J. Membrane Biol. 36, 83–95 (1977)

Current-Voltage Relationships and Voltage Sensitivity of the Cl^- Pump in *Halicystis parvula*

James S. Graves* and John Gutknecht

Department of Physiology and Pharmacology, Duke University Medical Center, Durham, North Carolina 27710, and
Duke University Marine Laboratory, Beaufort, North Carolina 28516

Received 3 January 1977; revised 11 April 1977

Summary. The current-voltage (I–V) relationships for internally perfused and nonperfused cells of *Halicystis parvula* were determined. In both types of cells the I–V curve shows a conspicuous region of negative slope, beginning at vacuole potentials around -30 mV and continuing to values of $+20$ to $+40$ mV. The negative slope in perfused cells is abolished by the metabolic inhibitors, darkness and low temperature. In order to determine the origin of this negative slope, we measured the voltage sensitivity of the unidirectional fluxes of Cl^-, Na^+ and K^+ in perfused cells. The results show that the Cl^- influx, which is mediated primarily by a Cl^- pump, increases as the vacuole potential is clamped at increasingly more *negative* values up to -50 mV, while the other fluxes measured changed in the directions predicted by the change in electrical driving force. The voltage sensitivity of the Cl^- pump quantitatively accounts for the negative slope of the I–V curve. Also, we observed a large transient outward current of 10–20-sec duration following an abrupt depolarization by voltage clamping. This transient current was reduced or abolished by low temperature, which suggests that it may be due to the voltage-sensitive Cl^- pump. Finally, we found an inverse relationship between the transprotoplasm resistance (R_m) and the PD under standard conditions, which suggests that the activity of the electrogenic Cl^- pump lowers R_m, i.e., it is a conductive pump.

An electrogenic pump is an active transport system which transports net ionic current and thus generates an electrical potential difference (PD) across a membrane. In separate theoretical models Finkelstein (1964) and Rapoport (1970) proposed that an electrogenic pump may be voltage sensitive and consequently contribute to the membrane conductance. There is experimental evidence for the existence of voltage-sensitive, electrogenic pumps in the marine alga, *Acetabularia* (Gradmann, 1975), the freshwater alga, *Nitella* (Spanswick, 1972), a molluscan nerve (Kostyuk, Krishtal & Pidoplichko, 1972) and the frog skin (Man-

* *Present Address:* Department of Physiology, Medical University of South Carolina, Charleston, South Carolina 29401.

del & Curran, 1973). In the frog skin direct flux measurements have been made to support this hypothesis, but the direction of voltage sensitivity and the anatomical complexity of the skin make the interpretation equivocal.

In the marine alga, *Halicystis parvula*, the inwardly directed Cl^- pump appears to be strongly electrogenic, accounting for nearly all of the -50 to -60 mV potential difference (*PD*) in internally perfused cells (Graves & Gutknecht, 1977). When connected to a voltage clamp device, the perfused cells of *H. parvula* allow the investigation of both the current-voltage relationships and the voltage dependence of the unidirectional fluxes of various ions. The existence of a negative slope region in the *I–V* curve of *H. parvula* suggested that the electrogenic Cl^- pump might be voltage sensitive, and we have investigated this possibility with direct determination of the voltage-dependent changes in direction and magnitude of the fluxes of Cl^-, Na^+ and K^+. In addition, the effects of metabolic inhibitors on the shape of the *I–V* curve were determined. Finally, we have measured the transprotoplasm resistance (R_m) at various cell-generated *PD*'s in order to investigate the question of whether or not the electrogenic Cl^- pump contributes directly to the membrane conductance.

Materials and Methods

Voltage Clamp Measurements

The current-voltage (*I–V*) relationships were measured in cells which were doubly impaled and connected to a perfusion system equipped with a voltage clamp device, as described by Graves and Gutknecht (1976). The circuit diagram for the voltage clamp is given by Graves (1974). The steady-state *I–V* curves were determined by adjusting the voltage to the desired level and then recording the current output of the voltage clamp for 1–3 min until it became stable. Normally, the experiment began at a hyperpolarized level and proceeded through zero in 10-mV steps, although other protocols gave similar results.

The effects of darkness and low temperature on the *I–V* curves were determined using the procedures described by Graves and Gutknecht (1977). After waiting for the inhibition to become complete (ca. 2–3 min), we measured *I–V* curves as described above.

Some experiments were designed to show the initial changes in current which occured after a rapid change in the vacuole potential. In these experiments the desired voltage was set while the voltage clamp was turned off (i.e., open circuit *PD* being recorded), and the voltage clamp was then switched on to generate a rectangular change in the vacuole potential.

Flux Experiments

The methods of measuring unidirectional ion fluxes in perfused cells are described by Graves and Gutknecht (1976). The effects of the vacuole potential on the ion fluxes were

determined by simply clamping the voltage at a desired level and collecting samples as usual. A transitional sample was discarded to allow the radioactivity in the perfusate to reach a new steady state after the change in voltage. In all experiments the flux was measured at 0 mV before proceeding to another voltage so that the flux at each subsequent voltage could be normalized to the initial flux at 0 mV. For influx measurements at least three 15-min samples were taken and averaged to compute the flux at each value of vacuole potential. For efflux measurements at least two 30-min samples were taken for each point.

Pulse Resistance Measurements

The transprotoplasm resistance (R_m) was measured in cells doubly impaled with microelectrodes. The methods and apparatus used for current pulse measurements of R_m in such cells are described by Graves and Gutknecht (1976). The steady change in voltage after 1 sec of hyperpolarizing current was used to calculate R_m. For this study R_m was measured in cells in which the *PD* was changing spontaneously under standard conditions (i.e., bathed in standard artificial seawater (ASW) at room temperature).

Results

Steady-State I–V Curves

Figure 1 shows typical steady-state *I–V* curves for *H. parvula* prior to perfusion and during vacuolar perfusion with ASW. In both cases, there is a conspicuous negative slope region. In all experiments vacuolar perfusion reduced the magnitude of both the maximum current and slope of the negative-going region of the curve (Table 1). When we attempted to clamp the vacuolar potential at values more positive than about

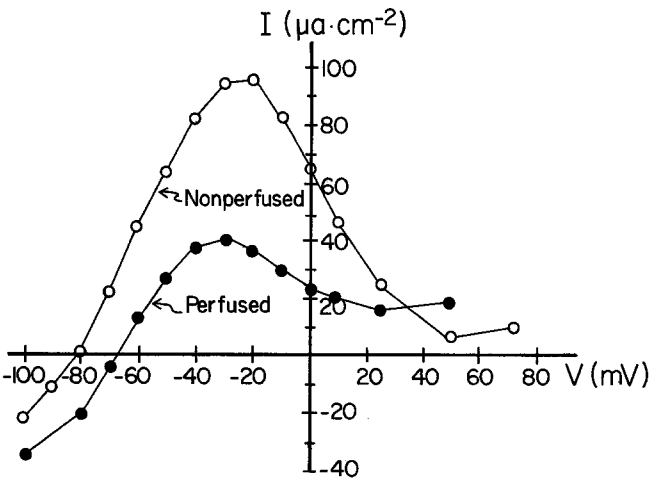

Fig. 1. Typical steady-state current voltage relationship for a perfused (●) and nonperfused (○) cell. Positive current means positive outward current

Table 1. Magnitudes of the negative slope and maximum current generated in perfused and nonperfused cells of *H. parvula*

Condition	Negative slope[a] ($\mu A \cdot cm^{-2} \cdot mV^{-1}$)	Maximum current ($\mu A \cdot cm^{-2}$)	Number of experiments
Perfused	0.30 ± 0.06	30.1 ± 8.1	6
Nonperfused	1.0 ± 0.5	71.5 ± 15.4	3

[a] Computed from region between -10 mV and $+10$ mV.
Data are expressed as mean $\pm$ SE.

$+50$ mV, the current oscillated with variable amplitude, and stable values could not be recorded. On the other hand, stable currents were recorded at negative vacuole potentials as high as -110 mV.

Voltage Dependence on Ion Fluxes

In cells perfused and bathed with ASW, the current at 0 mV is called the short-circuit current (SCC), which in *H. parvula* is almost entirely generated by an inwardly directed Cl^- pump (Graves & Gutknecht, 1976). Therefore, we investigated the possibility that the negative slope region of the *I–V* curve might be due to a voltage sensitivity of the Cl^- pump. Figure 2 shows that the relative Cl^- influx (i.e., normalized to the flux at 0 mV, which is about 350 $peq \cdot cm^{-2} \cdot sec^{-1}$) increases as the vacuole potential becomes more negative, which is in the opposite direction to the change predicted by the change in electrical driving force. However, this effect of vacuole potential on the Cl^- influx is in the direction necessary to explain the negative-slope portion of the *I–V* curve (*see Discussion*).

Table 2 shows the effect of various vacuole potentials on the Cl^- efflux and on the influx and efflux of Na^+ and K^+. In each case the flux changed in the direction predicted by the electrical driving force. The Cl^- efflux is particularly voltage sensitive, increasing by a factor of 2–3 between 0 and -25 mV, and this is consistent with it being primarily due to simple diffusion. The fluxes of Na^+ and K^+ show less voltage sensitivity, which is probably due to the fact that these fluxes have active components in both directions (Graves & Gutknecht, 1976). Thus, only the Cl^- influx changes in a direction opposite to the change in electrochemical driving force, and it is unlikely that any passive property of the system could account for this voltage sensitivity. Therefore, the data suggest that the Cl^- pump, or some integrally related membrane process, is voltage sensitive.

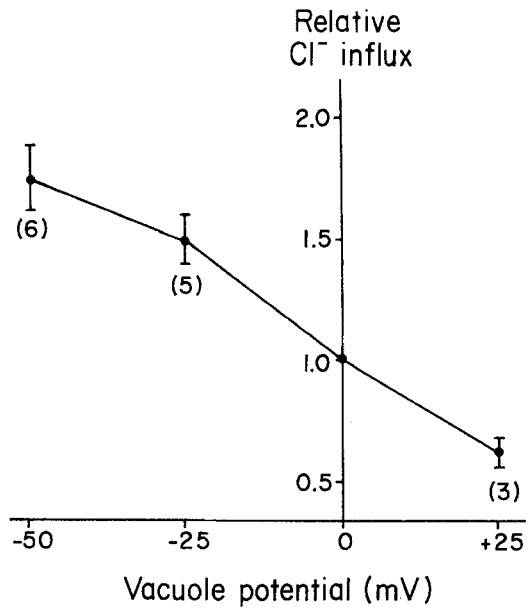

Fig. 2. Relative Cl^- influx at various clamped vacuole potentials in perfused cells. Each flux was normalized to the flux at 0 mV for that cell. Points and bars represent mean ±SE (number of experiments)

Table 2. Effects of clamped vacuole potentials on unidirectional ion fluxes in internally perfused cells of *H. parvula*

Type of Flux[a]	Ion flux at different vacuole potentials (pmole · cm^{-2} · sec^{-1})			
	+25 mV	0 mV	−25 mV	−50 mV
Cl^- efflux	15.3	52.8	134	351
	(0.3)	(1.0)	(2.5)	(6.6)
Cl^- efflux	27.2	99.1	341	–
	(0.3)	(1.0)	(3.4)	
Cl^- efflux	–	25.2	40.3	102
		(1.0)	(1.6)	(4.0)
Na^+ influx	–	33.1	76.1	–
		(1.0)	(2.3)	
Na^+ efflux	–	36.5	26.0	–
		(1.0)	(0.7)	
K^+ influx	–	0.88	1.35	–
		(1.0)	(1.1)	
K^+ efflux	–	1.65	1.32	–
		(1.0)	(0.8)	

[a] Single experiments.
Numbers in parentheses represent the flux normalized to the 0-mV control.

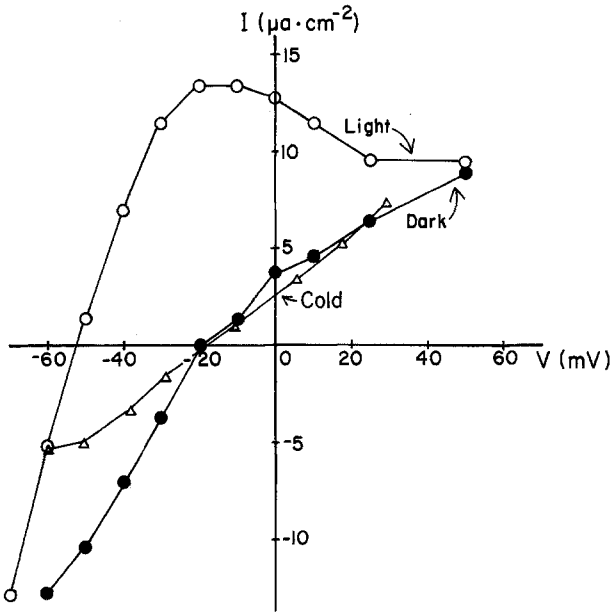

Fig. 3. Steady-state I–V curves for a perfused cell in the light (○) and in the dark (●). Also shown is the I–V curve for a different cell during inhibition by low temperature (ca. 4 °C) (△)

Effects of Metabolic Inhibitors on I – V Curves

If the negative slope region of the I–V curve is due to the voltage sensitivity of the Cl^- pump, then the negative slope should be abolished by the same metabolic inhibitors which inhibit the Cl^- pump (Graves & Gutknecht, 1977). Figure 3 shows the effect of two metabolic inhibitors, darkness and low temperature, on the shape of the I–V curve in perfused cells. In both cases the negative slope region is abolished, although some nonlinearity remains. Both darkness and low temperature also cause a decrease in the linear portion of the slope (i.e., an increase in R_m). Table 3

Table 3. Effect of darkness and low temperature on the transprotoplasm resistance (R_m)

Condition	R_m ($\Omega \cdot cm^2$)[a]		Number of experiments
	Mean	Range	
Control	685	350–1580	6
Dark	2362	1250–4000	4
Low temperature	4330	2220–6250	4

[a] Computed from the slope of I–V curves in the region near the cell PD.

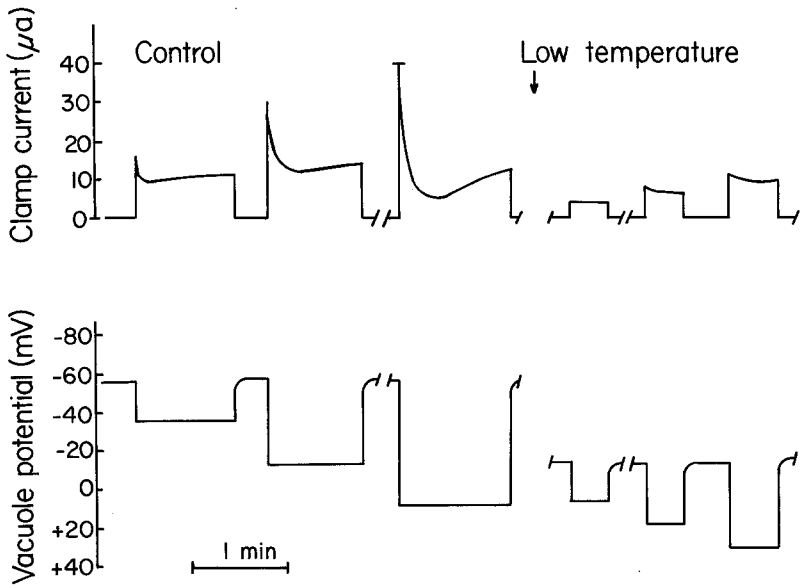

Fig. 4. Current and voltage traces during abrupt voltage clamp depolarizations before and during low temperature (ca. 4 °C) treatment of a perfused cell. The horizontal line on one of the peak currents indicates that the current was limited by the capabilities of the electrical system. Current is in the positive outward direction. The vacuole potential before and after depolarization is the open circuit *PD*. Cell surface area is 1.2 cm^2

summarizes the values of R_m computed from this slope for metabolically inhibited and noninhibited cells. While it is clear that R_m is substantially higher in inhibited cells, the values computed for these cells range so widely that precise quantitative comparisons are not possible.

Figure 4 shows recorder traces for the early currents which accompany an abrupt depolarization of a perfused cell, and it also demonstrates the effect of low temperature on these early currents. In an uninhibited cell there is a transient current spike the 10–20 sec immediately following the rapid depolarization. The magnitude of the peak current increases as the change in voltage is increased. Following the decreasing phase of this transient, the current rises to an intermediate, stable value within a few minutes. Metabolic inhibition of the cell with low temperature nearly abolishes the early transient, and the current response more closely resembles that of a simple ohmic resistor. In five experiments the inhibition of this transient by low temperature varied from 56% to 100% with a mean inhibition of 74%. The effects of other metabolic inhibitors on this early current response were not investigated. Because this early current appears to rely on metabolism, it seems likely that its origin involves an active current source, e.g., the Cl^- pump. In

similar experiments with *Acetabularia*, which also has an electrogenic Cl^- pump, Gradmann and Klempke (1974) observed a transient early current which had a magnitude and duration similar to that in *H. parvula*.

Relationship Between R_m and PD

During the course of early experiments with "intact" cells which were impaled with microelectrodes, we noticed that R_m was inversely related to the cell *PD*. Figure 5*A* shows the *PD* trace for a typical cell under standard conditions. An initial *PD* of −45 mV was followed by a gradual hyperpolarization to −82 mV and a concomitant decline in R_m. The trace shown in Fig. 5*B* is that of an atypical cell in which the *PD* spontaneously oscillated through zero before hyperpolarizing to the

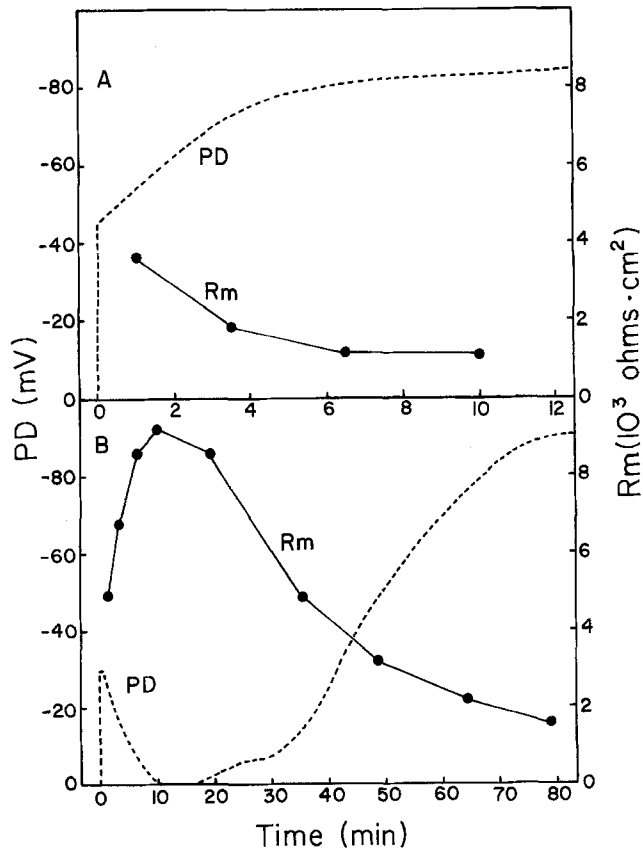

Fig. 5. Examples of spontaneous R_m and *PD* changes in two cells under standard conditions. Cells were impaled at zero time with the voltage-measuring microelectrode. *PD* was recorded continuously, and R_m was measured at various times by injecting a rectangular pulse of hyperpolarizing current. Note the different time scales for cells *A* and *B*

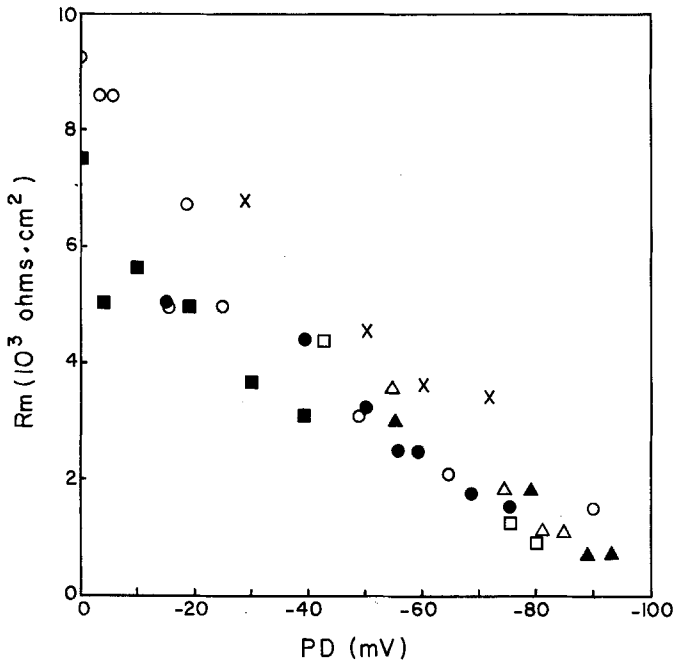

Fig. 6. Relationships between *PD* and R_m in seven nonperfused cells under standard conditions. Each symbol represents a separate cell in which the resistance was measured at a minimum of three different *PD*'s

normal level, and again R_m changed inversely with *PD*. A standard interpretation for such *PD* traces would be that time was required for the cytoplasm to seal around the microelectrode. However, R_m would have to *increase* during hyperpolarization to be consistent with this interpretation. A similar inverse relationship between R_m and *PD* was seen in other cells, as shown in Fig. 6. These results will be discussed further below.

Discussion

Origin of the Negative I–V Slope

The *I–V* curves for both perfused and nonperfused cells of *H. parvula* have a conspicuous region of negative slope. This negative-slope region begins around −30 mV and continues into the region of positive vacuole potentials (Fig. 1). Such unusual *I–V* relationships in *Halicystis* were first described by Blinks (1936), who observed that passing a depolarizing current beyond a certain threshold caused a dramatic swing of the cell *PD* through zero to values of +20 to +50 mV. It seems likely that this "reversal threshold" described by Blinks corresponds to the maximum current of the *I–V* curve, beyond which less current is required for increased depolarization.

We investigated the voltage sensitivity of the unidirectional ion fluxes in order to discern the origin of the negative-slope portion of the I–V curve. It is apparent that the Cl^- influx is sensitive to the vacuole potential in the direction opposite that predicted by the changes in electrochemical driving forces (Fig. 2). Because greater than 75% of this influx is mediated by an inward Cl^- pump (Graves & Gutknecht, 1976), it is probably this Cl^- pump which is voltage sensitive in the direction necessary to produce the negative slope of the I–V curve.

By making some reasonable assumptions about the passive ionic fluxes, we can show that the voltage sensitivity of the active influx is sufficient to account for the negative slope. Because the passive Cl^- efflux is large and voltage sensitive, it is reasonable to assume that Cl^- carries most of the passive current at all values of vacuole potential. Between 0 and -25 mV the Cl^- efflux increases by an average of 2.5-fold (Table 2), and we assume that the passive component of the Cl^- influx decreases by a factor of 2.5 over this voltage interval. This assumption is not strictly in accord with the prediction of the Goldman equation, but it is a reasonable approximation over this voltage range. With a mean passive efflux and influx of 59 pmoles $\cdot$ cm^{-2} $\cdot$ sec^{-1} at 0 mV (Table 2), this 2.5-fold change predicts that the *passive* current at -25 mV will be a net *passive* Cl^- efflux of 122 pmoles $\cdot$ cm^{-2} $\cdot$ sec^{-1}. To predict the slope of the I–V curve we can compare this change in passive Cl^- flux to the change in the active Cl^- flux between 0 mV and -25 mV. The unidirectional Cl^- influx increases by a factor of approximately 1.5 between these voltages (Fig. 2), and we use this observation to calculate the change in the active flux (ΔJ_{act}) by the expression:

$$\Delta J_{act} = (1.5 \cdot J^{in}_{V=0} + \Delta J^{in}_{pas}) - J^{in}_{V=0} \quad (1)$$

where $J^{in}_{V=0}$ is the unidirectional Cl^- influx at 0 mV (i.e., 354 pmoles $\cdot$ cm^{-2} $\cdot$ sec^{-1}, Graves & Gutknecht, 1976). The term, ΔJ^{in}_{pas}, is the voltage-dependent change in passive influx (i.e., $59-24=35$ pmoles $\cdot$ cm^{-2} $\cdot$ sec^{-1}), which decrementally affects the measurement of the voltage-dependent influx. The calculation from Eq. (1) yields 212 pmoles $\cdot$ cm^{-2} $\cdot$ sec^{-1}, and this change in the active influx is greater than the concomitant change in the net passive Cl^- efflux (i.e., 122 pmoles $\cdot$ cm^{-2} $\cdot$ sec^{-1}). The difference between the net active and passive Cl^- fluxes (converted to current units) divided by 25 mV gives a negative slope of 0.38 μA $\cdot$ cm^{-2} $\cdot$ mV^{-1}, which compares favorably with the mean negative slope of 0.30 μA $\cdot$ cm^{-2} $\cdot$ mV^{-1} computed from actual I–V curves (Table 1).

Further evidence supporting the idea that the Cl^- pump is responsible for the negative slope comes from studies with metabolic inhibitors. Both darkness and low temperature are inhibitors of the electrogenic Cl^- pump (Graves & Gutknecht, 1977), and both of these inhibitors abolish the negative slope region of the I–V curves of perfused cells (Fig. 3). Thus, both the negative slope and the Cl^- pump are strictly and rapidly dependent on energy metabolism, which is consistent with the existence of a cause-effect relationship between these two parameters.

The Cl^- Pump and Membrane Conductance

A voltage-sensitive source of current will, by definition, influence the measured conductance of a membrane or membrane system. This concept is included in theoretical models of membrane systems having voltage-sensitive, electrogenic ion pumps (Finkelstein, 1964; Rapoport, 1970). Although the voltage sensitivity of the Cl^- pump in *H. parvula*, over the voltages investigated, is in the opposite direction to that predicted in these models, the idea that it may be a "conductive pump" is still reasonable. We have shown that the transprotoplasm resistance (R_m) is inversely related to the cell *PD* as this *PD* changes spontaneously (Figs. 5 and 6). Because most of the *PD* is generated by the electrogenic Cl^- pump (Graves & Gutknecht, 1977), this inverse relationship suggests that as the activity of this pump increases (i.e., the absolute value of the *PD* rises) its contribution to the membrane conductance also increases (i.e., R_m decreases).

A reduction of R_m by the activity of a conductive pump invalidates the usual assumption that R_m is a measure of the resistance to passive ionic flow. Graves and Gutknecht (1977) use an indirect approach to estimate that R_m in the absence of the Cl^- pump is about $1800\,\Omega \cdot cm^2$. Furthermore, we calculated above the voltage-dependent change in passive Cl^- flux over the range of 0 to -25 mV (i.e., $122\,pmoles \cdot cm^{-2} \cdot sec^{-1}$), which we assume is the predominant passive current. Converting this flux to current units yields a value of $11.7\,\mu A \cdot cm^{-2}$ per 25 mV, and this represents a resistance of $2124\,\Omega \cdot cm^2$, which is similar to that calculated by the indirect method. The close agreement between these values, and our ability to calculate the "correct" pump-generated *PD* when this magnitude of R_m is used (Graves & Gutknecht, 1977), support the idea that the *measured* R_m is lower than the true resistance to passive ionic flow because of the activity of the conductive Cl^- pump.

Comparisons with Acetabularia

When the electrical properties of *H. parvula* are compared with those of another marine alge, *Acetabularia*, several striking similarities emerge. The Cl^- pump in *Acetabularia* is strongly electrogenic and produces about $-80\,mV$ of the $-170\,mV$ *PD*, the remaining $-90\,mV$ being produced by a predominantly K^+ diffusion potential (Saddler, 1970; Gradmann, 1970). The pump-generated *PD* of $-55\,mV$ in internally perfused *H. parvula* is superimposed on a diffusion potential of 0 mV, and this accounts for the difference in total *PD*, which is the most conspicuous difference between the electrical properties of these two organisms. The Cl^- influx in *Acetabularia* is 200–800 $pmoles \cdot cm^{-2} \cdot sec^{-1}$ (Saddler, 1970) which is similar to the predominantly active Cl^- influx of 100–650 $pmoles \cdot cm^{-2} \cdot sec^{-1}$ in perfused cells of *H. parvula* (Graves & Gutknecht, 1976). In both genera the measured R_m ($100\,\Omega \cdot cm^2$ in *Acetabularia*) is too low to account for the pump-generated *PD* by Ohm's law, and R_m increases during metabolic inhibition. Like *H. parvula*, the *I–V* curve of *Acetabularia* under standard conditions exhibits a conspicuous negative-slope region which is abolished by low temperature (Gradmann & Klempke, 1974; Gradmann, 1975). Both organisms also show similar early transient currents following an abrupt depolarization (Fig. 4, this paper; Gradmann & Klempke, 1974), which may be the result of hysteresis in the voltage-sensitive Cl^- pump. Therefore, the Cl^- pumps in *Halicystis* and *Acetabularia* have similar properties and thus may have similar molecular mechanisms.

Gradmann (1975) has constructed an analog circuit for the cell membrane of *Acetabularia*. Based primarily on electrical measurements Gradmann concludes that the negative slope region of the *I–V* curve is due to a voltage-sensitive Cl^- pump. His theoretical analysis proposes that the voltage dependence of the pump in the direction opposite to the electrical driving force is in a region about 200 mV more positive than the region of negative slope on the *I–V* curve. However, in *H. parvula* we have shown by direct flux measurements that the voltage sensitivity of the Cl^- pump which is responsible for the negative slope is in the same region of vacuole potentials as the negative slope (Fig. 2). Whether this difference between the two organisms has a biological or conceptual origin remains to be determined.

This work was supported by U.S. Public Health Service Grant HL 12157 and a N.I.H. predoctoral traineeship to J.S.G. We thank Drs. M.A. Bisson, L.J. Mandel, and D.F. Hastings for helpful discussions and Ms. S. Allison for critically reading the manuscript.

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