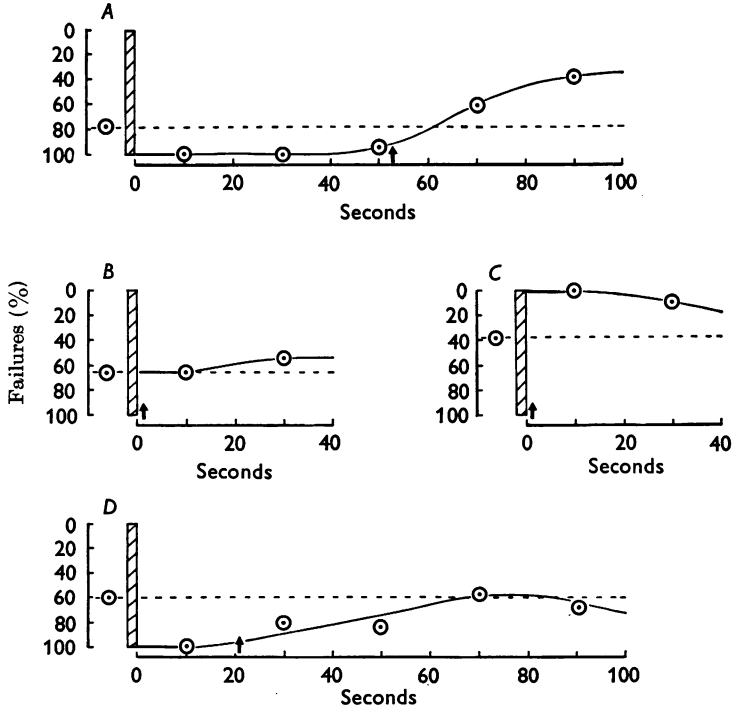

Fig. 7. The effect of sodium azide upon post-tetanic excitability. *A*. In control solution. *B*. 1 min after exposure to 5 mM sodium azide. *C*. 6 min after returning to the control solution. *D*. 43 min after returning to the control solution. The ordinates and abscissae and the significance of the hatched block, horizontal lines and arrows are the same as in Fig. 2.

antimycin A, but prolonged exposure (29 min) caused no further change and upon washing with control solution the change was not reversible.

Sodium azide was the most effective metabolic reagent used. In a concentration of 3–5 mM it abolished the post-tetanic depression of excitability so rapidly that it is probable that lower concentrations would have been equally effective. A typical experiment is illustrated in Fig. 7. In the control solution the excitability depression lasted about 60 sec and the first antidromic response was seen 53 sec after the end of the tetanus (Fig. 7*A*). After the preparation had been exposed for 1 min to sodium azide, there was no depression of excitability after a tetanus (Fig. 7*B*), and 5 min later another tetanus was followed by a phase of increased excitability (Fig. 7*C*). The preparation was then washed in control solution for 43 min. Even after this time, although some recovery had occurred, it was obviously incomplete (Fig. 7*D*).

DISCUSSION

Three problems will be discussed: (1) the validity and the limitations of the technique used to measure post-tetanic excitability of terminals; (2) the mechanism of P.T.H. revealed by the results; (3) the general applicability of this mechanism.

The evidence that post-tetanic hyperpolarization and depression of excitability are concomitant in axons and nerve cells is overwhelming (Gasser & Erlanger, 1930; Gasser & Grundfest, 1936; Grundfest & Gasser, 1938; Rudin & Eisenman, 1954; Wall, 1958). It seems unreasonable then to question whether the depression of excitability revealed in the present investigation is also due to hyperpolarization of the membrane of the nerve terminals; and this will be assumed for the remainder of the discussion. What is perhaps doubtful is the sensitivity of the technique of threshold testing to small differences in the magnitude and duration of the hyperpolarization, obscured as these must be by the spontaneous variations of threshold with time (Verveen, 1961). It is for this reason that most stress in the present investigation has been laid on abolition of the post-tetanic changes or their reversal (e.g. Figs. 3, 5) from depression to exaltation of excitability. Conversely, the small changes detected upon the exhibition of cardiac glycosides and antimycin A, while consistent, cannot be given the same weight as the investigations in which the post-tetanic depression of excitability was abolished.

The results so effectively corroborate the hypothesis that P.T.H. is due to a persistent increase in potassium permeability that little comment is required. In particular, the variation of P.T.H. with the extracellular potassium concentration (Figs. 2, 3) and with the magnitude and duration of applied currents (Figs. 4, 5) cannot be explained on any other theory so

far put forward. The finding (Fig. 2) that P.T.H. is at first increased by reducing $[K]_o$ is of course fully in accord with the permeability hypothesis since the membrane potential would initially be moved further from the potassium equilibrium potential. The later reduction in P.T.H. (Fig. 2*C*) can be explained by the depletion of intracellular potassium which would be brought about through the reduction of active ion transport (Hodgkin & Keynes, 1955) and by the loss of internal potassium because of an increased potassium gradient (Fenn & Cobb, 1934; Steinbach, 1940; Boyle & Conway, 1941). This change of P.T.H. with time, when $[K]_o$ is low, may also explain the fact that in rabbit and cat unmyelinated nerves, Holmes (1962) found that P.T.H. was increased when $[K]_o$ was lowered, yet Ritchie & Straub (1957) using the same preparation and procedure found that P.T.H. was decreased.

The failure of solutions with reduced sodium content to abolish P.T.H. was again in accord with the permeability hypothesis, although no explanation can be offered for the increase in P.T.H. which was found also by Ritchie & Straub (1957) under these circumstances. It is interesting to note that if sodium is replaced by lithium rather than by sucrose, which was used in the present experiments, P.T.H. is abolished (Ritchie & Straub, 1957; Connelly, 1959). This can be explained by the finding (Hurlbut, 1963) that, when lithium is substituted for sodium, frog nerves lose potassium as they gain lithium. In contrast, with sucrose as a substitute, the intracellular potassium is well maintained. The reduction in P.T.H. in the presence of lithium is probably due to the reduction of the potassium equilibrium potential, which is thus brought closer to the membrane potential.

The effects of the cardiac glycosides and metabolic inhibitors on P.T.H. (Figs. 6, 7) are consistent with both types of hypothesis and thus provide no conclusive evidence for either. The cardiac glycosides digoxin and ouabain were not very effective in reducing P.T.H. but did eventually cause nerve block. It is possible that these drugs may inhibit sodium transfer more than potassium transfer in this preparation and so cause nerve block while having little effect on V_k.

The metabolic inhibitors differed greatly in their effectiveness presumably reflecting the sources of energy for active ion transfer in the terminals. Antimycin A, which blocks aerobic metabolism (Slater, 1958), did not abolish P.T.H. at a concentration effective in C-fibres (Greengard & Straub, 1962) and this suggests that in motor nerve terminals anaerobic mechanisms may be sufficient to satisfy the energy requirements of the sodium pump. The relatively slow effect of glucose lack on P.T.H. (Fig. 8) was not unexpected since glycogen stores, presumably, would take a considerable time to deplete. The effect of sodium azide (Fig. 9) indicates that

this drug is probably a very effective inhibitor of active ion transport and its rapid action is compatible with the permeability theory if it is assumed that: (1) The potassium flux per impulse in nerve terminals is of the same order as that found in squid axons (4×10^{-12} mole/cm^2/impulse, Keynes, 1951). (2) The phrenic nerve terminals have the dimensions, to a first approximation, of a cylindrical synaptic gutter (90–150 μ long) (Gutmann & Young, 1944; Cole, 1957) with a cross-sectional area of 1–3 μ^2 (Thies, 1960). (3) $[K]_i$ is at most 150 mM.

It can then be calculated, assuming effective inhibition of the sodium pump, that a tetanus of the order of 1000 impulses would so deplete $[K]_i$ and consequently lower V_k that P.T.H. would be abolished. With a normally functioning pump, significant restoration of $[K]_i$ must therefore occur during the tetanus to maintain V_k, as has been shown in mammalian C-fibres (Keynes & Ritchie, 1963).

It will be appreciated that the evidence for a metabolic origin of P.T.H. in nerve fibres, based as it is on the abolition of P.T.H. in solutions with lithium substituted for sodium (Connelly, 1959) or on the inability of lowered $[K]_o$ to increase P.T.H. (Ritchie & Straub, 1957) can be differently interpreted or otherwise explained. There seems no reason, therefore, to consider in vertebrate or squid nerves any other hypothesis for P.T.H. generation than that of a prolonged increase in potassium permeability, as suggested by Meves (1961) following his experiments on frog nodes of Ranvier. It appears, however, that the hypothesis cannot be generalized to all excitable tissues, for in muscle there is good evidence that active extrusion of sodium ions can cause hyperpolarization of the membrane (Frumento, 1964; Mullins & Awad, 1965). There is probably a basic difference, however, between the mechanisms of active ion transfer in nerve and muscle, since drugs such as sodium azide appear to have completely opposite effects on sodium efflux in the two tissues (Hodgkin & Keynes, 1955; Hurlbut, 1958; Horowicz & Gerber, 1965). Thus the existence of an electrogenic pump in muscle adds no support to the hypothesis that P.T.H. in nerve is caused by a similar uncoupled efflux of sodium ions.

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