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POST-STIMULUS HYPERPOLARIZATION AND SLOW POTASSIUM CONDUCTANCE INCREASE IN *APLYSIA* GIANT NEURONE

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SUMMARY

1. Intracellular records from *Aplysia* giant (R 2) cell somata showed long lasting 4–10 mV hyperpolarizations after passage of outward current through a second intracellular electrode.

2. An increase in membrane slope conductance occurred simultaneously with the post-stimulus hyperpolarization (PSH).

3. Both the PSH and conductance-increase varied strongly with stimulus amplitude and duration.

4. Both the PSH and the conductance increase occurred in Ca-free medium containing tetrodotoxin, when action-potential production was completely blocked.

5. The PSH persisted in the presence of ouabain or DNP, with cooling, with removal of external K^+, and in media where all the Na^+ was replaced with Li^+, suggesting that it was not due to the activity of an electrogenic pump.

6. A reversal potential for the PSH was demonstrated by application of maintained inward current following the end of an outward-directed stimulus.

7. The PSH reversal potential varied with $[K]_o$, but not with $[Cl]_o$ or $[Na]_o$, suggesting that the PSH was mainly due to an increase in K conductance.

8. The PSH and the conductance increase were reduced strongly when all the Na^+ was replaced with Tris, and only slightly when Na^+ was replaced with sucrose.

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INTRODUCTION

Maintained depolarizing stimuli applied to the *Aplysia* giant cell commonly cause after-hyperpolarizations of considerable duration. Similar long-lasting hyperpolarizations following nerve activity have been reported for a variety of preparations (Table 1). This phenomenon may result from at least three classes of mechanisms: conductance changes, equilibrium potential changes, or the activation of a metabolic pump.

In the first class, conductance changes may either be mediated directly by the tetanus (Meves, 1961; Gage & Hubbard, 1966) or by depolarization without action potentials (Albuquerque & Grampp, 1968), or indirectly

TABLE 1. Examples of long-lasting post-tetanic hyperpolarizations

Source	Preparation	Reported mechanism: Electrogenic pump	Reported mechanism: K-conductance increase
Ritchie & Straub, 1957	Rab. symp. C-fibres	Possible	.
Connelly, 1959	Frog A-fibres	+	.
Straub, 1961	Frog A-fibres	+	.
Meves, 1961	Frog single node	.	+
Holmes, 1962	Rab., rat, cat C-fibres	+	.
Gage & Hubbard, 1966	Rat phrenic terminals	.	+
Nakajima & Takahashi, 1966	Crayfish str. receptor	+	.
Rang & Ritchie, 1968	Rab. vagus C-fibres	+	.
Den Hertog & Ritchie, 1969	Rab. vagus C-fibres	+	.
Baylor & Nicholls, 1969	Leech ganglion cells	+	.
Kuno *et al.* 1970	Cat DSCT neurones	+	.
Sokolove & Cooke, 1971	Crayfish str. receptor	+	.

through a recurrent inhibition system (Eccles, 1964). Hyperpolarization may result from an increased conductance to ions with equilibrium potentials more negative than the resting potential, from a decreased conductance to ions with equilibrium potentials more positive than the resting potential, or from a combination of both effects. We might expect the hyperpolarization to be accompanied by a change in the relation of membrane slope conductance to voltage. If, however, the hyperpolarization results from an increase in conductance to one ion having an equilibrium potential more negative than the resting potential and a simultaneous decrease in conductance to another ion having an equilibrium potential less negative than resting, then no net change of conductance need occur. In any case, a reversal potential should be demonstrable by the application of inward current following the stimulus. Further, by manipulating

the external ion concentrations it should be possible to vary the reversal potential (Nakajima & Takahashi, 1966; Rang & Ritchie, 1968).

In the second class, hyperpolarization may result from passive ion movements which shift the equilibrium potentials negatively. For example, diffusion of the electrode filling solution into the cell may occur; one should, therefore, examine the effect of changing the filling solution as a control. The extracellular accumulation of K shifts the membrane e.m.f. positively (Shanes, 1951; Frankenhaeuser & Hodgkin, 1956; Narahashi & Yamasaki, 1960; D. C. Eaton, 1971), and so could not explain post-stimulus hyperpolarization.

In the last class, metabolic pumps can hyperpolarize directly by the extrusion of cations, notably as the electrogenic Na pump, or indirectly by ion transports which shift the equilibrium potential (Ritchie & Straub, 1957). Application of ouabain, DNP, cooling, lowering external K and substitution of Li for Na are all procedures which inhibit pumps (see refs. in Table 1 reported as electrogenic pumps). Further, the hyperpolarization resulting from an electrogenic pump should show increased activity with Na injection (Kerkut & Thomas, 1965; Nakajima & Takahashi, 1966).

We will show that for the *Aplysia* giant cell, the hyperpolarization following depolarization is due to a slow K conductance change. Much of this material has been reported previously (Junge & Brodwick, 1970).

METHODS

Specimens of *Aplysia californica* of 3–6 in. body length were used. The parieto-visceral ganglion was removed and made immobile by embedding four sutured connectives into a wax-filled chamber and placing a silver retaining ring around the circumference of the ganglion. Almost all experiments were performed with the giant (R 2) cell (Frazier, Kandel, Kuperfermann, Waziri & Coggeshall, 1967). The ganglion was usually soaked in a 2 ml. solution of 10 mg pronase/ml. (B-Grade, Calbiochem Company) in normal *Aplysia* saline for 15–20 min, as an aid to impalement. However, both the post-stimulus hyperpolarization (PSH) and conductance increase described in this report were observed in preparations where the connective tissue capsule of the ganglion was dissected open without the use of pronase. The connective tissue capsule after treatment with pronase did not impede the actions of any of the ion substitutions or drugs tested, as most of the resulting effects were complete within 5 min after the start of perfusion. The only exception was tetrodotoxin, which usually blocked the action potentials in Ca-free solution within 15 min.

Transmembrane potential in the giant cell was monitored with a 5–20 MΩ micro-electrode inserted through the ganglion capsule and cell membrane, and stimulating currents were applied through a second micro-electrode (2–5 MΩ). Both micro-electrodes were filled with 3 M-KCl, and mounted in holders containing normal saline. Normal saline-agar bridges with chlorided silver leads were placed in the electrode holders. In some experiments the stimulating electrode was filled with 3 M-NaCl, to test for possible effects of K injection. No observable differences in PSH or conductance changes were seen when using NaCl instead of KCl. Membrane

potentials were recorded with respect to another normal saline-agar bridge placed in the external solution. The magnitude of the liquid-junction potentials developed at such an external bridge electrode have been shown previously to be less than 2 mV for the solutions used in this report (Geduldig & Junge, 1968), except for Na-free sucrose solution and Cl-free solution. Junction potentials at the external saline-agar bridge electrode were measured with respect to a 3 M-KCl-agar bridge, and were +6 mV for Na-free sucrose solution and +3 mV for Cl-free solution. Both the recording micro-electrode and external bridge electrode were connected to ELSA-2 amplifiers (Electronics for Life Sciences), and the difference in amplifier outputs was displayed on the oscilloscope. This differential recording method reduced the effects of amplifier drift and 60 c/s interference. Stimulating current was led from the bath with a chlorided silver wire, and measured across a 100 kΩ resistor between bath and circuit ground.

In some experiments a 'current clamp' was employed as follows: stimulating current as measured across the 100 kΩ resistor was subtracted from a command signal at the input of a 100 V operational amplifier (Philbrick Model MLF-100). The plus output of the operational amplifier was applied to the stimulating electrode; thus the feed-back loop acted to equalize command current and measured current.

TABLE 2. Composition of solutions (mM)

	Na	K	Ca	Mg	Cl	SO_4	$MeSO_4$	Sucrose	Total Tris
Normal saline	494	11	11	49	572	30	.	.	10
K-free	505	.	11	49	572	30	.	.	10
High-K	472	33	11	49	572	30	.	.	10
Cl-free	494	11	11	49	.	30	572	.	10
Na-free (Tris)	.	11	11	49	466	30	.	.	541
Na-free (sucrose)	.	11	11	49	78	30	.	673	10
Ca-free	494	11	.	49	562	30	.	.	26

This circuit aided in maintaining long stimulus currents at a constant level despite changes in stimulating electrode resistance. However, for most experiments the current was applied directly from a Tektronix model 161 pulse generator through a 250 MΩ series resistor.

The composition of the solutions used is shown in Table 2. The normal saline was made up of NaCl 494 mM, KCl 11 mM, $CaCl_2$ 11 mM, $MgCl_2$ 19 mM, $MgSO_4$ 30 mM, Tris 10 mM. The Tris (Tris-hydroxymethyl aminomethane) was neutralized to pH 7·7 with HCl in all solutions except Cl-free, where methanesulphonic acid was used. K-free solutions had an excess of 11 mM-NaCl above normal, and high-K solutions a deficit of 22 mM-NaCl below normal, to maintain tonicity. Cl-free solutions were prepared as follows: 50 g of methanesulphonic acid ($MeSO_4$) was added to 19 m-mole/MgO (MgO was added during the acid stage to prevent precipitation of Mg $(OH)_2$). Then 10 m-mole Tris, 494 m-mole NaOH and 11 m-mole KOH were added, and the pH was adjusted to 7·7 with $HMeSO_4$. After this 11 m-mole $CaCO_3$ and 30 m-mole $MgSO_4$ were added, and the solution was made up to 1 l. Na-free solutions were prepared by dissolving 541 m-mole Tris, neutralizing to pH 7·7 with HCl, then adding the usual amounts of KCl, $CaCl_2$, $MgCl_2$ and $MgSO_4$, and diluting to 1 l., or by dissolving 673 m-mole sucrose and adding the other constituents. Ca-free solutions also contained neutralized Tris as the substitute species (for a discussion of the calculations used in replacing Na and Ca with neutralized Tris, see Geduldig & Junge, 1968). Tetrodotoxin was added to Ca-free solutions from a stock solution of 10^{-3} g/ml.,dissolved in 10^{-4} N-HCl.

Solution changes were performed by continuous perfusion of the chamber (volume = 3 ml.) until 60 ml. of the new solution had passed through. This method was shown by dye-dilution experiments to result in a thorough exchange of solutions. The perfusion usually took about 3 min, and was stopped before obtaining recordings, to reduce vibration artifacts.

Almost all of the experimental tests in this report were 50 sec in duration. Most of the oscilloscope photographs included a period of resting potential less than 10 sec, a stimulus 13 sec in duration or shorter, and 27 sec or more of recovery. This period of recovery was usually sufficient for the membrane potential and slope conductance to return to unstimulated values, but to insure complete recovery we waited at least 2 min between the end of one test and the start of the next. Action potentials of constant overshoot were obtained for several hours.

Experiments were performed throughout the year, and were all conducted at 20–23° C, unless otherwise indicated.

RESULTS

Post-stimulus hyperpolarization and conductance increase. The effect of passage of about 2×10^{-7} A through the giant cell membrane for 10 sec is shown in Fig. 1. Following the burst of action potentials (peaks obscured by setting beam intensity low enough to photograph at this speed), the membrane potential was hyperpolarized 10 mV, and returned to the original resting level (-49 mV) after about 20 sec. The magnitude of the PSH varied with the stimulus parameters, but the greatest values obtainable in the various giant cells examined fell in the range of 4–10 mV. The duration of the PSH was also variable; the hyperpolarization usually disappeared after 15–20 sec, although in some cases it persisted for 40 sec after termination of the stimulus. Application of strong inward currents within the period of PSH did not alter its normal time course. A similar long-lasting hyperpolarization could be produced by a 10-sec train of brief (50 msec) depolarizing pulses applied at 10/sec, each pulse producing one action potential. Occasionally, it was possible to observe a membrane *depolarization* following a long *hyperpolarizing* stimulus. However, the phenomenon was more short-lasting than the hyperpolarization following a depolarizing stimulus, and was often not present. Hence this study was confined to the effects of depolarizing stimuli, to which the term 'PSH' refers exclusively.

The effects of a depolarizing stimulus on membrane slope conductance (g_s) is shown in Fig. 2. Membrane slope conductance was measured as $\Delta I/\Delta E$, where ΔI was the amplitude of a rectangular current pulse applied to the cell, and ΔE the change in potential produced by the current. The insets on the left side of Fig. 2 show conductance measurements starting from the resting potential and from various levels of hyperpolarization. The insets on the right side show the same measurements starting 0·5 sec after the end of a 10-sec stimulus. On the graph are plotted (lower line)

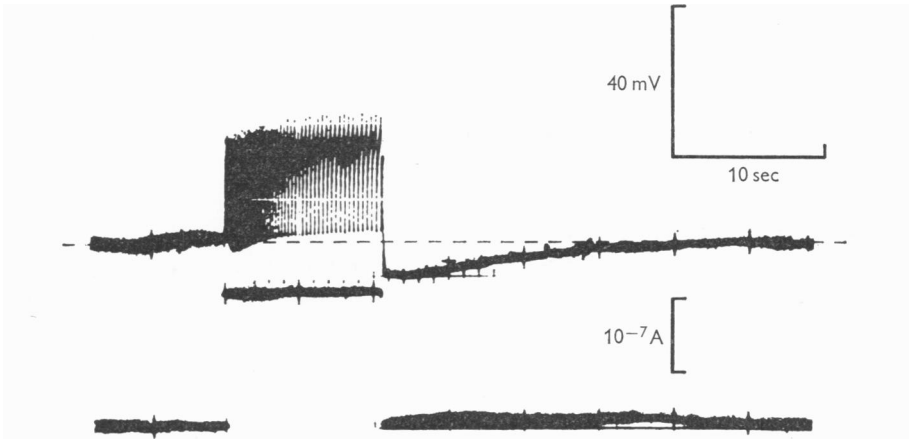

Fig. 1. Post-stimulus hyperpolarization in giant neurone of *Aplysia*. Upper trace: transmembrane potential, spikes during stimulus partially obscured. Lower trace: applied current.

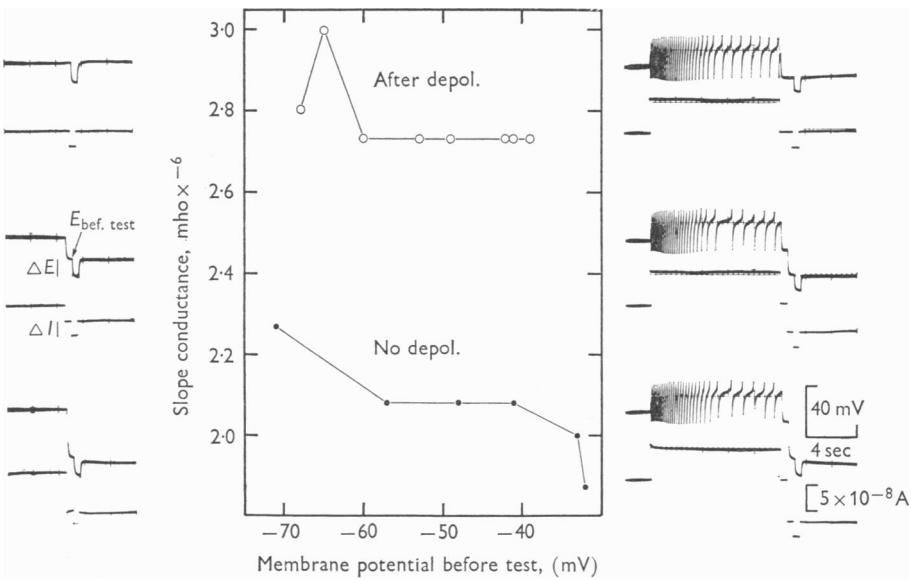

Fig. 2. Increase in membrane slope conductance caused by depolarizing stimulus. Insets on left: slope conductance measured as $\Delta I/\Delta E$, starting from different levels of hyperpolarization ($E_{bef. test}$). Insets on right: same measurements, starting 0·5 sec after a 10-sec depolarizing stimulus. Upper traces in insets show potential, lower traces current. Graph shows variation of slope conductance with starting potential level, with and without a preceding depolarization. Further discussion in text.

the slope conductance of the resting membrane as a function of membrane potential at the start of the conductance-measuring pulse ($E_{bef.\ test}$) and (upper line) the same relationship at 0·5 sec after the end of the depolarizing stimulus.

This comparison was necessary because of the anomalous rectification property of the *Aplysia* giant-cell membrane (Tauc & Kandel, 1964). As indicated in the bottom line of the graph in Fig. 2, the resting conductance increased with artificial hyperpolarization. Thus, the fact that the conductance was higher than the resting value at the end of a stimulus could have been due simply to the simultaneous occurrence of an abnormally hyperpolarized membrane potential, or PSH. That this was not the case was shown by the top line in the graph of Fig. 2: the slope conductance of the membrane after a stimulus was higher by about 40 % than that of the resting membrane at all values of potential just before the conductance-measuring pulse. It was thus evident that the increase in conductance produced by a stimulus was not a result of the post-stimulus hyperpolarization, and was in fact somewhat independent of membrane potential in the post-stimulus condition. On this basis, we decided to use slope-conductance measurements as an estimate of the change in membrane properties produced by a depolarizing stimulus.

The resting potential usually increased from an initial value of about -45 mV to -65 mV after 2 hr of recording, and the post-stimulus hyperpolarization (measured from the resting potential) showed a simultaneous decrease over the same period. The change in resting potential could have been due to (1) an increased concentration of internal K caused by loss of water from the cell (after a few hours, the cell soma appeared to shrink slightly); (2) entry of K into the cell from the micro-electrodes; or (3) increased activity of a pump, either by changing ion distributions or an electrogenic effect. All three of these effects would be expected to occur slowly.

Fig. 3 shows the time course of development of the PSH and conductance increase, tested with stimuli of increasing durations. Insets *A*, *B*, and *C* show the potential recordings (top traces) and applied currents (bottom traces). Conductance-measuring pulses were applied continuously before and after the stimulus. The post-stimulus potential and g_s, calculated as $\Delta I/\Delta E$ for the first conductance pulse after the stimulus, are plotted on the graph as functions of stimulus duration (current = 4×10^{-8} A). The values of post-stimulus potential and g_s at 0·1 sec duration (marked resting potential in Fig. 3) were the same as the values before the stimulus, to within the accuracy of measurement. The development of PSH apparently paralleled the growth of g_s with increasing stimulus duration, and both PSH and g_s changed most rapidly with shorter stimuli.

The variation of PSH and conductance increase with stimulus intensity (duration = 13 sec) are shown in Fig. 4. With currents of less than

5×10^{-9} A no measurable change of membrane potential greater than 1 mV or of conductance was produced by the stimulus. The development of PSH paralleled that of g_s with increasing stimulus intensity; the PSH, however, showed a greater tendency to saturate, or reach a limiting value,

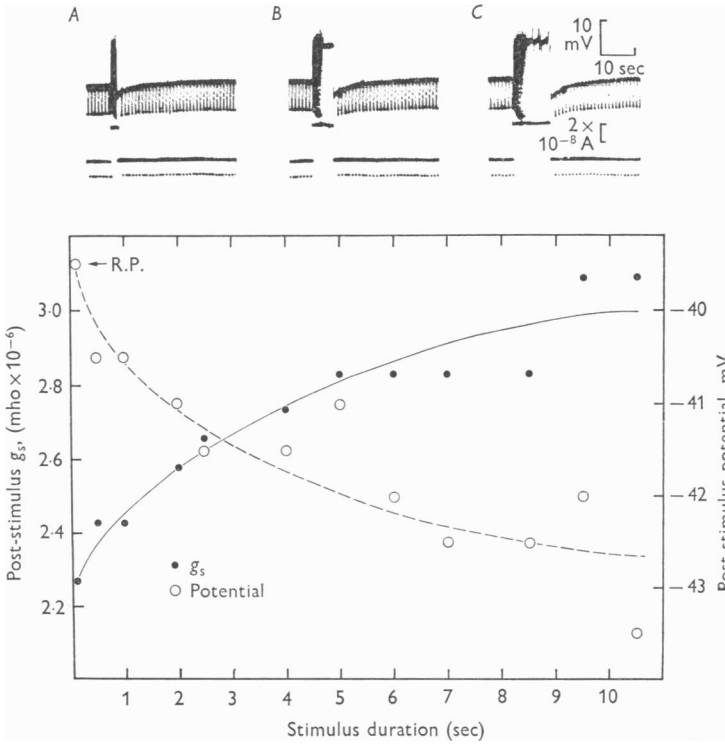

Fig. 3. Effect of stimulus duration on PSH and membrane slope conductance. Bottom traces of insets show depolarizing currents of increasing durations, preceded and followed by 1/sec hyperpolarizing pulses (ΔI) used to test conductance. Top traces show membrane potential changes (ΔE) produced by hyperpolarizing pulses, superimposed on PSH effect. Open circles in graph show potential immediately after depolarizing stimulus, as a function of stimulus duration. Filled circles show slope conductance, g_s, calculated at $\Delta I/\Delta E$ for the first hyperpolarizing pulse after the depolarizing stimulus.

than did g_s. The PSH might be expected to saturate if the membrane potential were approaching a reversal potential, even though g_s was still increasing.

Effect of blocking action potentials. Albuquerque & Grampp (1968) have observed post-stimulus hyperpolarization in the lobster stretch receptor when production of action potentials was blocked with tetrodotoxin. To see if PSH could be observed in *Aplysia* neurones in the absence of action

potentials, we applied Ca-free saline containing tetrodotoxin (Geduldig & Junge, 1968). The result is shown in Fig. 5. The first photograph shows a PSH of 9 mV and resting potential of −34 mV measured in normal saline. In the second photograph, a Ca-free solution containing tetrodotoxin, 10^{-5} g/ml., was applied to the cell. This procedure reduced the resting

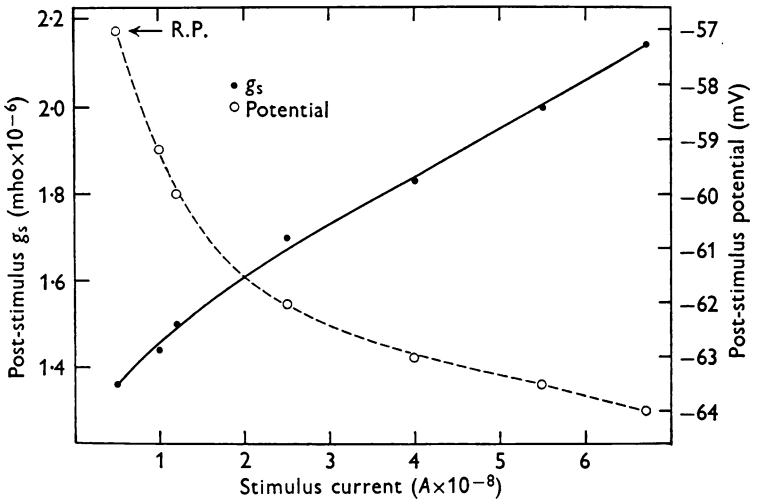

Fig. 4. Effect of stimulus intensity of PSH and membrane slope conductance. Procedure for obtaining points same as that for Fig. 3, but duration was held constant and depolarizing current varied. Different cell from that in Fig. 3.

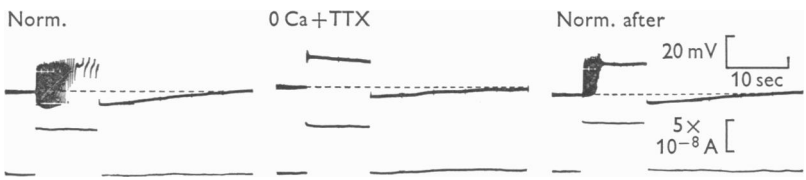

Fig. 5. Persistence of PSH in Ca-free solution containing tetrodotoxin. First photo: PSH in normal saline. Second photo: solution changed to Ca-free saline plus tetrodotoxin (TTX) 10^{-5} g/ml. Third photo: recovery upon returning to normal saline. Upper traces potential; lower traces current.

potential to −32 mV, and completely blocked the production of action potentials. The PSH, however, was 6 mV in this solution. The recovery in normal saline is shown in the third photograph; the resting potential became −35 mV, and PSH remained 6 mV.

In the top half of Fig. 6, taken from another cell, the post-stimulus potential is plotted as a function of stimulating current, in normal saline and in Ca-free solution containing tetrodotoxin, 10^{-5} g/ml. The resting

potential was reduced about 9 mV in the Ca-free tetrodotoxin solution, as indicated. In this experiment, the post-stimulus hyperpolarization, measured from the resting potential, was greater in the Ca-free tetrodotoxin medium over the entire range of currents tested. The two curves of PSH appear to approach the same limiting value at high stimulus intensities. The bottom half of Fig. 6 shows relative measurements of membrane slope conductance in normal saline and in Ca-free tetrodotoxin solution, plotted as functions of stimulus current. Absolute values of slope conductance were measured with small hyperpolarizing current pulses ($1{\cdot}5 \times 10^{-8}$ A, 0·5 sec) applied to the cell just before and just after the depolarizing stimulus. The resting (pre-stimulus) conductance as measured in this way increased about 30% in Ca-free tetrodotoxin solution. Consequently, in Fig. 6 the post-stimulus conductances were all divided by pre-stimulus values for comparison between solutions. It can be seen that membrane slope conductance increased with increasing stimulus current, both in normal saline and in Ca-free solution containing tetrodotoxin. Evidently the occurrence of action potentials was neither required for the post-stimulus hyperpolarization nor the accompanying increase in slope conductance. Hence, we adopted the term 'PSH' to describe the phenomenon in preference to 'post-tetanic hyperpolarization' or 'post-burst hyperpolarization'.

Effects of pump-inhibiting procedures. All but two (Gage & Hubbard, 1966; Meves, 1961) of the examples of PSH mentioned in the Introduction have been shown to be due to electrogenic pumps. In view of the fact that an active pump has been demonstrated in the *Aplysia* giant cell (Carpenter & Alving, 1968), the authors expected that this pump would contribute to the PSH we observed. That this was not the case is shown in Fig. 7. The first picture in the top row shows membrane potentials (upper trace) and currents (lower trace) in normal saline. Addition of 4×10^{-4} M ouabain (middle photograph) caused a depolarization of 6 mV within 3 min and the appearance of large 'anodal break' responses at the end of conductance pulses. The magnitude of the PSH, measured from the resting potential, was *increased* from 5 to 6 mV in the presence of ouabain. In the third photograph, the membrane potential was brought back to the original level by applying a maintained inward current. The break responses disappeared, and the size of the PSH returned to normal. Resting (pre-stimulus) conductance was apparently unaffected by ouabain. Thus, ouabain acted to depolarize the cell, presumably by inhibition of the pump mechanism, but the effect of ouabain could be offset by artificial hyperpolarization back to the normal resting potential. The drug did not block either the PSH or the simultaneous increase in slope conductance of the membrane.

The effect of cooling the preparation from 22 to 12° C by perfusion of

cold normal saline through the recording chamber is shown in Fig. 8. This procedure also produced depolarization of the cell, but, unlike ouabain, caused a large decrease in the resting (pre-stimulus) slope conductance. The PSH, measured from the resting potential, was enhanced by cooling

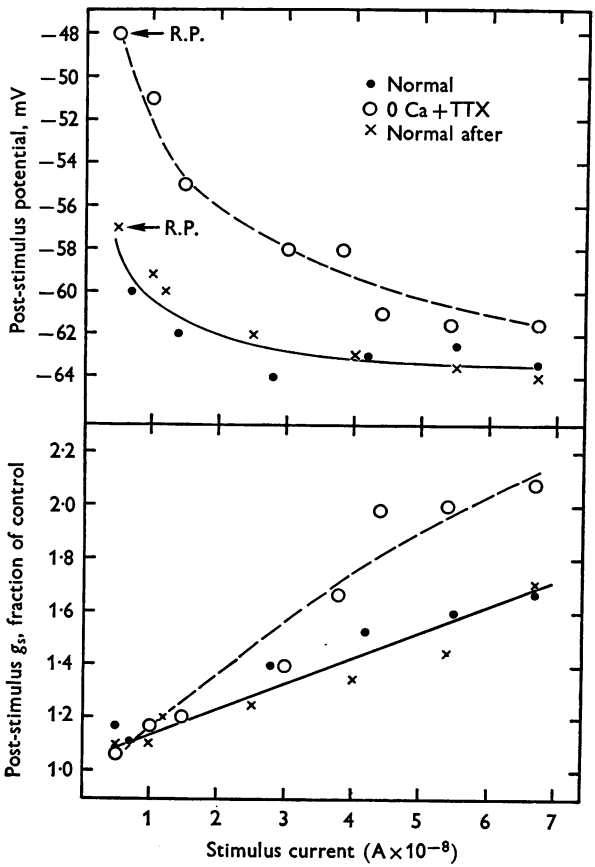

Fig. 6. Effect of Ca-free solution containing tetrodotoxin (TTX) on PSH and conductance increase. Top graph: PSH in normal: Ca-free + tetrodotoxin: normal plotted as a function of stimulus intensity. Resting potential was depolarized in Ca-free tetrodotoxin. Bottom graph: conductance (g_s) measured with small hyperpolarizing pulses, in same way as for Fig. 3. In the curves for each of the three solutions, post-stimulus conductance was normalized by dividing by pre-stimulus values, since the pre-stimulus value was elevated in Ca-free + tetrodotoxin. Different cell from that of Fig. 5.

and the simultaneous increase in slope conductance was not noticeably affected. A similar decrease in the giant-cell conductance upon cooling has been reported by Marchiafava (1970). As a further test of an electrogenic pump contribution to the PSH, we also applied DNP to the preparation. In one cell, the PSH was 3 mV in normal saline. Upon application of

1 mM-DNP in normal saline, a transient depolarization of about 10 mV occurred. However, in 3 or 4 min after changing the solution, the membrane potential had returned to the previous resting level. The PSH was 9 mV in the presence of DNP, and recovered to 4 mV in normal saline afterward. The resting membrane conductance in 1 mM-DNP was greater than in normal saline, an effect opposite to that seen with cooling. A similar increase in resting conductance with DNP has been seen in moth muscle (M. B. R. Eaton, 1971) and in cortical neurones (Godfraind *et al.* 1971).

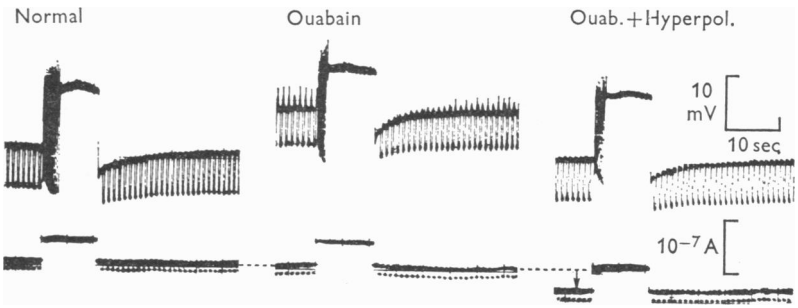

Fig. 7. Failure of ouabain to reduce PSH. First photo: PSH and conductance increase in normal saline. Second photo: solution changed to normal saline plus 4×10^{-4} M ouabain; cell depolarized, break responses at end of hyperpolarizing pulses. Third photo: additional constant hyperpolarizing current added to previous current stimuli; resting potential restored to normal. Upper traces potential; lower traces current.

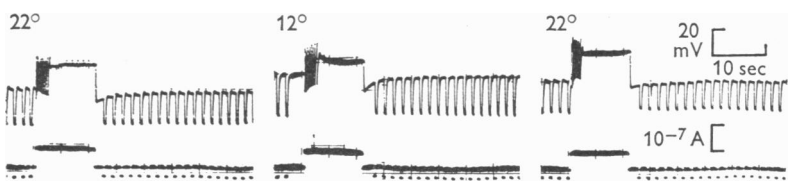

Fig. 8. Failure of cooling to reduce PSH. First photo: normal PSH at room temperature. Second photo: cold saline flowed into recording chamber for 5 min before measuring. Third photo: recovery. Upper traces potential; lower traces current.

Although we did not have a reliable indication of complete blockage of a metabolically driven pump, the common effect of depolarization by the three procedures used implied at least a partial block. The ouabain, DNP and cooling always had the effect of increasing PSH, measured from the resting potential. This behaviour should be contrasted with other known pump-dependent cases of PSH, where ouabain or DNP completely block the post-stimulus hyperpolarization (Holmes, 1962; Nakajima & Takahashi, 1966; Rang & Ritchie, 1968; Den Hertog & Ritchie, 1969; Baylor & Nicholls, 1969). In addition, we tried replacing all the Na in the external

medium with Li, a procedure which has been shown to interfere with electrogenic Na-pump activity (Ritchie & Straub, 1957; Nakajima & Takahashi, 1966; Rang & Ritchie, 1968; Livingood & Kusano, 1970). No significant reduction in either the PSH or conductance increase was produced by replacing Na^+ with Li^+. The results of these experiments with inhibitory agents for Na extrusion strongly suggest that PSH in *Aplysia* giant neurons is not due to an electrogenic pump.

Reversal potential for the PSH. If the phenomenon of PSH is due to an increase in membrane conductance for some ion or ions with equilibrium potentials more negative than the resting potential, then it should be possible to reverse the direction of change of the post-stimulus potential by artificially applying inward current after the depolarizing stimulus. In the normal condition, as the post-stimulus conductance decays with time, the membrane potential returns to the resting level. If an inward current is impressed which forces the membrane potential to the equilibrium potential for the PSH, then no change of the membrane potential with time should occur following the stimulus. Stronger inward currents should drive the final membrane potential to more negative levels than the initial-post-stimulus potential.

The demonstration of a reversal potential for PSH in the *Aplysia* giant cell is shown in Fig. 9. Part *A* shows the normal course of PSH. In Part *B*, an inward current was applied after the depolarizing stimulus, and almost no change of the membrane potential with time was seen. In Part *C* the direction of the potential change after the stimulus was reversed by applying still more inward current. To analyse experiments like this one, we defined E_{hold} as the final level of potential reached during the inward current, and ΔE as the difference between the potential immediately after the onset of the inward current and E_{hold}. In this way, the reversal potential for the PSH was taken as the value of E_{hold} where $\Delta E = 0$. In the cell of Fig. 10, ΔE was equal to zero at about -62 mV (resting potential $= -50$ mV). Such a reversal potential is not to be expected if the PSH is due to an electrogenic pump (Nakajima & Takahashi, 1966; Rang & Ritchie, 1968).

Effect of external K concentration. The reversal potential for the PSH varied with the external K-concentration, as shown in Fig. 10. The points were obtained by plotting ΔE *vs.* E_{hold}, as defined for the experiment shown in Fig. 9. Thus, positive values of ΔE occurred when E_{hold} was more negative than the potential at the onset of inward current. The curvature of the lines in the region $\Delta E > 0$ was probably due to the anomalous rectification shown in Fig. 2. The reversal potential, indicated by the line $\Delta E = 0$, became 13 mV more negative when K-free saline was placed around the ganglion, and became about 15 mV more positive in going from normal saline to a solution with K = 33 mM. This sensitivity of the

reversal potential to K concentration was typical of the cells examined in this way, and corresponds in this case to a variation of about 31 mV/ten-fold change in [K], or somewhat less than would be expected if the membrane in the post-stimulus condition behaves like a K electrode. Nevertheless, it strongly suggests that an increase in conductance to potassium occurs following a depolarizing stimulus.

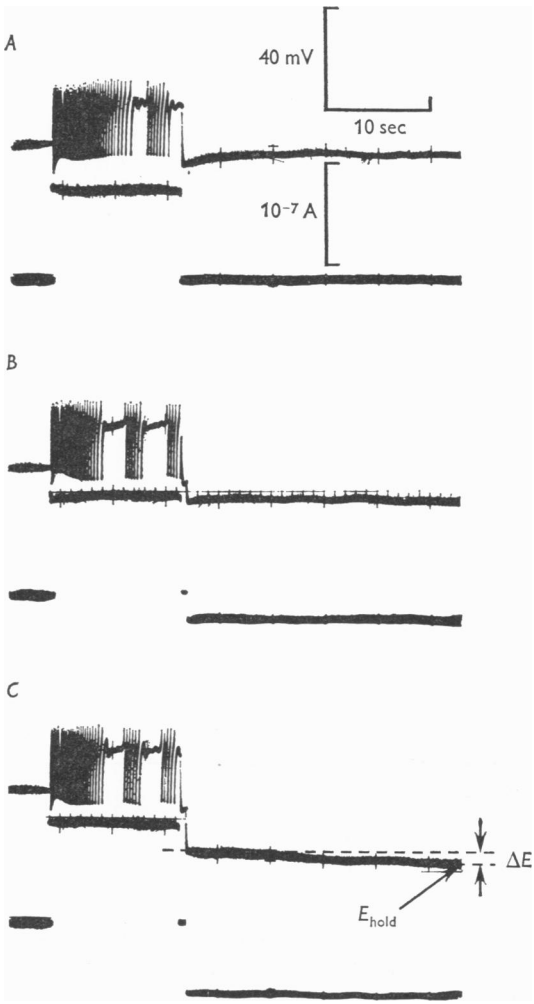

Fig. 9. Reversal potential for PSH. Following depolarizing current pulses (lower traces), long inward (hyperpolarizing) steps applied, amplitude of inward currents increasing in *A–C*. Membrane potential (top trace) tends toward a final value, E_{hold} at the end of the hyperpolarizing step. ΔE, the difference between potential at start of hyperpolarizing step and E_{hold}, is negative in *A*, goes through zero in *B*, positive in *C*.

The magnitude of the PSH in the absence of inward current varied with external K concentration. For the cell of Fig. 10, the resting potential and PSH, using a constant outward-current stimulus, were about −43 mV and 10·5 mV in normal saline (11 mM-K), −41 mV and 12 mV in K-free, and

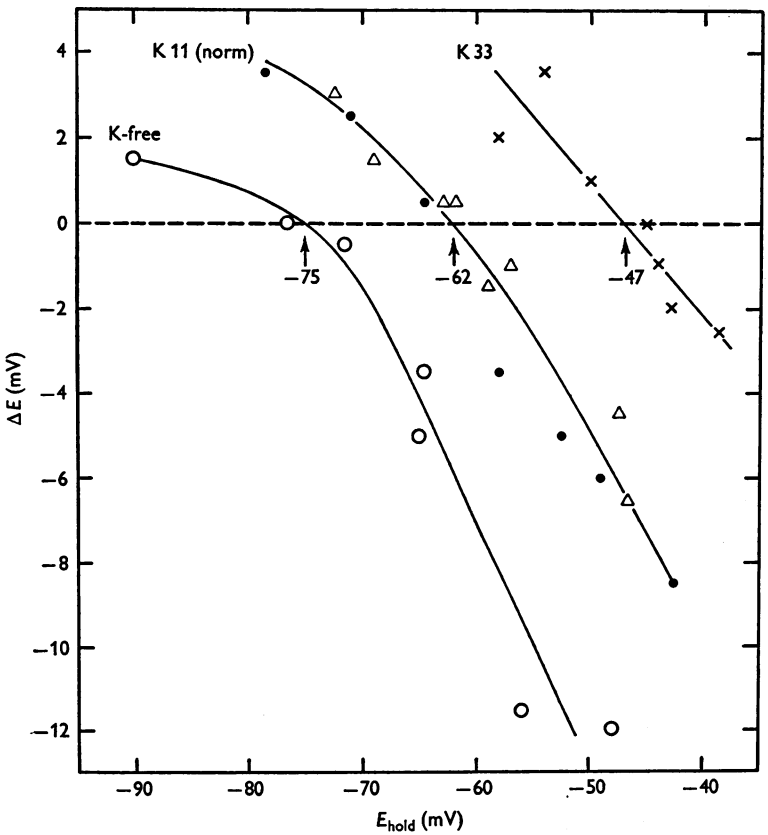

Fig. 10. Variation of PSH reversal potential with external K-concentration. Reversal potential taken as that value of E_{hold} for which $\Delta E = 0$, as defined for Fig. 9. Portions of graph below broken line correspond to normal (down-then-up) direction of PSH. Order of application of solutions ● ○ × △. Different cell from that of Fig. 9.

−45 mV and 0·5 mV in 33 mM-K. The observed inverse relationship of PSH and external K concentration would be expected for a K-conductance mechanism (Meves, 1961; Gage & Hubbard, 1966), but not for an electrogenic pump mechanism (Ritchie & Straub, 1957; Connelly, 1959; Straub, 1961; Kerkut & Thomas, 1965; Nakajima & Takahashi, 1966; Rang & Ritchie, 1968).

Effect of chloride replacement. The Cl-ion concentration of somatoplasm in *Aplysia* giant neurones has been reported as falling into two groups

having mean values of 27·7 and 40·7 mM (Brown, Walker & Sutton, 1970). Estimated values of E_{Cl} for these cells ranged from -49 to -70 mV. Since our resting potentials were about -35 to -55 mV, a change in Cl conductance could in some cases contribute to the production of PSH.

In order to determine if this was the case, we measured the reversal potentials for the PSH in normal saline and in a solution where all the Cl was replaced with methanesulphonate (Hagiwara, Gruener, Hayashi, Sakata & Grinnell, 1968; Sato, Austin, Yai & Maruhashi, 1968; D. C. Eaton, 1971). The reversal potentials were measured in the same manner

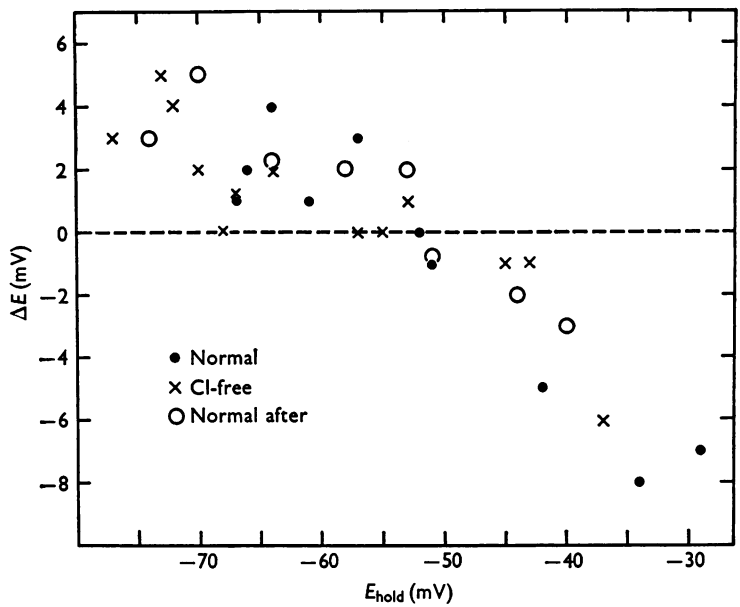

Fig. 11. Small effect of Cl replacement on PSH reversal potential. ΔE and E_{hold} measured in same way as for Fig. 9. Cl replaced with methanesulphonate.

as in Fig. 9. The variations of ΔE with E_{hold} in normal saline and in Cl-free solution is demonstrated in Fig. 11. The reversal potential was not affected by complete replacement of the Cl. Thus, it appears unlikely that a change in membrane conductance to Cl normally contributes to the PSH.

The magnitude of the PSH, measured from the resting potential, was somewhat reduced by replacement of external Cl with methanesulphonate. Since the Cl conductance in the R 2 cell is about 22 % of the total resting conductance (Brown *et al.* 1970), removal of the Cl shunt would be expected to increase the hyperpolarization due to an electrogenic pump (Rang & Ritchie. 1968). The failure of PSH to increase upon replacement of

external Cl may be taken as further evidence against an electrogenic-pump mechanism for the phenomenon.

Effect of Na replacement. Removal of all the Na in the bathing medium around an *Aplysia* giant cell usually results in a slight hyperpolarization (Sato *et al.* 1968; Geduldig & Junge, 1968), indicating that some Na conductance normally contributes to the resting potential. Na might also affect the PSH process by (*a*) the occurrence of a decrease in Na conductance simultaneous with the increase in K conductance, and (*b*) a partial sodium selectivity of the K-conductance channel.

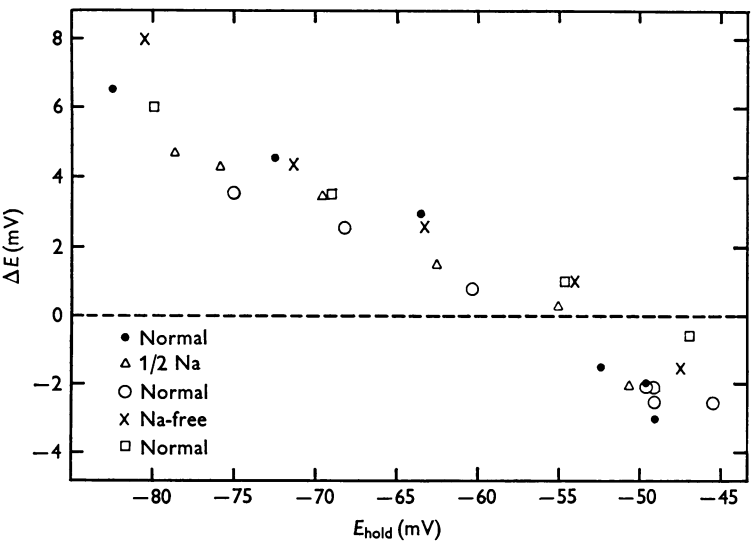

Fig. 12. Small effect of Na replacement on PSH reversal potential. ΔE and E_{hold} measured in same way as for Fig. 9. Na replaced with neutralized Tris.

To examine the possible contribution of Na to the PSH, we measured the reversal potential for the PSH in solutions where a part or all of the Na was replaced with neutralized Tris (Fig. 12). The values of ΔE and E_{hold} were defined as in Fig. 9. In this cell, neither the replacement of half nor of all of the Na in the external medium with Tris had any evident effect on the PSH reversal potential. This result indicates that a change in Na conductance does not contribute to the production of PSH. In a few cells, complete replacement of Na with Tris apparently abolished the reversal of the PSH; in these cases, ΔE was > 0 for all values of E_{hold}. This finding may reflect a general reduction of the PSH and post-stimulus conductance increase in Tris solutions, as will be discussed below.

Although the PSH reversal potential was not altered consistently by Na replacement, the magnitudes of the PSH and conductance increase

were always less in Tris-substituted Na-free solutions than in normal saline. When sucrose was used as a Na substitute, almost no reduction of PSH and conductance increase was seen. For example, in one cell the PSH and percentage conductance increase with a constant outward-current stimulus were 4 mV and 100% in normal saline, 1 mV and 33% in Na-free Tris solution, 4 mV and 89% in Na-free sucrose solution, and 6·5 mV and 67% in normal saline afterward. This result suggests that the reduction of PSH and conductance increase observed in Na-free tris solutions was due to an action of Tris on the PSH channel, and not simply to the absence of sodium. Tris buffers have been shown to exert direct effects besides the stabilization of pH in other preparations (Good, Winget, Winter, Connolly, Izawa & Singh, 1966; Ko, Gimeno & Berman, 1969).

DISCUSSION

The phenomenon of post-stimulus hyperpolarization in *Aplysia* giant neurones appears to result from an increased conductance to K. This mechanism is similar to that found for post-tetanic hyperpolarization in mammalian motor nerve terminals (Gage & Hubbard, 1966) and the frog node (Meves, 1961). Under voltage-clamp conditions, other examples have been found of long-lasting (order of seconds) increases in K-conductance which are activated by depolarization (sheep Purkinje fibres – McAllister & Noble, 1966; puffer-fish neurones – Nakajima & Kusano, 1966; frog muscle – Adrian, Chandler & Hodgkin, 1970). It is noteworthy that the K-conductance increase of frog muscle (Adrian *et al.* 1970), like that of the *Aplysia* giant cell, is not reduced by application of inward current immediately following the depolarizing stimulus.

A simple model which can account for the observed PSH in *Aplysia* giant cells is shown in Fig. 13. The resting potential, E_R, and resting conductance, g_R, are assumed to be in parallel with a PSH potential, E_P, and a PSH conductance, g_P. The PSH potential is taken as the reversal potential for PSH, as measured in Fig. 9. The values of g_P as a function of time are calculated as total membrane conductance minus g_R. Knowing the constants E_R, g_R, and E_P, and the time-varying function g_P, it is possible to calculate the post-stimulus potential, E_{PS}, as

$$E_{PS} = \frac{E_R g_R + E_P g_P}{g_R + g_P}.$$

Fig. 13 shows the result of this calculation for a cell in which all the above quantities were measured. The bottom graph shows measured values of g_s ($= g_R + g_P$), plotted as a function of time after the end of a 10-sec depolarizing stimulus. E_R was $-42{\cdot}5$ mV, g_R was $2{\cdot}3 \times 10^{-6}$ mho, and

E_P was -62 mV. The continuous line in the top graph shows calculated values of E_{PS}, and the circles are the observed post-stimulus potential. Thus, using the parallel-conductance model shown, the measured increase in membrane conductance fully accounts for the observed post-stimulus hyperpolarization.

The error in measured reversal potentials due to anomalous rectification may also be calculated using the model. If one assumes that the percentage increase of g_s with artificial hyperpolarization is the same as that shown

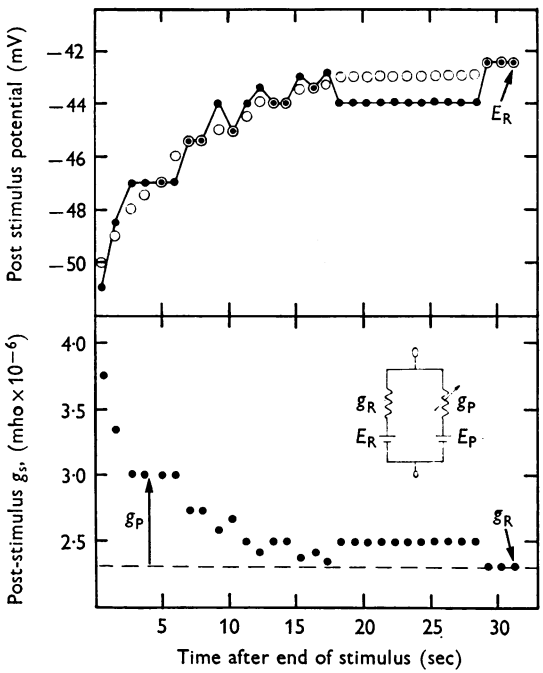

Fig. 13. Calculation of post-stimulus potential from measured post-stimulus conductance. See text for details.

in Fig. 2, then the model predicts curvature of the lines of ΔE *vs.* E_{hold} as seen in Fig. 10. With the assumption of this much rectification (up to 20 % increase in resting conductance with artificial hyperpolarization) the error in measured reversal potential is less than 1 mV.

Post-stimulus hyperpolarization in the absence of action potentials has been observed in other preparations (Albuquerque & Grampp, 1968; Strumwasser, 1968, Koike, Brown & Hagiwara, 1971). This result in the *Aplysia* giant cell may be taken as evidence against a recurrent inhibitory mechanism for the production of PSH: if any recurrent inhibitory pathways do exist for the giant cell (and no IPSPs have been observed in this

cell – Frazier *et al.* 1967), they should be blocked in Ca-free solutions containing tetrodotoxin. We were always able to observe PSH in such solutions.

Pressure-injection of Ca into the giant-cell soma of *Aplysia* causes an increase in K conductance (Meech & Strumwasser, 1970). The reversal potential for the Ca-induced hyperpolarization (−62 mV) was close to that which we observed for PSH (see Figs. 10, 11 and 12). Since some Ca-influx normally occurs during action potentials (Geduldig & Gruener, 1971) it seems possible that the PSH might be dependent on that Ca-influx. The failure of Ca-free medium to eliminate the PSH suggested that this was not the case. However, it should be noted that depolarization can cause release of internal stores of calcium in muscle (Winegrad, 1968) and in *Spirostomum* (Ettiene, 1970). Such a mechanism could yield a PSH in Ca-free external solutions.

The results of our experiments on replacement of Na with Tris suggest a direct pharmacological action of the compound on the PSH channel. A simple Na-dependence of the K conductance (Frankenhaeuser, 1962) cannot account for these results, since the PSH persists in sucrose-substituted Na-free solutions.

The post-stimulus increase in K conductance which we observed may be of importance to the phenomenon of slowing of repetitive discharge during constant-current stimuli. An electrogenic pump mechanism (Sokolove & Cooke, 1971) appears insufficient to explain the behaviour of *Aplysia* giant neurones, since we always observed slowing in the presence of pump-inhibiting procedures. A slow K conductance increase during depolarization has been advanced to account for the slowing of discharge rate of dorid neurones (Connor & Stevens, 1971).

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