A Contribution of an Electrogenic Na^+ Pump to Membrane Potential in *Aplysia* Neurons

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ABSTRACT The resting membrane potential (RMP) of *Aplysia* neurons is very temperature-dependent, and in some cells increases with increasing temperature by as much as 2 mv/°C. RMP at room temperature may significantly exceed the potassium equilibrium potential, which can be determined by measurement of the equilibrium point of the spike after potential. The hyperpolarization on warming is completely abolished by ouabain, replacement of external Na^+ by Li^+, removal of external K^+, and by prolonged exposure to high Ca^{++}, while it is independent of external chloride but is increased by cocaine (3×10^{-3} M). In an identified cell that shows a marked temperature dependence of RMP, both the potassium equilibrium potential and the membrane resistance were found to be relatively independent of temperature. The hyperpolarization on warming, which may increase RMP by as much as 50%, can most reasonably be ascribed to the activity of an electrogenic Na^+ pump.

INTRODUCTION

The active transport of Na^+ from the inside of cells is believed to be a factor of prime importance in the generation and maintanence of concentration gradients of various ions across the nerve cell membrane (11). The concentration gradients thus generated have been thought to be the direct and sole determinant of the resting membrane potential (45), which in turn is usually thought to be adequately described by the Hodgkin and Katz modification of the Goldman equation (21).

Extrusion of Na^+ was once held to be coupled in a one-to-one fashion to the entry of K^+ (45). However, such complete coupling of Na^+ and K^+ move-

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ments need not always be the case. If the pump activity transfers more Na^+ out of the cell than K^+ in, there will be a net movement of positive charge from inside to out. Such a movement of charge constitutes a current which will contribute directly to the membrane potential by an amount which will depend upon the membrane resistance (22). Such a system will be called an "electrogenic" Na^+ pump since it is a mechanism capable of generating directly a potential difference across the cell membrane in addition to that resulting from ionic concentration gradients (2).

Electrogenic Na^+ transport was first suggested to explain the hyperpolarization observed in frog and crab nerve upon tetanization (11). There have been a number of recent reports in which an electrogenic transport of Na^+ has been found after an artificial increase in intracellular Na^+ concentration in both nerve (25) and muscle (2, 12, 19, 26, 32). Posttetanic hyperpolarization in the crayfish stretch receptor (31) and a striking temperature dependence of membrane potential of lobster axons (38) have also been found to result from activity of an electrogenic Na^+ pump.

The resting membrane potential (RMP) of *Aplysia* neurons varies much more with temperature than is predicted by the Goldman equation (8, 10, 30). In many cells RMP increases with temperature by up to 2 mv/°C over the range of temperature to which the animal is normally subjected (8). The present experiments were begun to determine the mechanism of this marked temperature dependence of RMP.

A preliminary report has been given (4).

METHODS

Aplysia californica were obtained from Pacific Biomarine Supply Company (Venice, Calif.), and were kept in artificial sea water at 15–16°C. After dissection from the animal the visceral ganglion was pinned to a paraffin layer in a Lucite chamber and the five nerves to the ganglion mounted in suction electrodes. The chamber was constantly perfused with artificial seawater (Marine Magic, Lampert Kay, Inc., Los Angeles, Calif.) at 10–20°C. Once the experimental temperature changes were begun temperature was varied between 3°–25°C. Care was taken to avoid Na^+ loading of the nerve cells by not maintaining a temperature of less than 10°C for longer than 15 min at a time. Slow temperature changes, complete within about 5 min, were made by varying the length of the coiled polyethylene tubing through which the bathing solution flowed that was immersed in an ice bath. Rapid temperature changes were made by sudden application of 10 cc of water at room temperature to the ganglion which had been cooled to about 5°C after flow of the seawater had been stopped. Temperature was monitored as previously described (8).

Neurons were penetrated without removal of the connective tissue capsule with one or more microelectrodes filled with Na^+-free 3 M KCl and having a resistance of 0.5-2 megohms. All precautions previously described (8) to achieve accurate measurements of DC levels were observed.

In experiments in which ionic composition was varied an artificial seawater was

made containing 425 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 22 mM $MgCl_2$, 26 mM $MgSO_4$, and 2.5 mM $NaHCO_3$. Stock solutions of each salt were made with an osmolarity of 910 ± 10 milliosmols as measured on an Advanced Model 31L osmometer (Advanced Instruments, Inc., Newton Highlands, Mass.), and these solutions were combined in the ratio of 85% NaCl, 2% KCl, 3% $CaCl_2$, 6.5% $MgCl_2$, 3% $MgSO_4$, and 0.5% $NaHCO_3$ to give the above final concentrations. Na^+-free seawater was made by replacement of all NaCl with an equal concentration of LiCl, while in low Cl^- solutions all NaCl was replaced by Na^+ propionate. For changes in K^+ concentration, variations in the volume of KCl were compensated for by varying the volume of isosmotic NaCl.

In experiments in which the membrane potential was changed by applied current, two electrodes were independently placed in the cell and current passed through one electrode through a standard circuit (14). Ouabain was routinely used at a concentration of 4×10^{-4} M, and cocaine hydrochloride at 3×10^{-3} M. High and low gain recordings of each intracellular response, temperature, and extrinsically applied current were continually recorded on an Offner (Beckman Instrument Co., Offner Division, Chicago, Ill.) penwriter. Other recording procedures were as described previously (8).

Designation of identified cells is taken from Frazier et al. (15).

RESULTS

Fig. 1 shows the resting membrane potential (RMP) and antidromic action potential recorded from the giant cell (R_2) at four different temperatures. At 6°C the RMP is −48 mv and the afterpotential is clearly more negative (hyperpolarized) than the RMP. On warming the membrane potential increases and at 17°C is −56 mv. The afterpotential is now positive relative to RMP. Further warming results in failure of generation of a soma spike, presumably because of the hyperpolarization of the soma membrane (Fig. 1–4).

Results from a similar experiment on cell R_2 are shown in Fig. 2 where afterpotential and RMP are plotted as a function of temperature. Below 7°C the afterpotential of this cell was more negative than the resting potential, but as the temperature increased the afterpotential changed but little while the resting potential increased at an initial rate of about 2 mv/°C with a 13 mv total hyperpolarization over the 19°C temperature change.

These observations on afterpotentials suggest that the increase in RMP with temperature does not result from a permeability increase similar to that responsible for the afterpotential. The afterpotential in nerve and muscle has been attributed to an increase in K^+ permeability, and is assumed to approach the K^+ equilibrium potential (20). In order to determine whether, in fact, the afterpotential of *Aplysia* neurons is a K^+ potential, the effect of changing the concentration of K^+ was studied.

In all cells studied both RMP and afterpotential varied with external K^+ concentration. In three experiments the effect of changes in external K^+ on the equilibrium point of the afterpotential was determined in cell R_2. Two

electrodes were placed in the cell and current passed through one could set the internal potential at various levels. At each K^+ concentration antidromic spikes were elicited and recorded through the second electrode at a variety of levels of internal potential and the afterpotential equilibrium point, defined as the internal potential at which the afterpotential is exactly equal to the transmembrane potential, was determined.

Fig. 3 shows a plot of the equilibrium point of the afterpotential as a function of K^+ concentration from an experiment in which five different K^+ con-

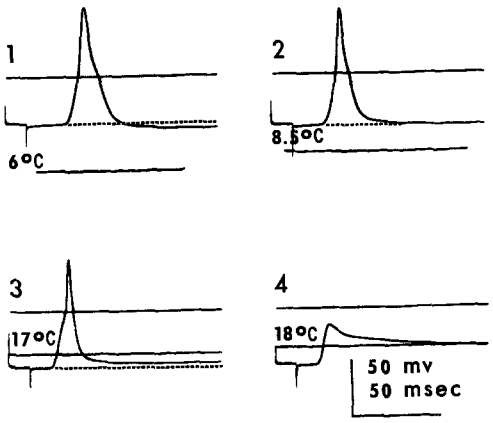

FIGURE 1. Antidromic action potentials recorded from cell R_2 at four different temperatures. The upper trace is a grounded base line which serves as reference for RMP and temperature changes. The lower trace in 1 and 2 and the middle trace in 3 and 4 record temperature changes and rise on warming. The antidromic action potential was elicited by stimulation of the right connective nerve. RMP changes are indicated by the increasing separation of the base line and intracellular response on warming. In 4 the hyperpolarization of the cell body is so great that a soma spike does not occur and only an axon spike is seen.

centrations were studied. The slope of 51 mv for a 10-fold change in K^+ at high K^+ concentrations approaches the theoretical 55.5 mv characteristic of a K^+ electrode at the temperature of 11 °C used in this experiment. We take this to indicate that the afterpotential of cell R_2 is primarily a K^+ potential and that the equilibrium point of the afterpotential is a valid measure of the K^+ equilibrium potential.

At K^+ concentrations greater than that normally found in seawater the RMP decreased with increased K^+ but with a slope less steep than that for the equilibrium point of the afterpotential. This suggests that the RMP is to some extent determined by concentration gradients of ions other than K^+. The rather unusual behavior of RMP at K^+ concentrations of less than that of normal seawater will be discussed later.

In five experiments, all on cell R_2, the equilibrium point of the afterpotential was studied as a function of temperature. The experiment was begun at an intermediate temperature, and then temperature was alternately raised and lowered. Each temperature was maintained for from 15–30 min, during which time the equilibrium point of the afterpotential was determined. On this schedule the equilibrium point of the afterpotential did not significantly

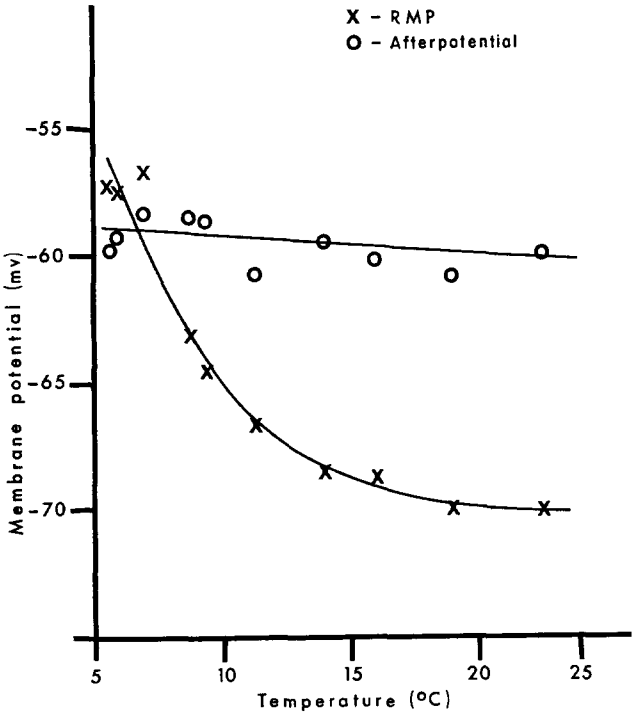

FIGURE 2. Plot of the variation of afterpotential and RMP with temperature in one experiment on cell R_2. Each point is an average of three measurements. Action potentials were elicited by stimulation of the left connective nerve.

change as a function of temperature, in spite of a consistent and maintained variation of RMP, spike overshoot, and spike width as is indicated for one cell in Table I.

Since Fig. 3 indicated that the equilibrium point of the afterpotential is a close approximation of the K^+ equilibrium potential, the results of Table I show that the K^+ equilibrium potential is relatively independent of temperature, at least for temperature changes maintained for 15–30 min. Furthermore the hyperpolarization on warming cannot be explained as an increase in potassium conductance for the RMP can exceed the K^+ equilibrium potential by a substantial amount. The relative temperature independence of the K^+ equilibrium potential also indicates that the K^+ concentration in the narrow

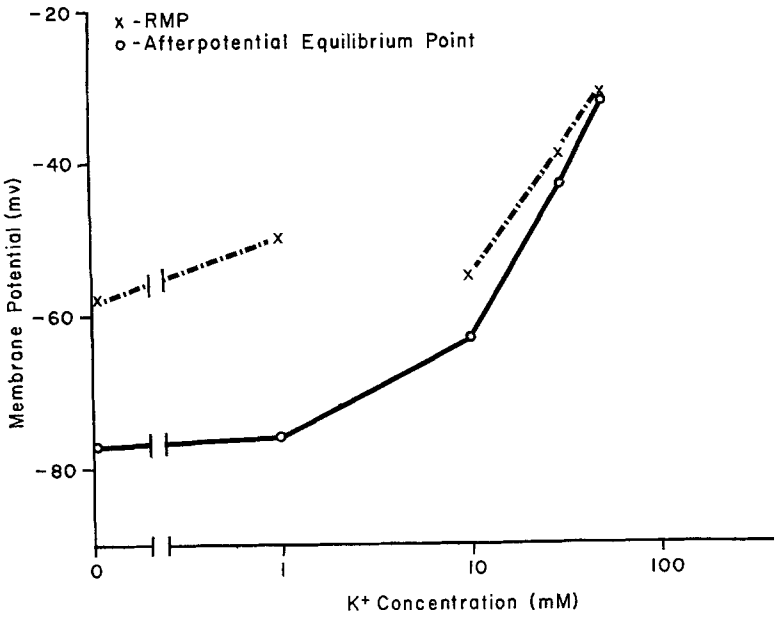

FIGURE 3. Plot of the variation of afterpotential equilibrium point and RMP with external K^+ concentration. The experiment was performed on cell R_2 at a temperature of 11°C. The slope for a 10-fold change in K^+ concentration is 51 mv between 30–50 mM K^+ and 45 mv between 10–30 mM K^+ for the equilibrium point of the afterpotential, and 35 mv between 30–50 mM K^+ and 32 mv between 10–30 mM K^+ for RMP. The break in the line connecting the RMP points is to indicate the onset of an additional effect of low K^+ on RMP, and will be discussed later.

intercellular clefts does not vary in a consistent and maintained fashion with temperature.

Another possible explanation for hyperpolarization on warming might be an increased permeability to Cl^- ions, or perhaps a Cl^- pump as has been suggested by Strumwasser (42). In experiments on six cells the RMP shift with temperature was compared in normal seawater and in seawater in which all NaCl was replaced with Na^+ propionate, where propionate is presumed to be an impermeant anion (36). In none of these experiments did the decrease in

TABLE I

	5°C	8°C	11°C	18.5°C
1. RMP, *mv*	−48	−57	−58	−63
2. Equilibrium point of afterpotential, *mv*	−59	−59	−59	−58
3. Spike height, *mv*	111	119	118	115
4. Spike overshoot, *mv*	+63	+61	+60	+51
5. Spike half-width, *msec*	9.6	6.6	5.6	2.7

Cl^- alter the magnitude of the RMP shift, and thus it is unlikely that Cl^- is involved in the temperature dependence of RMP.

If the hyperpolarization on warming were a result of a conductance change one should be able to measure a change in membrane resistance on changing the temperature. However, the current-voltage (I-V) relations of most *Aplysia* neurons are not simple, and in many cells there is little or no linear portion of the current-voltage curve, due primarily to the presence of anomalous rectification (43). The presence and amount of anomalous rectification is very temperature-dependent[1]. Furthermore, the ionic mechanisms responsible for pacemaker generation are temperature-dependent (8) and thus complicate resistance measurements in cells specialized for pacemaker activity. There are some cells, however, which do have a reasonably linear I-V relationship at all temperatures, which are not pacemakers, and which occasionally do not receive so much synaptic input at higher temperatures as to make I-V measurements impossible. In such a cell, in which the predominant temperature-dependent process is the RMP hyperpolarization on warming, one can test whether this hyperpolarization is a result of a conductance change.

Fig. 4 A shows I-V relations from one such cell at four different temperatures. In spite of a considerable increase in the amount of synaptic activity, this cell showed a maintained increase in RMP of 6 mv when warmed from 5° to 20°C. As a result the coordinates for the different temperatures are shifted along the I-V curve. In this cell there is no detectable change in membrane resistance with temperatures ranging from 5° to 20°C. Fig. 4 B shows the form of the RMP shift seen in this cell upon a sudden temperature change. Since this cell demonstrates a temperature dependence of RMP without significant variation in the slope of its I-V relations at several temperatures, we conclude that the hyperpolarization on warming cannot be explained as being caused by a conductance change to any ion.

Hyperpolarization with increases in temperature could also be a result of active transport of Na^+ ions. In order to test this possibility the RMP shift was compared in normal seawater and in seawater containing ouabain, known to be a specific inhibitor of active Na^+ transport (7, 37).

Fig. 5 shows penwriter records of the results of such an experiment in cell R_2, where in both A and B the upper trace records intracellular potential while the lower trace records temperature. In A-1 the RMP at 5°C is −45 mv but upon a sudden warming it rapidly increases to about −54. On cooling the RMP returns to its previous level. Part B illustrates the results of a similar temperature change in this cell 8 min after the addition of 4×10^{-4} M ouabain to the seawater. At 5°C the RMP is −42 mv and is thus not greatly different from the control. However, warming now produces no hyperpolarization.

[1] Carpenter, D. O. Unpublished observations.

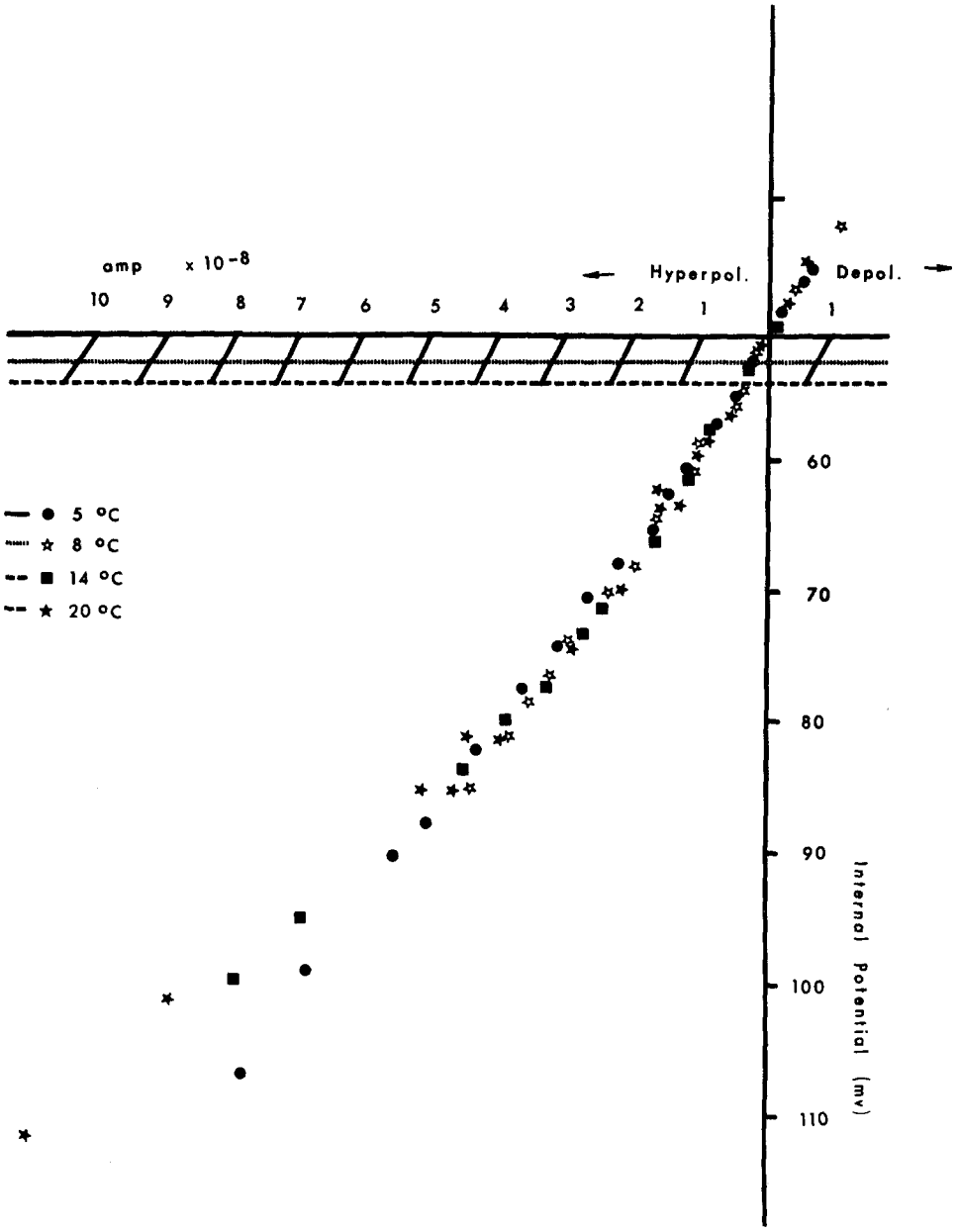

FIGURE 4 A. Current-voltage relations in cell R_2 at four temperatures. This cell was selected to show the absence of a significant resistence change with temperature. On warming from 5°–20°C the cell showed a maintained hyperpolarization of 6 mv. Consequently the coordinates at each temperature are shifted down the I-V curve obtained at 5°C by the amount of hyperpolarization at that temperature. The change in current ordinates is drawn and the one appropriate for each temperature is indicated. An increase in anomalous rectification with temperature is seen at extreme hyperpolarizations, but no significant resistance change with temperature is apparent at internal potentials less than −90 mv.

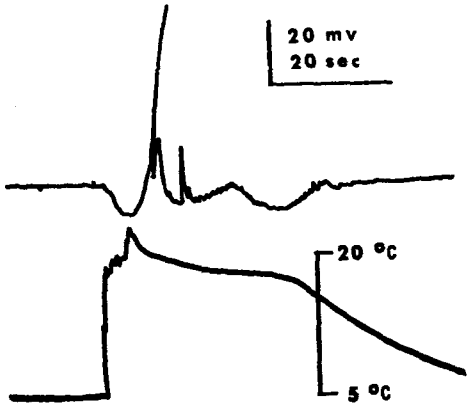

FIGURE 4 B. Penwriter records of a rapid temperature change in the same cell as in part A to show the extent of the hyperpolarization on warming. The upper trace is the intracellular potential, while the lower trace shows the temperature change.

Instead the increased synaptic activity evoked by warming leads to a depolarization sufficient to cause a rapid discharge.

Fig. 6 A shows the effect of sudden temperature changes on the membrane potential and firing pattern of one of the left upper quadrant pacemakers. As the temperature was suddenly increased the membrane hyperpolarized by an amount similar to that observed in silent cells, such as cell R_2 in Fig. 5 A. However, the cell then spontaneously depolarized and began to discharge in its characteristic periodic pattern. A similar transient hyperpolarization on sudden warming could be seen in all pacemaker cells, whether their discharge pattern was periodic or regular. The transient hyperpolarization in pacemaker neurons suggests that the changes in active Na^+ transport occur with less delay after a temperature change than does the change in passive permeability to Na^+ ions, which is probably the mechanism of the pacemaker potential (13). Such a difference in the time course of these two processes with temperature change can explain the anomalous firing frequency transients observed in many preparations (8).

Fig. 6 B shows that the transient hyperpolarization in pacemaker neurons is completely abolished by ouabain. In place of the hyperpolarization the cell now slowly depolarizes and begins to discharge after the temperature increases. After the first step increase in temperature there is still a tendency for discharge in bursts but further increase in temperature causes a regular firing pattern.

The effect of ouabain on pacemakers spontaneously discharging in bursts was studied in 16 cells. In the majority this pattern was abolished completely and the cells either fired regularly or became silent. However, it was always possible to restore a pattern of discharge in bursts by application of a hyper-

polarizing current through a second microelectrode. It thus seems unlikely that this firing pattern is dependent upon the Na^+ pump except in that it requires a certain negative internal potential, and a considerable portion of this normally generated by the Na^+ pump. Many cells of all types became com-

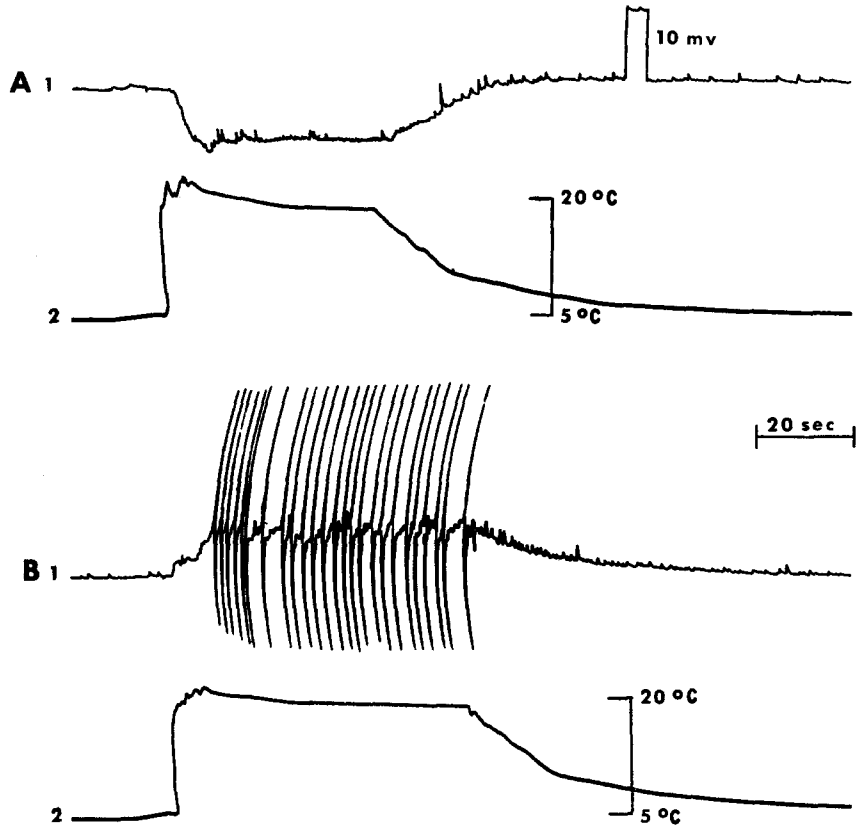

FIGURE 5. Penwriter records showing the effect of a rapid temperature change upon the intracellularly recorded response in cell R_2 before (A) and after (B) application of 4×10^{-4} M ouabain. The spike distortion in B-1 results from the high gain recording on the penwriter. RMP in A-1 is −45 mv at 5°C, and in B-1 is −42 mv at 5°C.

pletely silent after 10–15 min in ouabain, presumably as a result of inactivation of spike generation by the resulting depolarization, since spontaneous discharge could be restored by applied hyperpolarization. The effect of ouabain on pacemaker pattern and spike generation could be mimicked by an applied depolarization.

The effect of ouabain on the RMP shift and on the rate of spontaneous discharge has been studied in over 35 cells in the visceral ganglion. In every case in which hyperpolarization on warming was observed, it was markedly re-

duced or abolished within 2–10 min of exposure to 4×10^{-4} M ouabain. In those few cells which did not demonstrate a clear hyperpolarization on warming because of pacemaker activity or a large synaptic input, it was always possible to demonstrate a marked increase in discharge at the higher tempera-

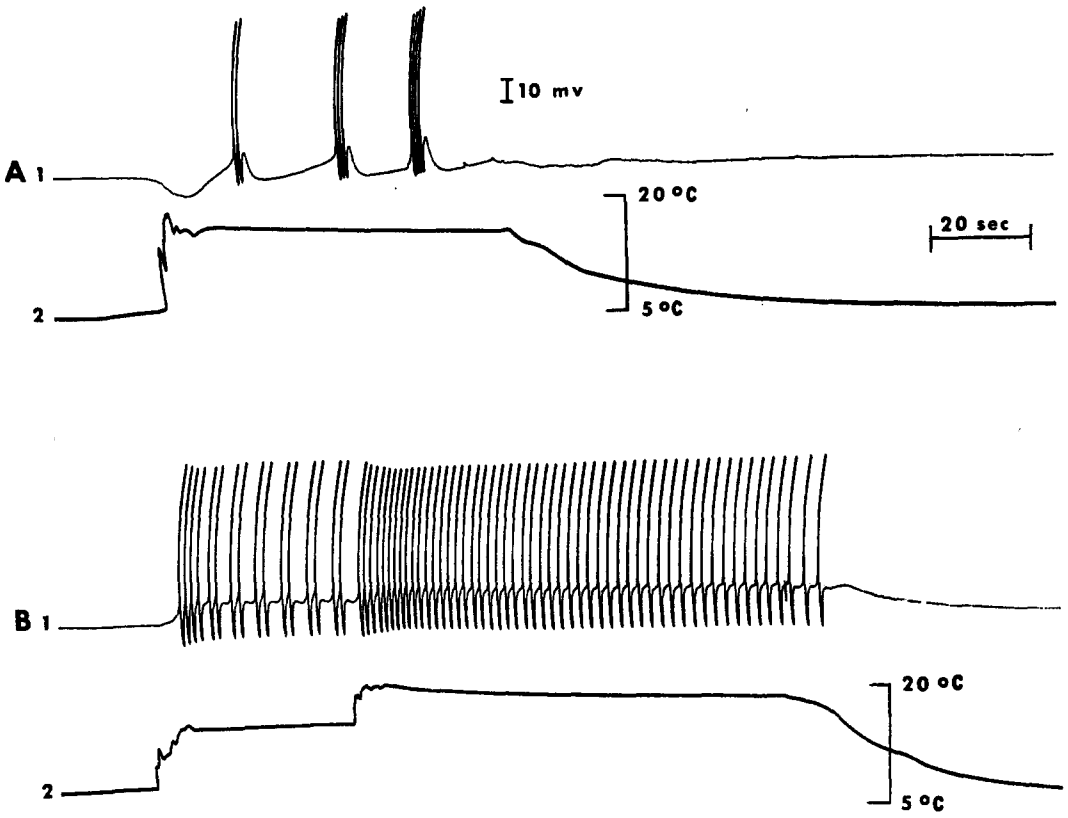

FIGURE 6. Penwriter records showing the effect of a rapid temperature change upon the intracellularly recorded response of one of the left upper quadrant bursting pacemaker neurons before (A) and after (B) application of 4×10^{-4} M ouabain. RMP is −51 mv at 5°C in A-1, and −45 mv at 7°C in B-1.

tures after ouabain application indicating that a hyperpolarization resulting from active Na^+ transport is characteristic of all cells but may be occasionally obscured by other events. The abolition of the hyperpolarization by ouabain was not readily reversible even with prolonged washing.

If the hyperpolarization on warming is a result of active transport of Na^+ ions, it should be abolished by prolonged exposure to a solution in which Li^+ replaces Na^+. Although Li^+ can enter the passive Na^+ channels it cannot be actively transported out again (28). The effect of replacement of Na^+ with Li^+ was tested in seven cells, and is illustrated in Fig. 7 in records from cell

R_2. A-1 and 2 show intracellular and temperature traces on slow cooling in normal seawater, and illustrate the marked depolarization which results. The RMP shift was never immediately affected by exposure to Li^+ seawater but was in all cases completely abolished after 20–30 min, as illustrated in Fig. 7 B. This cell, which was previously silent at 20°C, now discharges rapidly in

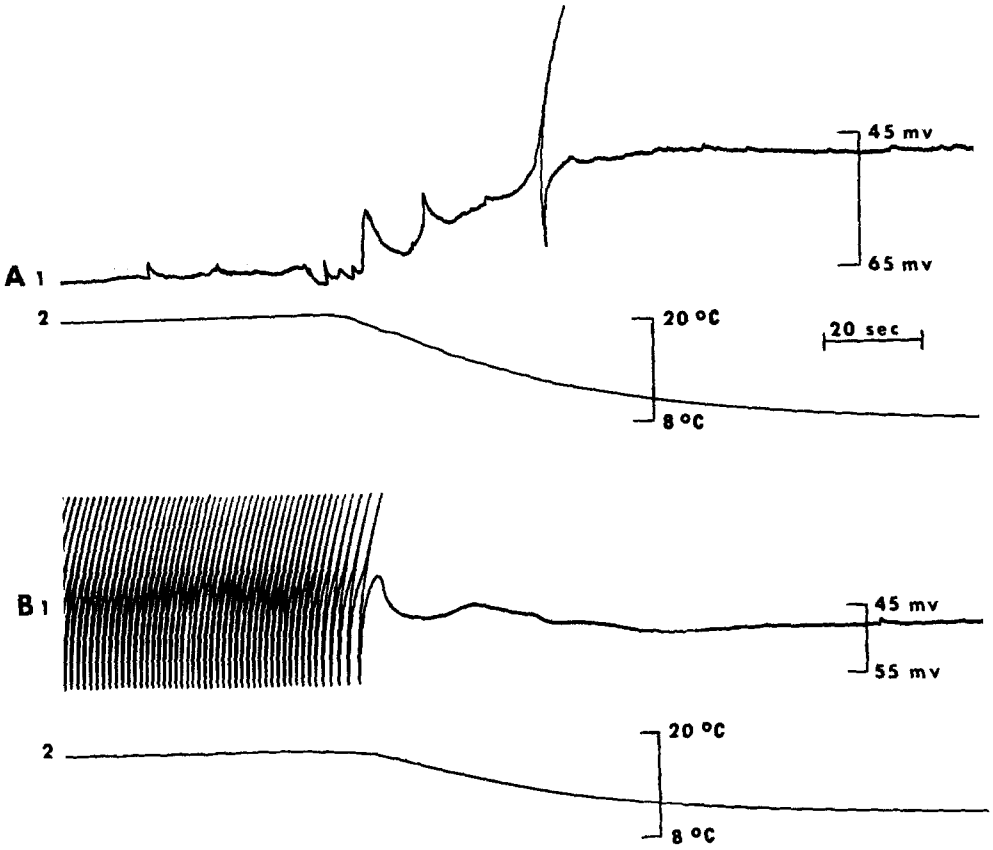

FIGURE 7. Penwriter records from an experiment on cell R_2 showing the effect of a slow temperature change upon RMP and spontaneous activity in cell R_2 in normal seawater (A) and in seawater in which all NaCl has been replaced with LiCl (B).

the presence of Li^+ and on cooling the discharge ceases without a trace of depolarization. Note that the RMP in the cold is not changed from the control, which would indicate that there has not been a significant accumulation of intracellular Li^+.

Active transport of Na^+ in red blood cells is inhibited by exposure to high concentrations of Ca^{++} (23, 24). The inhibition presumably takes place on the inside of the cell membrane (23). In 10 cells the effect of 4 times normal Ca^{++} (40 mM) was studied. In the majority the RMP shift was reduced or completely

abolished within 60 min. The inhibition was readily reversible by prolonged washing and/or chelating agents.

In nine cells the effect of removal of all K^+ from the bathing seawater was studied, and typical results are shown in Fig. 8 from two cells recorded simultaneously. In both cells in normal seawater (A) a rapid temperature change resulted in nearly 20 mv hyperpolarization which was maximal within less than 4 sec of the onset of the temperature change. Part B shows a similar temperature shift after 45 min in seawater containing no K^+. The hyperpolarization is reduced or absent in both cells. Record C was taken less than 5 min after a return to normal seawater and demonstrates the almost immediate recovery on exposure to K^+.

The K^+ requirement for pump activity explains the initially puzzling observation that while replacing normal seawater with one containing no K^+ at 5°C resulted in a hyperpolarization as expected from the Goldman equation, the same ionic change at 20°C caused a depolarization. However, the value of RMP in seawater containing no K^+ was found to be nearly the same at 5° and 20°C. These results are consistent with the postulate that under the conditions of these experiments the membrane obeys the Goldman equation only at low temperature (5°C or less) and/or when the electrogenic component of the Na^+ pump is abolished, as by seawater containing no K^+.

The somewhat peculiar variation of RMP with K^+ concentration seen in Fig. 3 can also be explained in terms of different effects of changing K^+ concentration on Na^+ transport and the K^+ concentration gradient. At the temperature at which the experiment in Fig. 3 was performed (11°C) the Na^+ pump would be partially active. At normal or higher K^+ concentrations the RMP varies as is to be expected from a cell which obeys the Goldman equation and where the K^+ concentration gradient is an important determinant of RMP. A decrease in K^+, however, would reduce the Na^+ pump activity as well as change the concentration gradient. The hyperpolarization on warming was essentially abolished after perfusion with seawater containing one-tenth (1 mM) the usual amount of K^+, and thus the depolarization observed in going from normal to one-tenth K^+ almost certainly reflects the abolition of Na^+ pump activity. Further decrease in K^+ causes only a hyperpolarization as predicted by the Goldman equation since the pump is already inactive.

Cocaine has been found to increase the negativity resulting from active Na^+ transport in Na^+-loaded frog muscle (2), presumably by increasing the resistance to passive K^+ movements (39). The effect of 3×10^{-3} M cocaine on membrane resistance of *Aplysia* neurons was tested in five cells. In four cells a constant current pulse was repetitively applied while going from seawater to cocaine seawater. In one cell, illustrated in Fig. 9, the effect of cocaine on the I–V relation at 23°C was determined. The membrane slope resistance is greater at every point along the I–V curve in the presence of cocaine than

in the control. In both the control and with cocaine the slope resistance measured by large hyperpolarizing pulses is considerably less than that for small pulses near the resting potential. This effect has been called anomalous recti-

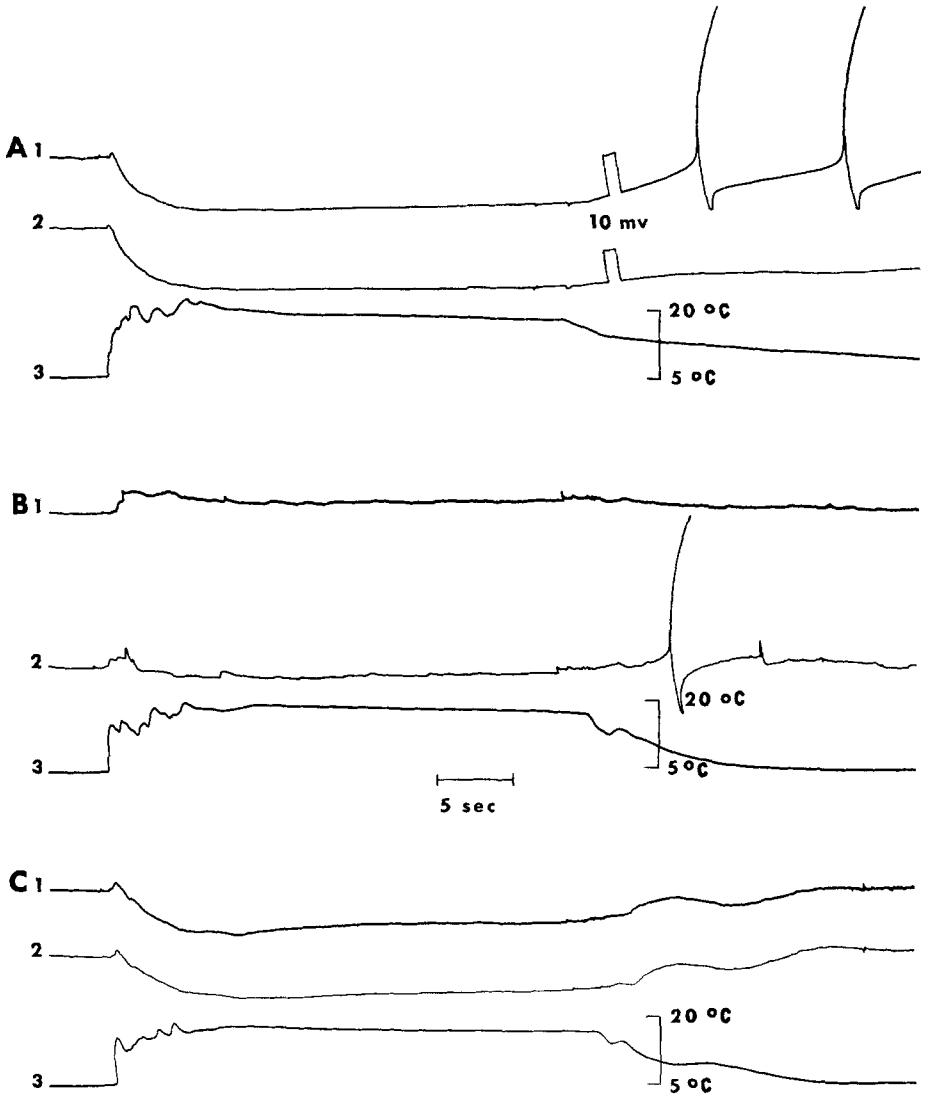

FIGURE 8. Simultaneous recordings from two cells in normal seawater (A), seawater containing no K^+ (B), and less than 5 min after return to seawater containing the usual amount of K^+ (C). In each series the upper trace is from cell R_{14} and the middle trace from an unidentified cell in the lower right quadrant. The hyperpolarization on warming in cell R_{14} is abolished and even turned over and that in the second cell is greatly reduced in the absence of external K^+. The depolarization on warming in B-1 results from synaptic excitation and an increase in the passive Na^+ permeability. Initial RMP is −50, −60, and −60 mv for cell 1 and −41, −48, and −49 mv for cell 2 in A, B, and C respectively.

fication and has been shown to result from an increased K^+ conductance both in muscle (1) and in *Aplysia* neurons (44). The increase in resistance with cocaine is particularly large in the anomalous rectification portion of the curve. In Fig. 9 the chord resistance for the reasonably linear portion of the curve near the resting potential was increased by 30% by cocaine, while the increase is 50% in the region of anomalous rectification. The results of the experiment illustrated in Fig. 9 suggest that, as in other preparations, cocaine

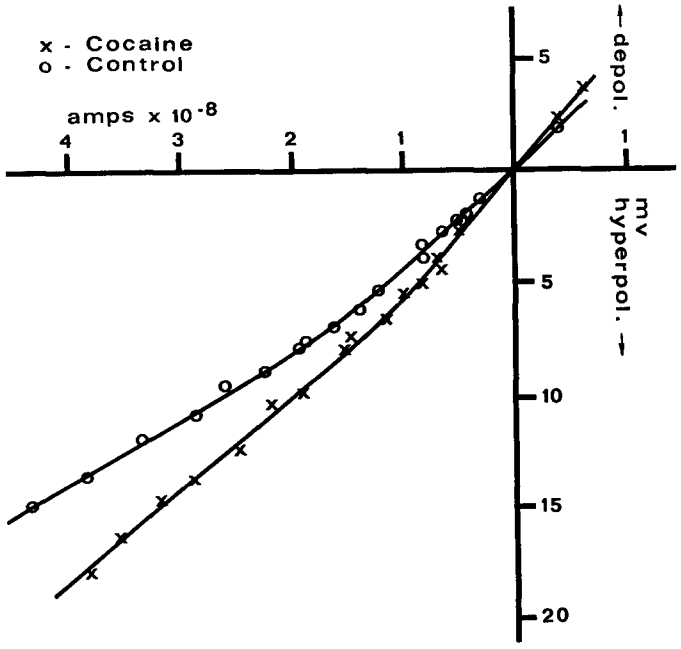

FIGURE 9. Current-voltage relations in cell R_2 at 23°C in seawater and in the presence of 3×10^{-3} M cocaine.

acts in *Aplysia* neurons to increase the resistance to the passive flow of ions and specifically to the movements of K^+.

Fig. 10 shows the effect of cocaine on the RMP shift in an unidentified cell. A temperature change in normal seawater led to an acceleration of discharge which nearly obscures any hyperpolarization. As illustrated in record B, in the presence of 3×10^{-3} M cocaine, warming produced a clear-cut hyperpolarization. Record C shows return to the control state after washing for 30 min. Record D shows a temperature shift after the electrode was withdrawn from the cell and demonstrates that there is no DC shift with temperature in the recording equipment. Cocaine could not be shown to increase the RMP shift in every cell, but did so in the majority of those studied. It was particularly effective in cells such as those illustrated in Fig. 10 where there was only a small shift initially.

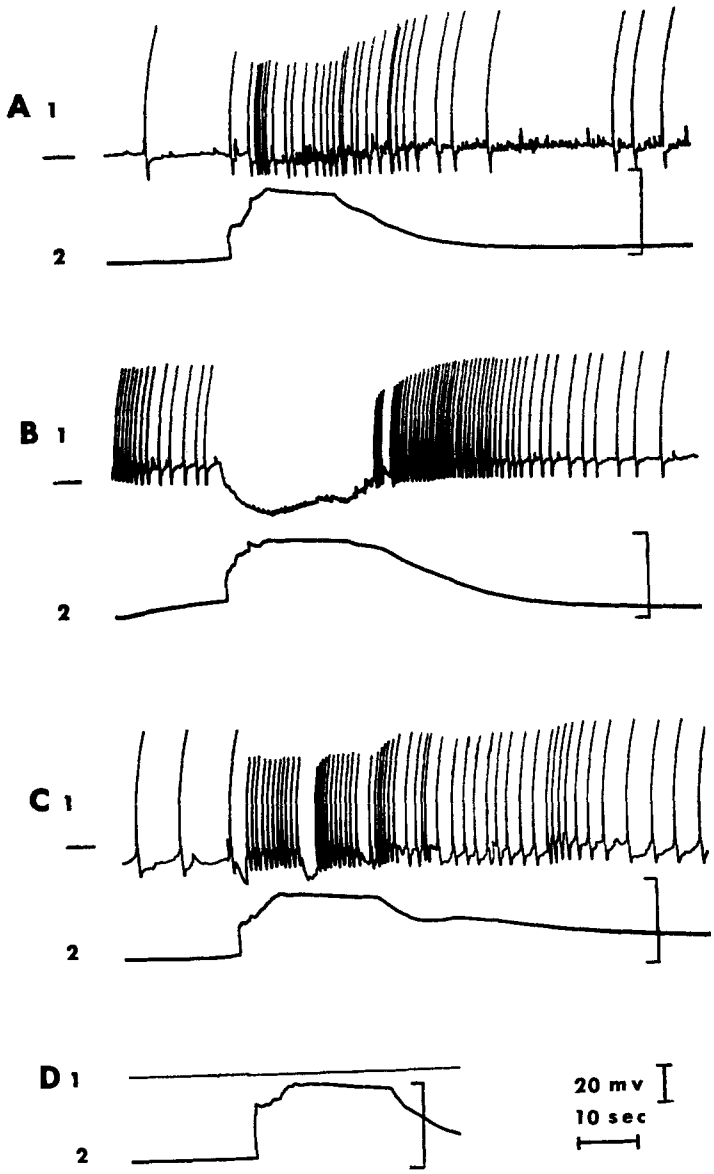

FIGURE 10. The effect of a rapid temperature change in a very active unidentified cell before (A), during (B), and after (C) exposure to 3×10^{-3}M cocaine, showing that the magnitude of the hyperpolarization on warming is increased in the presence of cocaine. The temperature calibrations all indicate 5° and 20°C. The RMP in A-1 is −43 mv, and the dash preceding B-1 and C-1 indicates −43 mv. In D the effect of the temperature change was tested immediately after withdrawing the electrode from the cell. D-1 shows that no shift in the DC level is seen when the electrode is not inside a nerve cell.

DISCUSSION

These experiments have demonstrated that RMP of *Aplysia* neurons may increase by up to 50% of its value at 5°C when warmed to room temperature. The increase in RMP on warming can most reasonably be explained as resulting from activity of an electrogenic Na^+ pump.

The evidence implicating active Na^+ transport is principally the inhibition by ouabain, for inhibition by the cardiac glycosides is believed to be specific for (7, 37), and has become a generally accepted test of active Na^+ transport (18). The ionic changes in the perfused seawater which caused reduction or abolition of the hyperpolarization are also those which have been found in other preparations to reduce Na^+ transport. Removal of external K^+ is known to decrease Na^+ efflux in red cells (16) and squid axon (27, 29). The abolition of the hyperpolarization on warming when Na^+ is replaced by Li^+ is consistent with the observation in muscle that Li^+ can substitute for Na^+ along the passive channels but is actively transported out of the cell at a very low rate (28). High Ca^{++} also is known to interfere with active transport of Na^+ in red cells (23, 24).

Since a potential change such as that observed in these experiments could result from a change in passive membrane conductance, the effect of temperature upon the I–V relations of these neurons is an important test of active transport. The observation that no conductance change is measured as a function of temperature in cells which show clear hyperpolarization on warming is consistent with this explanation for the origin of the potential shift, and is clear evidence that the hyperpolarization does not result from a conductance change.

Finally the degree of temperature dependence is also consistent with the effect of temperature on Na^+ transport (35). Passive ion conductances, although temperature-dependent, have been found to have Q_{10} values in the range of 1.1 to 1.4 in squid axon (22) while Na^+ transport has a Q_{10} of greater than 3 in both squid axon (22) and muscle (41). Electrogenic Na^+ transport is reduced or abolished by cold in Na^+-loaded muscle fibers (19, 32).

The Na^+ pump could generate a potential by either an electrogenic mechanism or by a chronic, temperature-dependent depletion of K^+ in the extracellular clefts with a resulting change in the K^+ equilibrium potential. The speed with which this potential is generated (20 mv in 4 sec in Fig. 8) argues for the electrogenic mechanism. Furthermore, the K^+ equilibrium potential, as indicated by the equilibrium point of the afterpotential, was found to be relatively temperature-independent over the time course used in these experiments. Thus it is most likely that the potential is generated by a net transfer of positive charge across the cell membrane.

The experiments with cocaine provided further support for the electro-

genic nature of this effect. The available evidence indicates that cocaine acts to increase resistance to the passive flow of ions (2, 39). If the pump is not completely coupled to K^+ influx, there is a component of current flow across the membrane which is not accounted for in the Goldman equation. After the initial generation of potential on warming there will be a return inward current, equal to the outward current generated by the electrogenic Na^+ pump, which is carried along the passive channels of K^+, Na^+, and Cl^-. The increase in the hyperpolarization on warming in the presence of cocaine is consistent with the view that the portion of active Na^+ transport not coupled to K^+ movement functions as a constant current source across the membrane resistance. Therefore, when this resistance is increased, as by cocaine, the voltage generated increases. Unfortunately the present experiments provide no information as to the extent of the uncoupling between Na^+ and K^+ transport.

Evidence is presented elsewhere (9) supporting the view that the electrogenic transport of Na^+ seen in these experiments is dependent upon the activity of the enzyme, Na^+-K^+–activated adenosinetriphosphatase (Na^+-K^+ ATPase), which is thought to be responsible for coupled Na^+-K^+ transport (6, 18, 34). However, it must be pointed out here that some of the present observations are best understood by a knowledge of the ionic requirements of this enzyme. The absence of K^+ in the external medium is known to decrease Na^+ efflux (16, 29), and this fact has been used as evidence for a coupling of Na^+ and K^+ transport (27). The present experiments have demonstrated a requirement for K^+ in the external medium for an electrogenic transport of Na^+, when by definition K^+ is not being actively transported into the cell. However, Na^+-K^+ ATPase requires K^+ for activation (33) and the site at which K^+ is needed is thought to be on the external cell membrane (17). The inhibition of Na^+ transport in both red cells and in *Aplysia* by high Ca^{++} can also be explained by the effect on this enzyme. The Na^+-K^+ ATPase requires Mg^{++} for activation (40) and is inhibited by high Ca^{++} (35).

Recently it has been suggested (5) that there are two types of Na^+ transport, one dependent upon Na^+-K^+ ATPase and coupled and another which is electrogenic and not dependent upon Na^+-K^+ ATPase. It is unlikely that such is the case in *Aplysia* neurons, for on every test the electrogenic potential behaved as would be expected on the basis of present knowledge of active transport and the enzyme. In addition to the effects of removal of K^+ and high Ca^{++} discussed above, both active transport (17) and Na^+-K^+ ATPase (3) require Na^+, and Li^+ cannot substitute for Na^+ (3, 28). Both are specifically inhibited by the cardiac glycosides (7, 18). Furthermore the relative potencies of three cardiac glycosides for inhibition of the hyperpolarization on warming in these cells have been found to be in agreement with their rela-

tive effectiveness in inhibiting both active transport and Na^+-K^+ ATPase (9).

The present experiments provide an explanation for the transient potential changes observed when temperature is changed in these cells. However, the hyperpolarization on warming is usually well-maintained for long periods of time, although there may be some initial adaptation (8, 30). The mechanism of the maintained potential changes is under investigation.

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