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MEASUREMENT OF ELECTROGENIC-PUMP CURRENT IN *APLYSIA* NEURONES WITH CONSTANT-CURRENT AND CONSTANT-VOLTAGE TECHNIQUES

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SUMMARY

1. The responses of the abdominal and pleural giant cells to warming and cooling were studied using constant-current and constant-voltage techniques.

2. The potential change upon warming from about 7 °C to 22 °C was reversed by application of maintained inward current. The reversal potential was -77 ± 12 mV.

3. The membrane conductance increased with warming, but was not affected by pump-blocking agents.

4. The electrogenic-pump current was found to be about 16 nA for a model in which the pump acted across the membrane ionic conductance. This model could explain the reversal of the warming response with hyperpolarization.

5. In voltage-clamped cells, the response to warming was converted from an outward to an inward current by hyperpolarization. The reversal potential for the current response was 31 ± 7 mV more negative than resting, or about -80 mV.

6. The pump currents measured under voltage-clamp conditions were the same as those calculated for the hyperpolarizing responses in unclamped cells.

INTRODUCTION

The presence of electrogenic, or potential-producing, ion pumps has been demonstrated in nerve cell bodies in a variety of species, including snail ganglion cells (Kerkut & Thomas, 1965; Moreton, 1969), *Anisodoris* ganglion cells (Gorman & Marmor, 1970), *Aplysia* ganglion cells (Carpenter & Alving, 1968; Carpenter, 1970), the crayfish stretch receptor (Nakajima & Takahashi, 1966; Sokolove & Cooke, 1971), lobster cardiac ganglion cells (Livengood & Kusano, 1970), leech ganglion cells (Baylor & Nicholls, 1969; Jansen & Nicholls, 1973), cervical sympathetic ganglion cells (Kosterlitz, Lees & Wallis, 1970), and dorsal spinocerebellar tract neurones (Kuno, Miyahara & Weakly, 1970). The contribution of the pump to normal resting

potential may be studied by application of pump-blocking agents or by warming to room temperature from a level at which the pump is inactivated (Carpenter & Alving, 1968; Baylor & Nicholls, 1969; Gorman & Marmor, 1970; Livengood & Kusano, 1970; Sokolove & Cooke, 1971).

The first voltage-clamp demonstration of active production of current by an electrogenic pump was made by Thomas (1969), using Na-injection to stimulate the pump. Subsequently, Kononenko & Kostyuk (1976) showed that large injections of sodium produce currents which are very potential-sensitive and may even reverse at sufficiently negative potentials. Relatively little attention has been paid to voltage-clamp studies of electrogenic currents in normal unstimulated neurones. This report contains calculations of electrogenic-pump currents in the normal resting membrane of an *Aplysia* nerve cell body, as well as direct measurements of this current under voltage-clamp conditions.

MATERIALS AND METHODS

Aplysia californica of 10–20 cm length were collected in local tidepools and maintained for up to 3 weeks in cooled sea water aquaria. Survival was greatly improved by feeding every other day with lettuce. Removal of the visceral or pleural ganglion was carried out after the animal had been relaxed with a 30 cc injection of 1 M $MgCl_2$ into the abdominal cavity. The ganglion was treated for 15–20 min with a 1% solution of pronase (B-grade Calbiochem) as an aid to impalement; control experiments in hand-dissected ganglia gave comparable results to those obtained in pronase-treated preparations.

Usually the giant cell (R2) in the visceral ganglion, and occasionally its anatomical homologue in the left pleural ganglion (Hughes & Tauc, 1963) was used. These cells behaved identically in our experiments. The ganglion was pinned firmly in a silastic-lined chamber (3 ml). Recording electrodes had resistances of 2–5 MΩ and were filled with 3 M KCl. Stimulating electrodes had resistances of 2–3 MΩ and were filled with 3 M K-acetate to prevent injection of Cl^-. The external reference electrode was a calomel half-cell (Beckman model 39270). Transmembrane potentials were measured differentially between the reference electrode and a follower connected to the recording electrode. With these electrodes in the bath warming from 7 to 22 °C produced less than 1 mV d.c. potential shift, but potential shifts up to 5 mV were seen when a Ag–AgCl wire was used as reference. Currents were measured across a 100 kΩ series resistor in the bath ground lead.

The voltage-clamp circuit is shown in Fig. 1. A command voltage, V_c was added to the external potential at the negative input of the control voltage amplifier (CVA: Tektronix Type 1A6). The negative output was connected to the stimulating electrode through a 100 kΩ feedback attenuator. The initial holding potential was adjusted to the resting level by setting the CVA to zero current output with the d.c. position control. While this procedure did not give us absolute membrane potentials, we could easily measure changes from the resting level. The current, potential and temperature were recorded on a Brush Model 2222-1707-120 recorder. Currents were smoothed with an RC circuit (τ = 66 ms), since large high-frequency fluctuations were seen similar to those observed in snail neurones by Thomas (1969). Temperature was recorded with a thermistor placed within 2 mm of the soma.

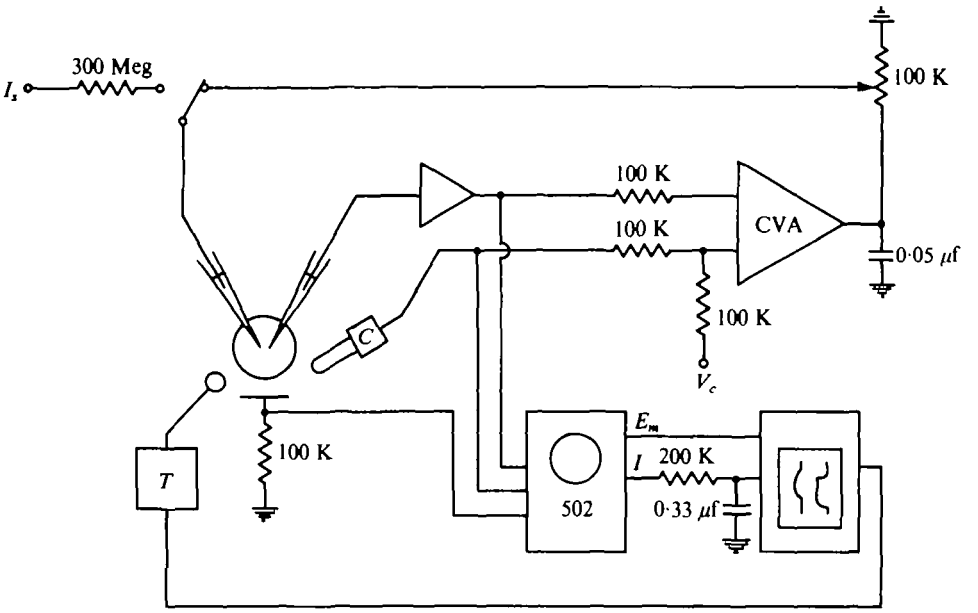

Fig. 1. Experimental arrangement. Switch may be connected to 300 MΩ resistor for constant-current stimulation or to other position for voltage clamp. I_s = Stimulating current in constant-current mode. T = Thermistor bridge. C = Calomel reference electrode. CVA = Control Voltage Amplifier. See text for details.

The composition of the normal bathing fluid was: NaCl 494 mM, KCl 11 mM, $CaCl_2$ 11 mM, $MgCl_2$ 30 mM, $MgSO_4$ 19 mM, Tris-HCl (pH 7·7) 10 mM. K-free solutions were prepared by increasing the $[Na]_o$ to 505 mM. Rapid warming was obtained by running room-temperature saline into the bath. Rapid cooling was obtained by switching the bath inlet to a large (2 l) beaker containing both liquid and frozen saline. In this way, the temperature could be changed by 15 °C within a minute or so.

RESULTS

Constant-current experiments

The effect of warming an R2 cell from 7 °C to 22 °C is shown in Fig. 2. In part A the potential was −50 mV initially and the response to warming was a hyperpolarization of about 3 mV. In part B an inward current of 54 nA was applied throughout. This current hyperpolarized the cell to −80 mV, and the warming response was virtually blocked. In part C an inward current of 108 nA was applied, hyperpolarizing the cell to −110 mV. In this condition the warming response was reversed, becoming a depolarization of 8 mV. Part D was obtained after about 5 min treatment with K-free solution, which has been shown to inhibit the activity of electrogenic pumps (Carpenter & Alving, 1968; Thomas, 1969; Moreton, 1969). In this condition the warming response at rest was converted to a depolarization of 5 mV. This is thought to result from an increased g_{Na}/g_K ratio upon warming (Carpenter & Alving, 1968; Marchiafava, 1970). In part E, an inward current of 54 nA was applied, and the warming response was augmented to 12 mV. Part F shows the recovery of the

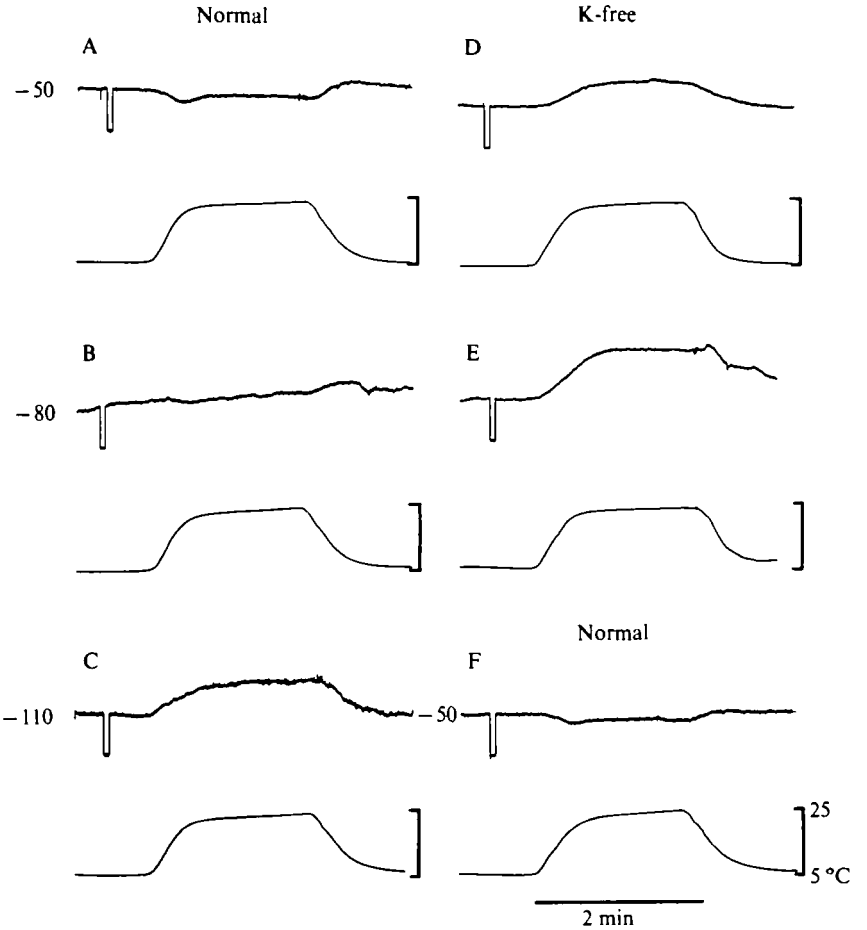

Fig. 2. Effects of warming on membrane potential in normal and K-free solutions. Top traces membrane potential; lower traces temperature. (A–C) Normal saline. (D, E) K-free. (F) Normal after D and E. (A, D) Initial potential = Resting potential. (B, E) Initial potential hyperpolarized to −80 mV by constant inward current. (C) Initial potential: −110 mV. (F) Initial potential = Resting potential. Potential calibration = 10 mV.

hyperpolarizing response in the unpolarized cell 15 min after returning to normal saline.

In order to analyse these results in non-voltage clamped cells it was necessary to obtain current–voltage (i–v) relationships under the various conditions tested. The i–v curves for the cell discussed above are shown in Fig. 3. It is apparent from inspecting these graphs that no large curvature occurs at potentials more positive than −80 mV; this was also reported by Carpenter & Alving (1968) and Junge (1976). The resting potentials recorded at 7 °C in both normal and K-free solutions were −50 mV, while those at 22° were −45 mV in K-free solution and −53 mV in normal saline. The conductance g, defined as $i/\Delta E$, was slightly potential-dependent, but had values close to 1·8 μmho at 7 °C and 2·0 μmho at 22 °C, and did not depend on the presence of potassium. Thus, apparently, pump activity does not contribute directly to measured membrane conductance. Brodwick & Junge (1972) demonstrated a similar lack of effect on conductance when the pump was inhibited by ouabain.

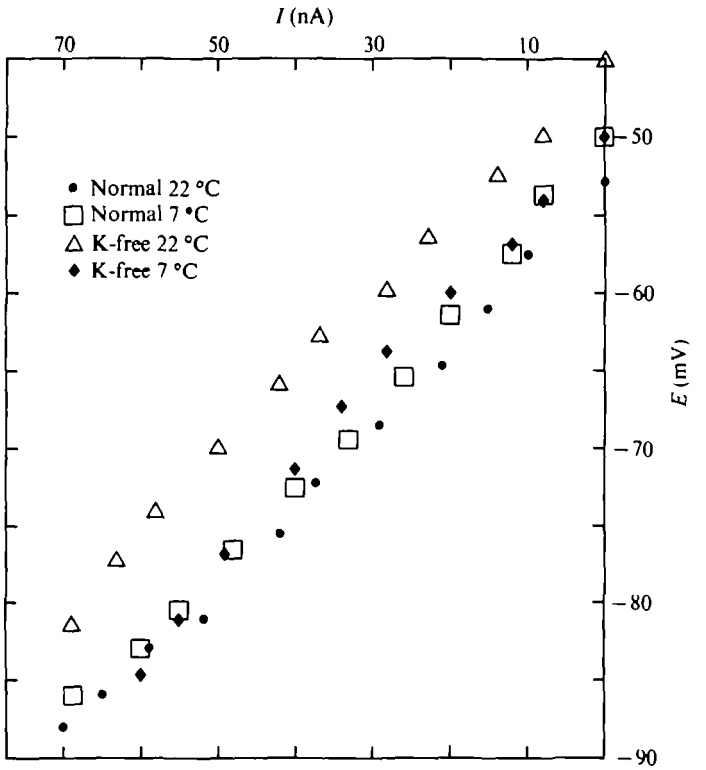

Fig. 3. Current–voltage relationships in normal and K-free solutions, measured with 1 sec hyperpolarizing pulses.

Knowing how the conductance and potential varied with temperature in the absence of pump activity, it was possible to use the data from experiments such as that of Fig. 2 to calculate the net current due to the electrogenic pump. This method is shown in Fig. 4, where the top of each diagram corresponds to the cell exterior. The electrogenic pump is symbolized by a constant-current generator in parallel with the membrane; artificial inward currents are indicated by schematic electrodes. The membrane conductance and equivalent potential are dependent on temperature, but not on external potassium. In part D, the effect of warming in K-free solution when the pump is inhibited is seen as a depolarization of 5 mV and an increase in conductance of 0·2 μmho. In part A, assuming the same equivalent batteries as in D, the measured response implies that the pump must generate 16 nA (this amount of current is required to produce the difference between -45 mV and the measured potential at 22 °C of -53 mV). In part B, the same equivalent batteries and conductances can be used to explain the blockage of the warming response with artificial hyperpolarization. The increase in conductance with warming makes the applied current less effective in hyperpolarizing the cell, at the same time as the pump current is activated. These opposing effects on the membrane potential just cancel. In E the increase in conductance results in a larger depolarization than in D. Part C shows that application of a larger artificial inward current results in a net depolarization upon warming. In this condition the model is able to account for the direction but not the

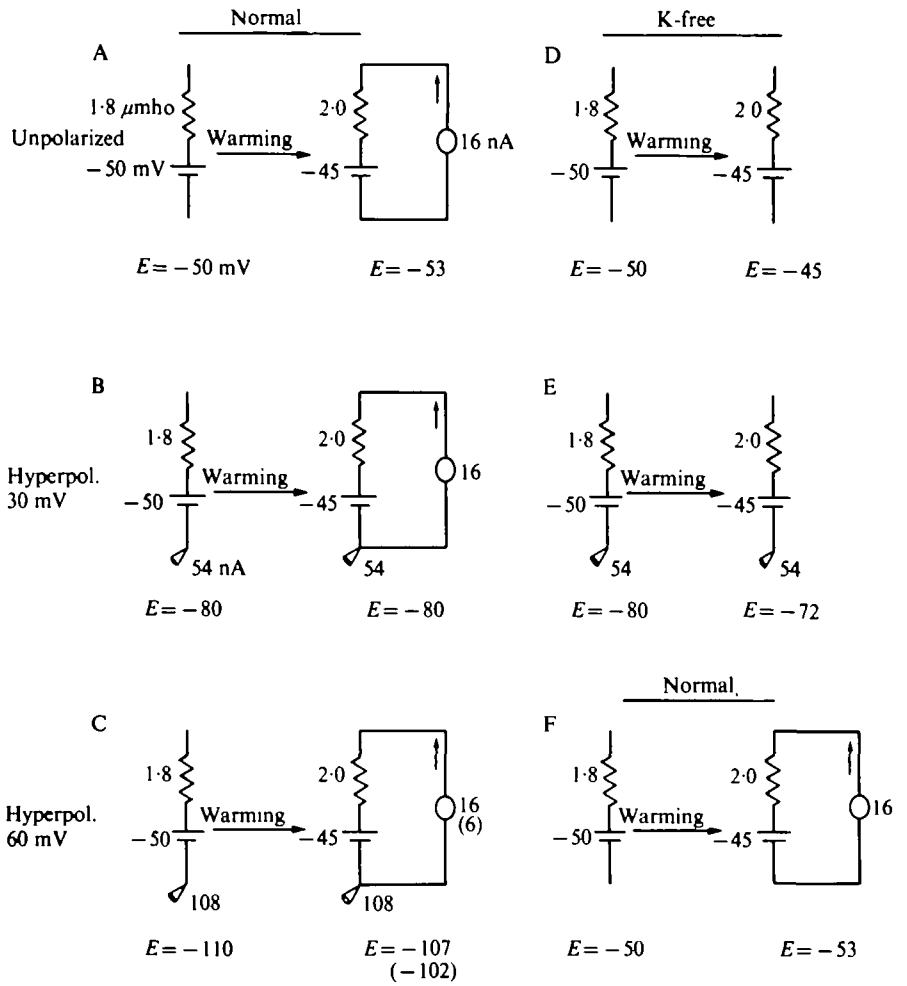

Fig. 4. Equivalent-circuit analysis of the results in Fig. 2. Conductance and potential in the model assumed to vary only with temperature. Electrogenic pump considered to be current source acting across the membrane ionic conductance. Letters correspond to those in Fig. 2. Top of each circuit = cell exterior. Applied inward currents shown by schematic electrodes at bottom. See text for further details.

magnitude of the observed depolarization (8 mV). The appropriate potential (−102 mV) obtains by reducing the pump current to 6 nA, as shown in parentheses in Fig. 4, Part C. This result might indicate some potential dependence of the pump current. It should be emphasized, however, that the membrane i–v curve in normal saline is strongly rectifying in this range of potentials, and that the rectification is temperature dependent (Eaton & Brodwick, 1976).

In another cell, the warming response with the pump inhibited by 0·5 mM strophanthidin was a depolarization from −46 to −45 mV and an increase in conductance from 1·6 to 2·3 μmho. The warming response in normal saline was a hyperpolarization from −46 to −52 mV. The electrogenic current calculated for this neurone from the model was again 16 nA. Most other cells subjected to warming showed hyper-

polarizing responses, but one with an unusually low conductance (0·7 μmho) showed a depolarization of 11 mV. Excluding this cell, the average response to warming in unpolarized cells was a hyperpolarization of 5·1 ± 2 mV (n = 5) (mean ± S.D.). All of these cells showed reversals of the responses at some hyperpolarized level. These reversal potentials were −77 ± 12 mV (n = 5).

The general equation describing these warming responses and those in Fig. 2 is

$$\Delta E = E_w - E_c + i_{ext}\,(1/g_c - 1/g_w) - i_p/g_w, \quad (1)$$

where ΔE = potential change on warming; E_w, equivalent potential at 22 °C; E_c, equivalent potential at 7°; g_w, conductance at 22°; g_c, conductance at 7°; i_{ext}, applied artificial current; i_p, pump current. With no artificial current applied, the pump current is then given by

$$i_p = g_w(E_w - E_c - \Delta E).$$

Constant-voltage experiments

The experiments in unclamped cells indicated that a current-source model of the electrogenic pump could explain the effects of warming on membrane potential. To test this model further, we used the voltage-clamp technique to measure the pump currents directly. The results of an experiment of this kind are shown in Fig. 5. In part A the cell was clamped at the resting potential, and an outward current of 19 nA was noted upon warming from 7° to 22 °C. In part B the potential was held at 40 mV more negative than in A, and the warming response became an inward current of about 4 nA. In part C the potential was 80 mV more negative than rest, and warming produced an inward current of 31 nA. Parts D–F show the results of analogous experiments with the same cell in K-free solutions. In D, warming at the resting potential produced an inward current of about 10 nA. In E the potential was clamped at 40 mV below rest in K-free solution, and the warming response was about 8 nA. In F the potential was 80 mV below rest, and the inward current increased to 16 nA. The current due to the electrogenic pump could be estimated by subtracting the current observed when the pump was blocked from that measured with the pump active. In part D, the inward current resulting from conductance and equivalent potential changes alone was 10 nA. Presumably this current was also present in part A, so the pump would have to produce a net current of 29 nA to give rise to the net outward current of 19 nA. Subtracting the current in E from that in B yielded a pump outward current of 4 nA when the potential was 40 mV more negative than rest. Performing the same subtraction of the current in F from that in C yields an *inward* pump current. We consider this result probably due to the K-sensitive inward rectifier (Marmor, 1971), which was active in this range of potential, rather than to any reversal of the electrogenic pump (see Discussion).

In another cell, when ouabain was used as the pump-blocking agent, the electrogenic pump current measured at the resting level was 6 nA. The average pump current for all cells clamped at their resting levels was 18 nA. This was about the same as the current predicted from the warming responses of unclamped cells (16 nA) and was within the range of currents measured for this cell in a study of pump stoichiometry by Cooke, Leblanc & Tauc (1974).

All of the cells tested under voltage clamp conditions except one showed outward

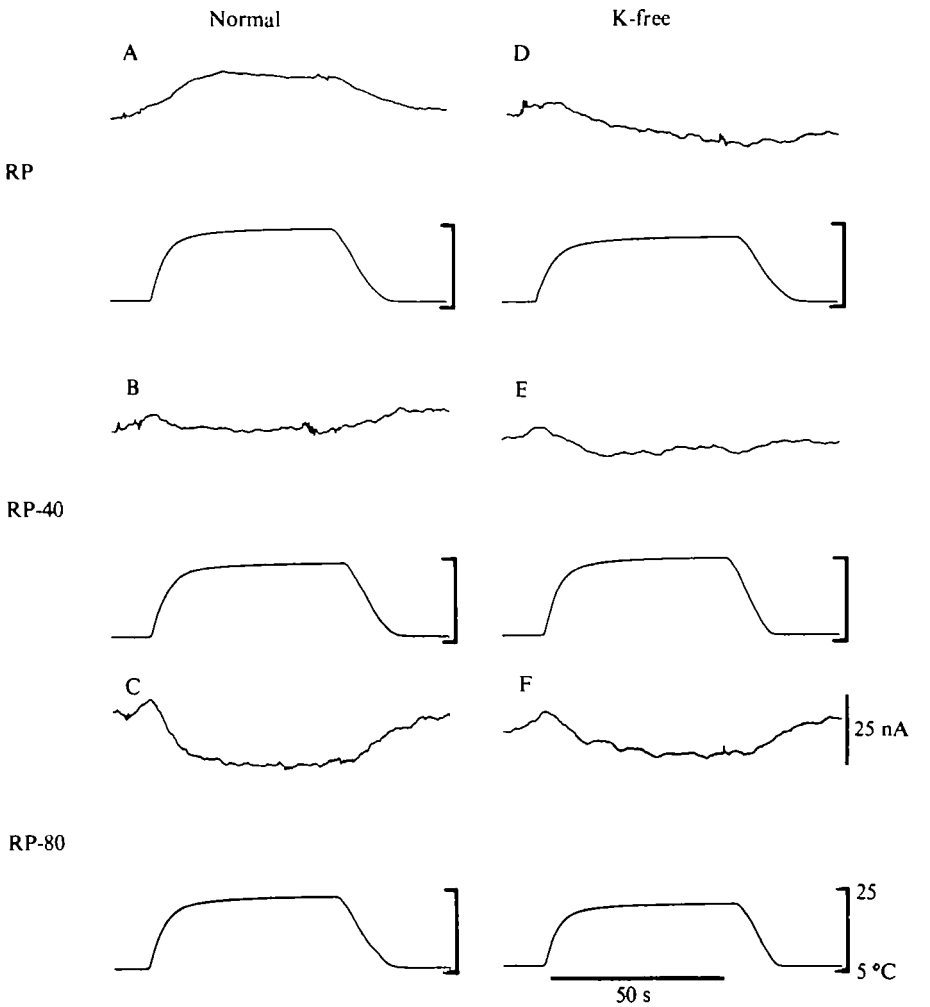

Fig. 5. Voltage-clamp currents in normal and K-free solutions. Top traces current; lower traces, temperature. (A–C) Normal saline. (D–F) K-free. (A, D) potential held at resting level. (B, E) Potential held at 40 mV more negative than rest. (C, F) Potential held at 80 mV more negative than rest. Upward deflexion = outward current.

current upon warming at the resting potential. The inward current seen in this cell might be expected to give rise to a depolarization upon warming without potential control, but this experiment was not attempted. The average warming response, excluding the cell showing inward current, was 28 ± 15 nA ($n = 5$) (mean $\pm$ S.D.). The reversal potential for the currents was 31 ± 7 mV ($n = 4$) more negative than rest, or about -80 mV. This agrees well with the reversal potential for the hyperpolarizing responses in unclamped cells. The results obtained with voltage clamp support the model of the electrogenic pump as a conductanceless current source. The pump contribution to normal resting potential ($\sim$ 8 mV) is that expected to result from the measured pump current ($\sim$ 16 nA) acting across the known ionic conductance ($\sim 2\,\mu$mho).

Table 1. *Comparison of electrogenic current, membrane resistance, and pump contribution to resting potential in several preparations*

Source	Preparation	ΔRP due to pump, mV	R_m, Ω-cm²	I_p, nA/cm²	I_p, pmol/cm².sec
Hodgkin & Keynes, 1956	Squid axon	1·4	10^3	1000–2500	10–25
Carpenter, 1970	*Aplysia*	22	10^5	220	2·2
Marmor, 1971	*Anisodoris*	20	10^5	20–400	0·2–4
Casteels, Droogmans & Hendricx, 1971	Taenia coli	22	$2·5 \times 10^4$	900	9
Christofferson, 1972	*Helix*	7	10^4	1000	10
This paper	*Aplysia*	8	$3·5–8 \times 10^4$	100–240	1–2·4

DISCUSSION

The electrogenic currents measured in our voltage clamp experiments were consistent with those calculated for unclamped cells. In normal saline the direction of the membrane current reversed at potentials similar to the reversal potentials for the warming responses in unclamped cells. When the pump was blocked with K-free or ouabain-containing solutions, the membrane current under voltage-clamp conditions was always inward, and increased with increasing negativity of the holding potential. This is the expected behaviour of a membrane held at a fixed potential, more negative than rest, in which warming increases the conductance.

That a decrease in inward current in the model was required to explain the observed behaviour in the cell of Fig. 2 was equivocal, as mentioned. Since the inward rectifier is K-dependent (Marmor, 1971), the membrane conductance at large negative potentials might have decreased significantly in K-free solutions, compared to normal. In the cells treated with strophanthidin, the evidence is somewhat stronger for a potential dependence of the pump current, since the membrane conductance is unaffected by strophanthidin in this range of potentials. The voltage-clamp experiment in Fig. 5 indicates that pump current is reduced at a membrane potential 40 mV more negative than rest, compared to that at the resting level. This may be valid even though obtained with K-free solution, since the i–v relationship is linear in this range of potential.

The measured currents due to the electrogenic pump in our formulation may be translated into a current density for comparison with other studies. The area of the soma considered as a sphere varies from 0·005 cm^2 to 0·011 cm^2 in adult animals. If an infolding factor of $14\times$ is assumed for the somatic membrane (Eaton, Russell & Brown, 1975; Miller, 1976) the effective membrane area becomes 0·07–0·16 cm^2. Thus an electrogenic current of 16 nA, or 0·166 pmol/sec, yields a density of 1–2·4 $pmol/cm^2.sec$. This is compared to electrogenic currents in other preparations in Table 1. It is clear that the size of the pump-dependent component of membrane potential depends both on the specific pump current density and the membrane resistance. Thus, the squid axon has only a small metabolic component of potential, although it has the largest pump current, because of its very low membrane resistance.

The fact that the presence or absence of pump activity does not affect the membrane conductance (Fig. 3) lends support to the model of the pump as an infinite-impedance current source. In the model, the pump current is directed outwardly which results in an *inward* current through the ionic conductance. The physical realization of this process might be the transfer of Na^+ outwardly through conductanceless channels, with the resultant establishment of a counter-EMF (inside negative) similar to the inside-negative EMF produced by outward diffusion of K^+-ions in the resting cell, according to the theory of Nernst (1889). In the case of the electrogenic pump, the outward driving force would be maintained by an active process, rather than by a concentration gradient.

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