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Studies on the Perfused Plasmalemma of *Chara corallina*: I. Current-Voltage Curves: ATP and Potassium Dependence

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Summary. The electrical properties of the *Chara* cell membrane have been studied using a perfusion method based on that of Williamson, R.E. 1975. *J. Cell Sci.* **17**:655. The vacuole, tonoplast, and inner cytoplasm are removed by a brief rapid perfusion. Electrical properties of the plasmalemma indicate that it remains intact after this perfusion.

The membrane potential difference after perfusion and with no ATP was close to the potassium equilibrium potential; the current-voltage characteristic had a slope that was time- and voltage-dependent, indicating that the steady-state potassium conductance increased with depolarization. At -125 mV the membrane conductance of the plasmalemma depended on $[K^+]_o$. This dependence was inhibited by perfusing with 2.0 mM ATP or by clamping at a more negative membrane potential. The addition of ATP to the perfusion medium of unclamped cells caused a hyperpolarization of *ca.* 50 mV, presumably by activating the proton pump. In clamped cells, perfusion with ATP caused currents of *ca.* 20 mA m^{-2}, whose magnitude depended on pH_o. ATP induced membrane conductance changes which were variable. 2.0 mM ADP inhibited the proton pump. The intersection points of current-voltage characteristics can set limits on the stalling potential; the resulting stoichiometry of the proton pump appears to be 1.5–2.0 H^+ per ATP.

Since Tazawa (1964) developed the technique of vacuolar perfusion of charophyte cells, it has been used to study many properties of the cytoplasmic layer. However, the study of the plasmalemma alone requires the removal of the tonoplast.

Williamson (1975) developed a perfusion technique which removed the tonoplast of *Chara* internodal cells so that the cytoplasmic streaming mechanism could be studied. This method has been modified to allow the study of the electrical characteristics of the plasmalemma (Smith & Walker, 1978). Independently, a method for the removal of the tonoplast of charophyte cells was developed by Tazawa, Kikuyama and Shimmen (1976). Their method has been used to study cytoplasmic streaming, the membrane potential difference (ψ_M), and excitability of the plasmalemma (Shimmen, Kikuyama & Tazawa, 1976; Tazawa et al., 1976; Fujii, Shimmen & Tazawa, 1979; Kikuyama, Hayama, Fujii & Tazawa, 1979; Shimmen & Tazawa, 1977; Tazawa, Fujii & Kikuyama, 1979). Their method differs from that of the writers in that they perfuse at a much slower rate, relying on the Ca^{2+}-chelating properties of EGTA in the perfusion medium (PM) to disintegrate the tonoplast. The two methods and the results they give will be compared later in this paper. Perfusion of the cell has also been used by Sanders (1980*a*, *b*) and by Reid (1980) in studies of chloride transport.

The mechanisms that determine ψ_M in *Chara* cells include K^+ and Na^+ diffusion (Hope & Walker, 1961) and an electrogenic mechanism that is probably a proton pump (Oda, 1962; Hope, 1965; Kitasato, 1968; Spanswick, 1972, 1974; Richards & Hope, 1974; Walker & Smith, 1975; Keifer & Spanswick, 1978). The contributions made by these mechanisms to the membrane conductance (g_m) are not yet clear.

The present paper reports studies of ψ_M and g_M of the perfused plasmalemma, its current at constant voltage, and its current-voltage characteristic (i/ψ curve). These studies were undertaken in an effort to demonstrate the existence of the proton pump, to determine its stoichiometry, and to investigate the contribution of the mechanisms mentioned to the conductance of the membrane.

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Materials and Methods

General

Chara corallina (Klein *ex* Will), (=*Chara australis* [R.Br.]) was cultured outdoors in a large cement tank in a shaded situation.

Before experiments, internodal cells were isolated from neighboring cells and stored for 5 to 15 days at 22–24 °C in storage medium (SM) containing (mM): $Ca(OH)_2$, 0.2; KOH, 0.2; NaOH, 0.4; adjusted to pH 6.5 with MES[1] 2.0. Fluorescent tubes illuminated the isolated cells for 16 hr per day. SM was sometimes buffered to pH 5.5 with MES.

The outside medium (OM) resembled SM and contained (mM): $Ca(OH)_2$, 0.2; KOH, 0.2; NaOH, 0.4; sorbitol, 253; adjusted to pH 6.5 with buffer ~2.0. MES was used as the buffer for pH 4.5 to 6.5, MOPS for pH 7.0 and 7.2, HEPES for pH 7.8 and 8.0, TAPS for pH 8.5, and CHES for pH 9.5 in experiments in which pH was varied.

PM contained (mM): KOH, 120; Na-EGTA, 5.0; $MgSO_4$, 1.0; adjusted to pH 7.9 with MES, ~120. These concentrations were chosen so that $[Ca^{2+}]$ was less than 10^{-7} M (Williamson, 1975) and $[K^+]$ was comparable to that previously measured for the cytoplasm (Vorobiev, 1967; Tazawa, Kishimoto & Kikuyama, 1974).

The osmotic pressure of OM solution was adjusted to give incipient plasmolysis for each batch of cells: the osmotic pressure of PM was adjusted to give incipient "dimpling" of the perfused membrane bathed in OM. Dimpling resembles plasmolysis visually, but results from steady outward water flow passing by osmosis across the plasmalemma. The compositions of the media were chosen so that as far as possible the only permeant or transported ions present were K^+, Na^+ and H^+. The media were stored frozen and their pH was checked before each use. The K^+ activities of PM and OM were measured with an Orion K^+ specific ion electrode and a reference electrode consisting of a glass pipette with a tip diameter of 200 μm. It was filled with the test solution and was connected to a calomel half cell by an agar bridge with 3-M KCl. The electrode was calibrated with stock KCl solutions, in which the K^+ activity was taken to be equal to the tabulated KCl activity (Robinson & Stokes, 1955).

Using the measured K^+ activities (on a millimolar scale) of 0.25 ± 0.04 (3) and 65 ± 1 (4) for OM and PM, respectively, ψ_K was calculated as −140 mV. The K^+ concentration of OM was measured with a flame photometer and found to be 0.25 mM. The extraneous 0.05 mM K^+ was assumed to come from contamination.

ATP was obtained from Boehringer Mannheim. The stock solutions of ATP were prepared so that upon addition of an aliquot to PM, there was only a small change in the free $[Mg^{2+}]$ of 0.5 mM. The adenylate stock was added to PM to give final concentrations of (mM): Na_2 ATP, 2.0; ADP, 0.2; and K_2HPO_4, 3.0. The concentrations of ATP and ADP are comparable to those that are thought to be in *Chara* cells in vivo (Walker & Smith, 1975; Spanswick & Miller, 1977; Reid, 1980). The concentration of phosphate in *Chara* cytoplasm is uncertain and it has been argued that it may range to 30 mM (Reid, 1980). 3 mM phosphate was chosen with a view to inhibiting chloroplast activity through depletion of photosynthetic metabolites (Heber, 1974). ATP, ADP and PO_4^{3-} were added to fix the free energy of ATP hydrolysis (ΔG_{ATP}).

[1] *Abbreviations:* MES, 2[N-morpholino] ethanesulfonic acid; MOPS, morpholine propanesulfonic acid; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; TAPS, tris(hydroxymethyl)methylamino-propanesulfonic acid; CHES, 2[N-cyclohexylamino] ethanesulfonic acid; EGTA, ethyleneglycol-bis-(β-amino-ethylether)-N,N′-tetra-acetic acid; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine-3′-monophosphate; cyclic AMP, adenosine-3′,5′-cyclic monophosphate; AMP-PNP, β,γ-imido-adenosine-5′-triphosphate; CTP, cytidine 5′-triphosphate; UTP, uridine 5′-triphosphate; GTP, guanosine 5′-triphosphate; NADH, α-nicotinamide adenine dinucleotide; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl-urea.

Perfusion Chamber and Perfusion Method

The method of perfusion was similar to that described by Williamson (1975) with the addition that the center part of the cell was bathed in OM so that the electrical properties of that part of the plasmalemma could be measured.

An internodal cell was blotted dry and mounted on a perspex block (Fig. 1), so that the cell passed through three compartments. To ensure that solution and electric current could not leak from one compartment to the next, the cell was sealed at the junctions with silicone grease. A coverslip was placed over the central compartment to permit the observation of the streaming cytoplasm with a 100X oil-immersion objective. The portion of the cell in the central compartment was 3.7 mm long and had a surface area of 10 mm^2. One end of the cell was sealed into a plastic ring 12 mm high and 14 mm in diameter that had an opening in its base through which the cell could pass. The ring was filled with PM to a depth of 4 mm. Normally, the central compartment had OM flowing through, the volume being replaced once per minute (0.2 mm s^{-1}). When the composition of OM was changed during an experiment, the flow rate was increased temporarily to 7 mm s^{-1}.

After perfusion, small volumes of stock solution could be added to the PM in the plastic ring and mixed thoroughly by a stirrer that alternately sucked and expelled solution (Fig. 1). This stirrer also maintained a continual movement of PM within the cell as a result of the alternating pressure differences between the media in the two end compartments. This reduced to some extent the thickness of the unstirred layers within the cell.

Two sets of experiments were performed to investigate the effects of incipient plasmolysis on the electrical characteristics of intact cells. Internodal cells were pretreated for 5 to 15 days in SM of pH ranging from 6.0 to 9.5. First, using the method described by Walker (1960), microelectrodes were inserted into turgid cells. After ψ_M and g_M were measured, OM of the same pH and cation concentration replaced SM. After allowing sufficient time for the cell to reach incipient plasmolysis, ψ_M and g_M were again measured.

Second, measurements of ψ_M and g_M made with inserted microelectrodes were compared with those obtained from cells that were mounted on the perfusion block with the ends intact and in PM.

Electrical Techniques

To measure ψ_M, a glass pipette electrode of tip diameter 200 μm filled with OM was placed in the central compartment and another filled with PM was placed in the outflow end compartment. These electrodes were connected to calomel half cells by agar bridges with 3 M KCl. Current (i_c) was passed through electrodes consisting of vinyl tubing glued to glass tubes that contained Cl-coated Ag wire and were filled with 3 M KCl. The vinyl tubing of each current electrode was filled daily with OM or PM as appropriate (Fig. 1). The electrodes were filled in this way so that KCl solution did not leak into the bathing solutions.

The external electrode method of measuring ψ_M and g_M as used by other workers (Shimmen et al., 1976; Kikuyama et al., 1979) uses only two compartments and requires the subtraction of the series resistance for the cell sap and bathing solutions. This is not necessary in our perfusion system because there is no current flow between the two compartments from which the PD is measured (Fig. 1).

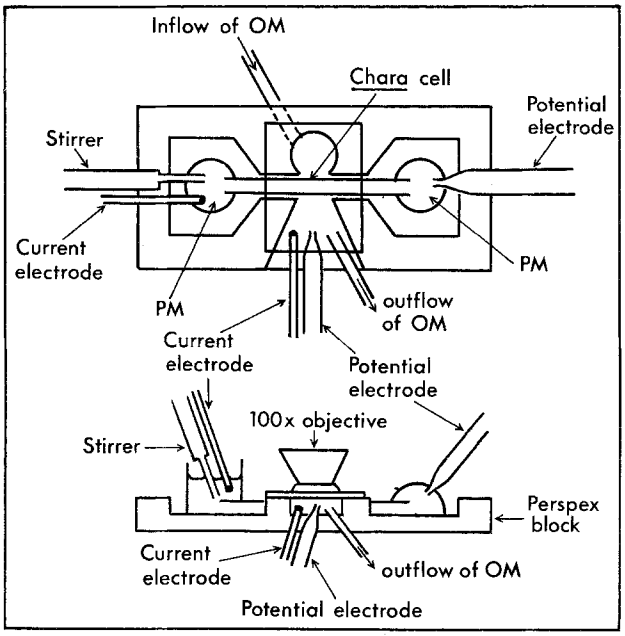

Fig. 1. The perfusion apparatus

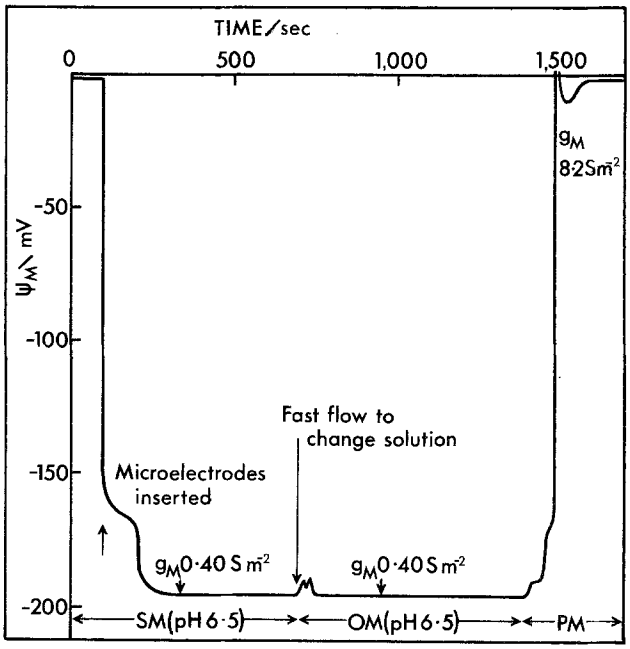

Fig. 2. Response of ψ_M to incipient plasmolysis caused by OM (pH 6.5) and pM. Values of ψ_M were measured with inserted microelectrodes

The electrical set up was similar to that already described (Walker, Beilby & Smith, 1979). It was used to measure ψ_M and either to record i/ψ curves by applying a current ramp or to clamp ψ_M at a desired value and record i/ψ curves by adding a train of voltage pulses to the clamp command voltage. The pulses were 300 msec wide, rising in amplitude from ± 6 to ± 90 mV in 30 sec. Recordings were taken in the second half of each pulse so that the transient current which charged the membrane capacitance was not recorded. The value of i_c was recorded as a function of ψ_M on an X-Y recorder and both were separately recorded as functions of time on a two-pen recorder.

The voltage-clamp circuit passed current from the current electrode in the end compartment, through the cell membrane from interior to outside, and then to the current electrode in the central compartment (Fig. 1). If the external circuit passed a positive current in this direction, it would cause ψ_M to become less negative. This is the direction of flow which we define as positive. Such a positive current, produced by the voltage clamp, resulted when the command was more positive than the resting membrane potential. A membrane current resulting from outward transport of positive ions would tend to hyperpolarize the membrane and would thus result in a positive clamp current flowing.

Results

Effects of Incipient Plasmolysis on ψ_M and g_M

Figure 2 shows that incipient plasmolysis, caused by exchanging SM (pH 6.5) with OM (pH 6.5), had no effect on ψ_M or g_M measured in intact cells with microelectrodes. Similar results were obtained using OM and SM with pH 6.0, 7.0, 7.8, 8.5 and 9.5. Replacing OM (pH 6.5) with PM depolarized the membrane and increased its g_M. For three cells, ψ_M went to -2 ± 1 mV and g_M increased to 7 ± 2 S m^{-2}. This value of g_M is of the same order of magnitude as the minimum estimated tonoplast conductance (Walker et al., 1979). This establishes that PM will reduce ψ_M to near zero, which is the basis of the experiments below.

Figure 3*A* and *B* illustrate the comparison of g_M and ψ_M, respectively, made on intact cells stabbed with microelectrodes and cells mounted on the perfusion block with external electrodes. There was no difference in ψ_M measured by the two techniques, except at pH 8.5. The g_M of cells mounted on the perfusion block at incipient plasmolysis was higher at most pH values than that for turgid intact cells.

For an intact plasmolyzed cell mounted in the perfusion block, the measured PD would be the sum of the PD's in the end and center compartments. Since the end compartment is bathed in PM, the PD in this compartment would contribute little to that measured by the electrometer. Similarly, the measured resistance would be the sum of the resistances of the parts of the cell membrane in the central and end compartments. The resistance in the central compartment is at least 20 times greater than that in the end compartment (calculated from Fig. 2 results). Consequently, the PD and conductance measured would be those of the membranes in the central compartment. After the ends and the tonoplast have been removed, g_M and ψ_M should accurately reflect the electrical characteristics of the plasmalemma in the central compartment.

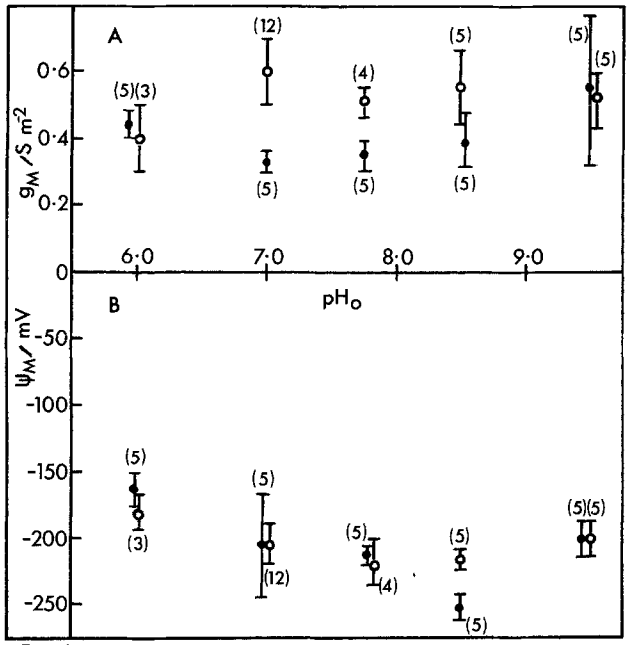

Fig. 3. Values of g_M (*A*) and ψ_M (*B*) for intact plasmolyzed cells measured with external electrodes (○) and intact turgid cells measured with inserted microelectrodes (●). Cells were pretreated for 5 to 15 days at the pH shown and electrical properties measured at that pH. The number of cells used is in parentheses

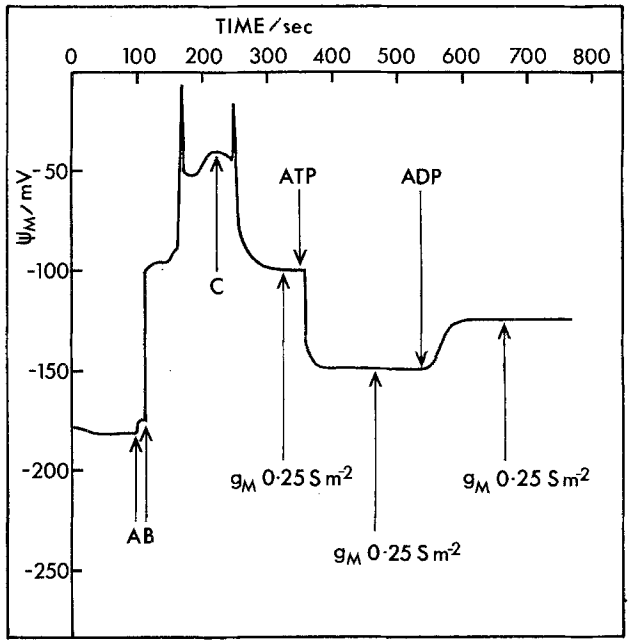

Fig. 4. A recording ψ_M of a cell during perfusion. The two ends were removed at *A* and *B*. Flow of vacuolar organelles was initially observed in the same direction as perfusion but in the opposite direction to cytoplasmic streaming. At *C* cytoplasmic organelles could be observed being swept along with the perfusion flow

Sequence of Events during Perfusion

Table 1. Effects of perfusion and ψ_c on i_c and g_M

	ψ_c/mV	i_c/mA m^{-2}	g_M/S m^{-2}
Intact cell	−180	+12.8±0.8 (111)	0.60±0.01 (112)
Perfused cell	−123±1 (94)	0	0.74±0.03 (86)
Perfused cell	−180	−20±1 (46)	0.28±0.01 (60)

Figure 4 illustrates the change in ψ_M during an early experiment (10th December, 1975) in which perfusion was rapid throughout. Routinely, the direction of perfusion was opposite to that of cytoplasmic streaming at the site of observation. After the ends of the cell were removed (Fig. 4, *A* and *B*), ψ_M became more positive while vacuolar bodies could be observed being swept with the perfusion flow as cytoplasmic streaming continued. After about 100 sec, the membrane commenced to repolarize (Fig. 4, *C*) and at the same time cytoplasmic organelles could first be observed being swept backwards by perfusion flow at the same level as cytoplasmic streaming. Streaming of organelles ceased within a further minute and ψ_M stabilized. The addition of ATP resulted in the commencement of organelle movement. At precisely the same time, ψ_M became more negative by 50 mV. ADP depolarized the membrane by 25 mV. Since a stirrer was not used, the adenylate concentrations are not well known.

In later experiments it was found that a higher success rate could be achieved by using an initial slow vacuolar perfusion followed by a short rapid perfusion that effectively disintegrated the tonoplast. The total time for perfusion was usually less than 500 sec.

The conclusion that the tonoplast is lost during perfusion is based on the movement of cytoplasmic organelles and on the electrical results. The tonoplast is thought to rupture or commence to disintegrate when organelles can first be observed; at the same level as organelle streaming, being swept backwards by the perfusion flow. The two-way flow of organelles at this level indicates a breakdown of the barrier between the cytoplasm and vacuole. At the same time, ψ_M starts to go more negative and shortly afterwards organelle streaming ceases completely. Only the flow of unattached organelles in the opposite direction to that of streaming could be observed. This flow of organelles could be precisely controlled by altering the pressure gradient on the PM. The perfusion of ATP causing hyperpolarization and commencement of organelle streaming is strong evidence that PM is in direct contact with the plasmalemma.

With successful perfusions the electrical properties of the plasmalemma remained in the normal range

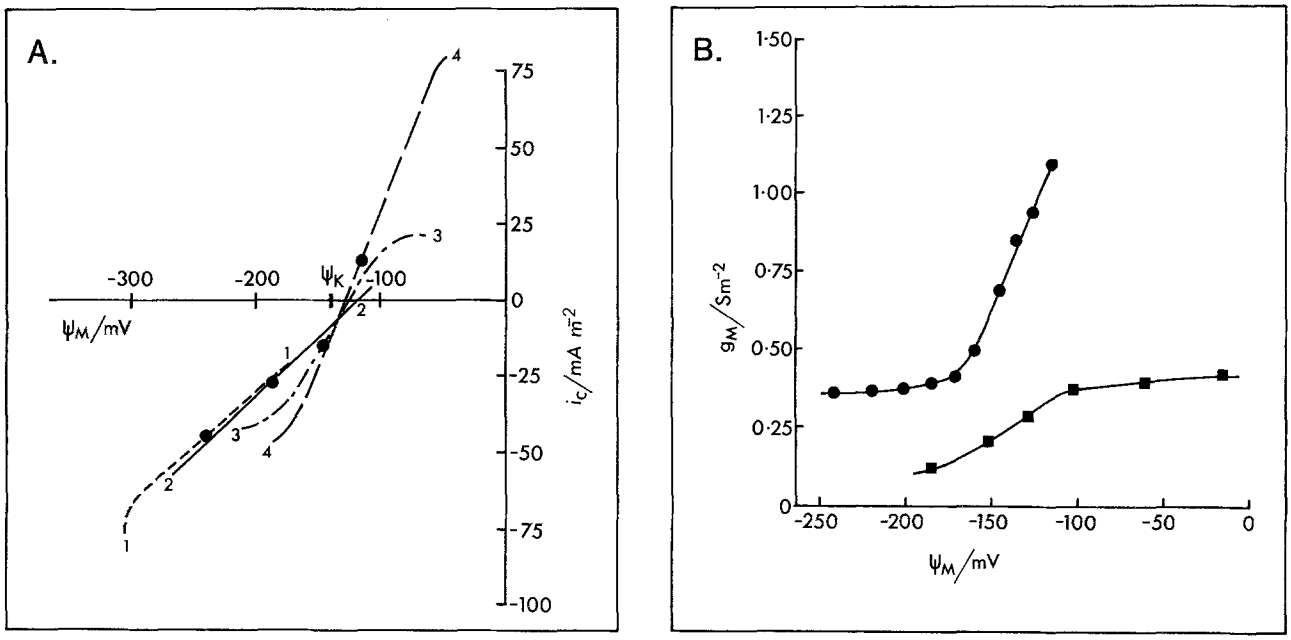

Fig. 5. (*A*): The i/ψ curves of a perfused cell clamped at (mV): −240 (*1*), −185 (*2*), −145 (*3*) and −115 (*4*). The i/ψ curves were recorded after i_c had reached a steady value. (*B*): Values of g_M for two perfused cells clamped at voltages from −240 to −115 mV (●) and −187 to −15 mV (■)

for up to several hours. An unexplained sudden rise in g_M, a depolarization or a large negative i_c was taken to indicate the rupture of the plasmalemma. Throughout the life of a successful preparation the chloroplasts remained in place and retained the microscopical appearance that characterizes those in normal whole cells.

Effect of Perfusion on the Plasmalemma

Before perfusion the cells had a resting potential of -201 ± 2 mV (111). The cells were voltage clamped at −180 mV before perfusion, not clamped during perfusion but were again clamped when ψ_M had stabilized at a constant value. Table 1 shows the values of g_M and i_c at these clamp potentials (ψ_c).

Time and Voltage-Dependent Conductance

The slope of the i/ψ curve (g_M) depended on ψ_c. This is illustrated in Fig. 5*A*, where the i/ψ curves for one cell show a progressive change of slope as ψ_c is varied. The dependence of slope on ψ_c is given in Fig. 5*B*. The curves in Fig. 5*A* all intersect at −138 mV, −8 mA m^{-2}.

The time-dependence of the effect of ψ_c on g_M has not been investigated; it is indicated by the data on i_c following the clamping of the perfused preparations at −180 mV (from a mean of −123 mV). There was a fall in i_c from −28 mA m^{-2} (46) to −20 mA m^{-2} (46), with a half-time of 85 ± 12 sec (46); this was accompanied by a fall in g_M.

This effect complicates studies of the effects of adenylates, inhibitors, etc; to avoid it we routinely measured the electric properties of the perfused preparations at the fixed value of −180 mV as well as at the resting potential without ATP (resting PD).

Effects of Adenylates on i_c and g_M

(a) ATP. In cells not voltage-clamped, the perfusion of ATP caused a hyperpolarization of the order of 50 mV and the commencement of organelle streaming, while the addition of ADP inhibited the ATP-induced hyperpolarization (Fig. 4).

In the following experiments, ψ_M was clamped at −180 mV. Addition of various concentrations of ATP to the PM produced changes in i_c of up to +20 mA m^{-2} (Fig. 6). Concentrations of ATP as low as 50 μM produced a change in i_c of $+7 \pm 2$ mA m^{-2} (3) at −180 mV and started slow organelle movement. At low concentrations of added ATP it is difficult to be sure of the ATP concentration in the layers adjacent to the plasmalemma, since chloroplasts, mitochondria, phosphatases and ATPases may alter the

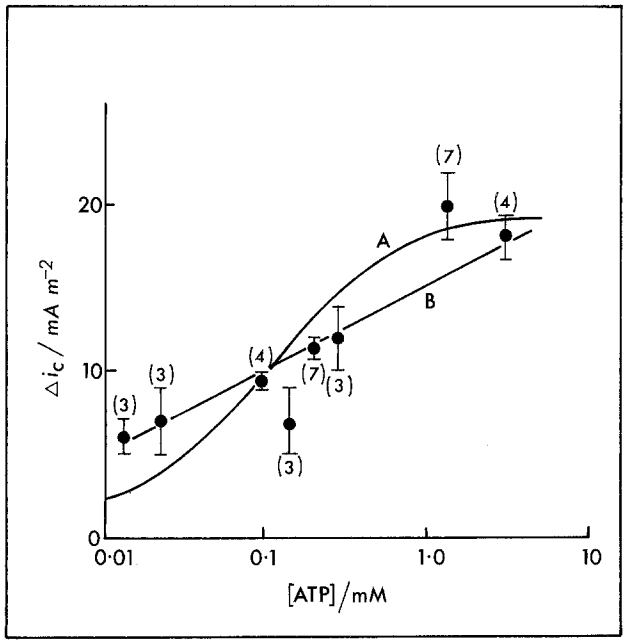

Fig. 6. The Δi_c at -180 mV caused by the perfusion of ATP. *B* is the line of best fit. *A* is the line derived from the Michaelis-Menton equation with K_M 0.1 mM and V_M 20 mA m^{-2}. The number of cells used is in parentheses

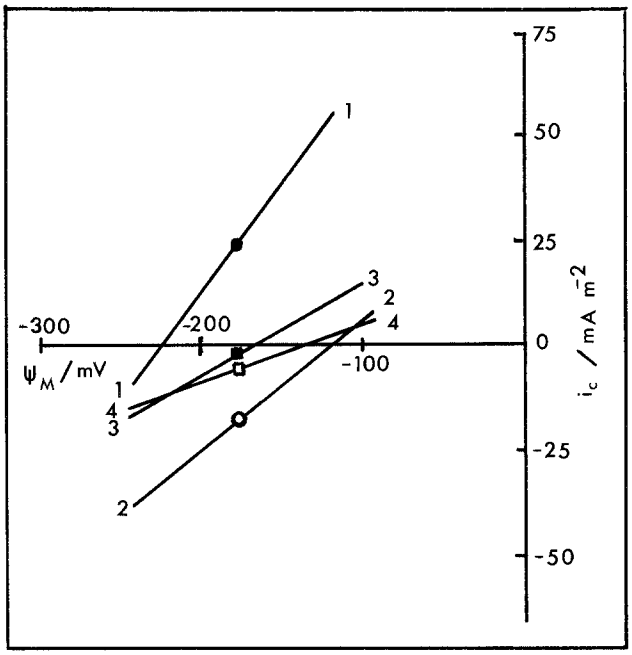

Fig. 7. The i/ψ curves of a *Chara* cell clamped at -180 mV before perfusion (*1*), after perfusion (*2*), +AS (*3*) and +AS+2 mM ADP (*4*). Symbols on the lines mark the clamp potential

ATP concentration. So 50 μM DCMU, a photosynthetic inhibitor, and 100 μM oligomycin, a mitochondrial ATPase inhibitor, were each perfused through five illuminated cells clamped at -180 mV before the addition of ATP. There was no measurable change in g_M or i_c in either case. The addition of 50 μM ATP caused a change in i_c of $+6\pm2$ mA m^{-2} in the presence of DCMU and $+5\pm3$ mA m^{-2} in the presence of oligomycin. These results are not significantly different from those where no inhibitor was used, indicating that the added adenylate concentrations are not significantly altered by organelle activity.

(b) ADP. After 2 mM ATP had been added, the addition of ADP at a concentration of 0.2 mM or less had no effect on i_c or g_M. 2.0 mM ADP inhibited the ATP effect on i_c by 50% and decreased g_M from 0.33 ± 0.02 S m^{-2} (34) to 0.24 ± 0.01 S m^{-2} (24). Further additions of ADP caused little change in i_c or g_M.

The addition of 2.0 mM ADP to cells clamped at -180 mV (without ATP) caused organelle movement to commence at a slower rate than seen with ATP. The value of i_c also changed by $+5\pm1$ mA m^{-2} (3). Further addition of ATP did not cause a change in i_c.

(c) Other Adenylates. NADH, CTP, UTP and GTP are present in plant cells and may supply energy for certain reactions. To test the effect of these nucleotides, the cells were clamped at -180 mV and 2 mM of each were perfused through the cells before ATP. None of these compounds caused a measurable change in g_M. NADH caused no change in i_c, while CTP, UTP and GTP caused changes of (mA m^{-2}): 0.3 ± 0.1 (4), 0.3 ± 0.1 (4) and 2.8 ± 0.8 (4), respectively. The addition of ATP after GTP caused a change in i_c of 12.0 ± 0.8 mA m^{-2} (4).

Neither cyclic AMP (30 μM) nor AMP (0.5 mM) had any effect on i_c or g_M of perfused preparations clamped at -180 mV, with or without ATP.

Effects of Adenylates on i/ψ Curves

Figures 7, 8 and 9 show comparisons of i/ψ curves of three cells in OM (pH 6.5) that were voltage clamped at -180 mV. Removal of the tonoplast caused a decrease in the slope of the i/ψ curve of each cell – from Curves *1* and *2*. Perfusion with PM containing adenylates (ATP, 2 mM; ADP, 0.2 mM; phosphate, 3 mM) – referred to subsequently as perfusion with AS – had a varied effect on the i/ψ curves – from Curves *2* and *3*. Figure 7 shows that AS caused a decrease in the slope of the i/ψ curve while Fig. 8 shows no change in the slope and Fig. 9 shows an increase in the slope. In each cell the addition of ADP caused a decrease in the slope of the i/ψ curve – from Curves *3* and *4*.

Intersection points for pump activated (Curve *3*) and pump inhibited (Curve *4*) i/ψ curves in Figs. 7,

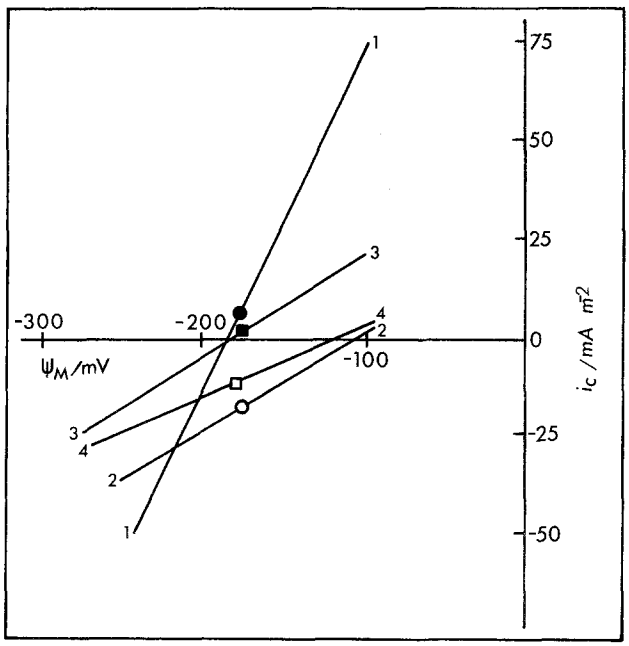

Fig. 8. The i/ψ curves of a *Chara* cell clamped at -180 mV before perfusion (*1*), after perfusion (*2*), +AS (*3*), and +AS+2 mM ADP (*4*). Symbols on the lines mark the clamp potential

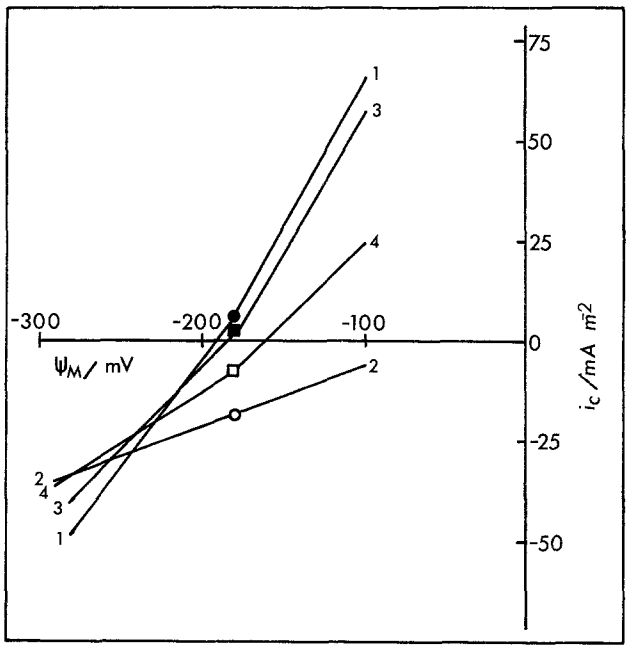

Fig. 9. The i/ψ curves of a *Chara* cell clamped at -180 mV before perfusion (*1*), after perfusion (*2*), +AS (*3*), and +AS+2 mM ADP (*4*). Symbols on the lines mark the clamp potential

Table 2. Effects of change in pH_o from 6.5 to 5.5 on i_c and g_M, at -123 mV

	i_c/mA m^{-2}	Δi_c/mA m^{-2}	g_M/S m^{-2}	Δg_M/S m^{-2}
Perfused	0 (24)	-1.7 ± 0.3 (8)	0.55 ± 0.02 (24)	0 (8)
Perfused+AS	20 ± 1 (24)	$-3\ \pm1$ (10)	0.48 ± 0.05 (24)	0.12 ± 0.05 (10)
Perfused+AS+2 mM ADP	8 ± 2 (4)	-0.5 ± 0.4 (4)	0.46 ± 0.05 (4)	0 (4)

Table 3. Effects of change in pH_o from 6.5 to 5.5 on i_c and g_M, at -180 mV

	i_c/mA m^{-2}	Δi_c/mA m^{-2}	g_M/S m^{-2}	Δg_M/S m
Perfused	-14.5 ± 0.4 (23)	-0.7 ± 0.2 (17)	0.22 ± 0.05 (23)	-0.01 ± 0.02 (17)
Perfused+AS	-0.5 ± 0.4 (20)	-2.5 ± 0.3 (17)	0.30 ± 0.06 (23)	-0.03 ± 0.03 (13)
Perfused+AS+2 mM ADP	-9.8 ± 0.5 (13)	-0.3 ± 0.1 (13)	0.24 ± 0.07 (13)	-0.02 ± 0.04 (13)

8 and 9 are (mV): -225, ca. -310 and -240, respectively. The mean intersection point with OM (pH 6.5) was -269 ± 5 mV (20) and with OM (pH 5.5) was -228 ± 7 mV (3).

Effects of pH_o

For perfused cells clamped at the resting PD (-123 ± 2 mV (35)), a change from pH_o 6.5 to 5.5 caused changes (Table 2) in i_c (Δi_c) and changes in g_M (Δg_M); the values of i_c and g_M before the change in pH are included in Table 2.

Figure 10 shows the dependence of i_c and g_M on pH_o of cells perfused with AS and clamped at the resting PD.

For perfused cells clamped at -180 mV in pH_o 6.5, initial values of i_c and g_M, and the changes caused by changing pH_o to 5.5 are shown in Table 3.

Figure 11 is an example of the effect of changes in pH_o on i_c for a perfused cell voltage clamped at -180 mV. A change in pH_o had a considerable effect on i_c only when the cell was perfused with AS. The addition of ADP inhibited this pH dependence.

Effects of External Cations

Figure 12 shows the dependence of g_M and i_c on $[K^+]_o$ for cells clamped at the resting PD (-128 ± 1 mV (14)). Changes in $[K^+]_o$ affected both i_c and

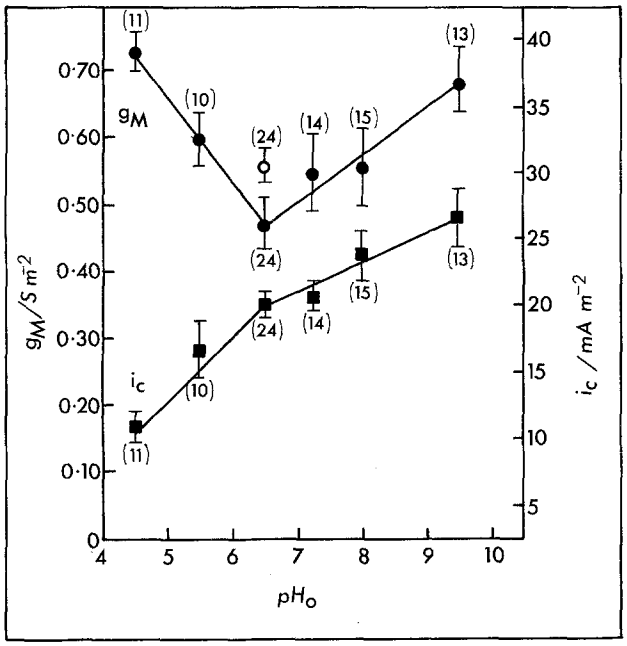

Fig. 10. Effect of pH$_o$ on i_c (■) and g_M (●) after perfusion with AS. Cells were clamped at the resting PD (−123±2 mV (24)) and the steady i_c and g_M recorded after a change from pH 6.5. The g_M before perfusion with AS is shown (○). The number of cells used is in parentheses

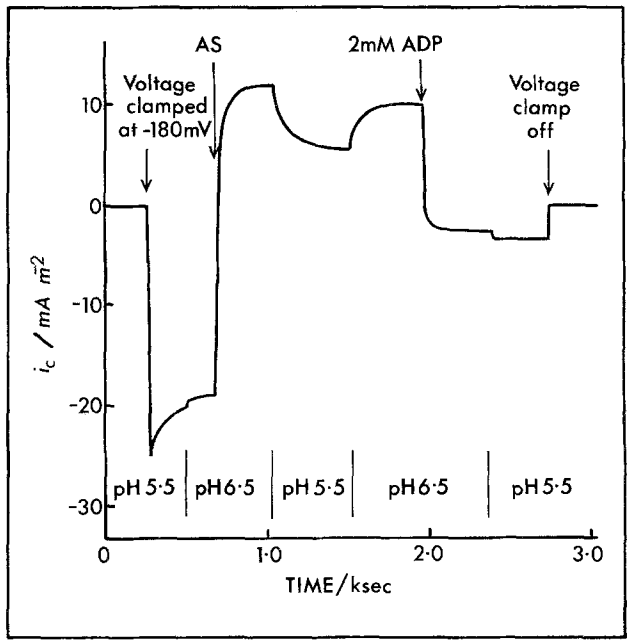

Fig. 11. Effect of adenylates and pH$_o$ on i_c of a perfused cell clamped at −180 mV

g_M more before perfusion with AS than after it. The initial values and changes caused by changes in $[K^+]_o$ from 0.05 to 1.5 mM are shown in Table 4.

A change in $[Na^+]_o$ from 0.4 to 1.4 mM, at the same resting PD with $[K^+]_o$ constant at 0.2 mM, caused the changes in g_M and i_c shown in Table 5.

A change in $[K^+]_o$ from 0.05 to 1.5 mM for cells voltage clamped at −180 mV caused a change in i_c from −16.1±0.6 mA m^{-2} [9] of −0.7±0.2 mA m^{-2} (9) and no measurable change in g_M. After the perfusion with AS, a change in $[K^+]_o$ from 0.05 to 1.5 mM caused no measurable change in g_M or i_c. A change in $[Na^+]_o$ from 0.4 to 1.4 mM, with $[K^+]_o$ constant at 0.2 mM, caused no measurable change in i_c or g_M.

Discussion

Methods of Measuring Electrical Properties

Experiments like those of Fig. 2 show that incipient plasmolysis, brought about by bathing the cells in OM, does not alter ψ_M or g_M. Further, the measurement of these quantities using external electrodes gave results only a little different from those obtained with microelectrodes inserted into intact cells (Fig. 3). The slight difference, that g_M is higher with external electrodes, is in the opposite direction from the difference found between the two methods by Tazawa, Kikuyama and Nakagawa (1975). Using *Nitella*, they showed

Table 4. Effects of change in $[K^+]_o$ from 0.05 to 1.5 mM, at −128 mV

	i_c/mA m^{-2}	Δi_c/mA m^{-2}	g_M/S m^{-2}	Δg_M/S m^{-2}
Perfused	0.9±0.3 (10)	−12±2 (10)	0.50±0.03 (10)	0.22±0.06 (10)
Perfused+AS	17.4±0.6 (6)	−3.0±0.5 (6)	0.38±0.01 (6)	0.08±0.04 (6)

Table 5. Effects of change in $[Na^+]_o$ from 0.4 to 1.4 mM, at −128 mV

	i_c/mA m^{-2}	Δi_c/mA m^{-2}	g_M/S m^{-2}	Δg_M/S m^{-2}
Perfused	0 (4)	−1.5±0.5 (4)	0.5±0.1 (4)	0 (4)
Perfused+AS	17±2 (4)	0 (4)	0.4±0.08 (4)	0 (4)

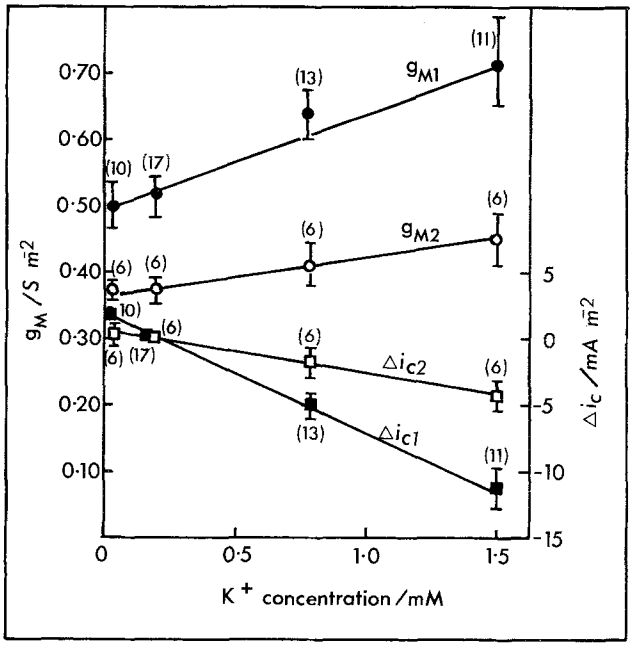

Fig. 12. Effect of $[K^+]$ on g_M (g_{M1} is without AS (●); g_{M2} is with AS (○)) and i_c (Δi_{c1} is without AS (■); Δi_{c2} is with AS (□)). The value of Δi_c is the difference in i_c caused by the change in $[K^+]$ from 0.2 mM. The cells were clamped at the resting PD (-128 ± 1 mV (14)) and the number of cells used is in parentheses

that g_M was higher with microelectrodes and concluded that this was the result of damage to the membrane. Our results do not confirm this, and we do not reach the conclusion of Tazawa et al. (1975) that the external electrode method is the better. It seems equally good, but has the disadvantage that the osmotic pressures of the external electrode solutions need to be accurately balanced to prevent water flow.

Condition of the Cell after Perfusion

(a) Without ATP. The electrical properties of perfused cells indicate that the plasmalemma remains intact after perfusion, retaining electrical characteristics comparable to those of the plasmalemma in vivo. Without ATP, ψ_M stabilizes at a value (-123 mV) not far from the estimated ψ_K. It is quite normal to find intact cells with values of ψ_M near this value – the depolarized state. The conductance of the perfused cell is also in the normal range for cells in this state (*see* Walker, 1960).

(b) With ATP. Perfusion with ATP caused hyperpolarization (Fig. 4). Values of i_c of cells perfused with AS and clamped at -180 mV were generally lower but in some cases similar to those required to clamp the cell at -180 mV before perfusion (Figs. 8, 9). The latter results indicate that ψ_M is maintained by similar mechanisms in the plasmalemma perfused with ATP and in the intact cell.

The slope of the i/ψ curve (g_M) of the intact cell was usually greater than that of the cell perfused with ATP; Curve *1* and Curve *3*, respectively, in Figs. 7 and 8. However, in a few cases the i/ψ curves were not very different (Fig. 9). The cause of this variability is not understood.

Other workers have also reported that g_M of the perfused plasmalemma with ATP is significantly lower than that of intact hyperpolarized cells (Shimmen et al., 1976; Tazawa et al., 1976; Shimmen & Tazawa, 1977; Kikuyama et al., 1979; Tazawa et al., 1979). They interpreted this as a change of the plasmalemma caused by perfusion with media containing EGTA; but there seems no particular reason to single out EGTA as the cause.

Passive Permeabilities and Resting Potential

Walker and Hope (1969) reported changes of i_c after a change of ψ_c, with half-times also of the order of one minute. These changes in whole *Chara* cells seem to be essentially similar to those reported here for perfused preparations. They argued that because of the change in transport number, a positive current flowing to the inside of a cell would cause a decrease in $[K^+]_o$ just outside the plasmalemma while a positive current flowing to the outside would cause an increase in $[K^+]$ just outside the plasmalemma; and that such changes in ion concentration produced the observed drifts in i_c. Our results do not agree with this interpretation.

First, if the drift in i_c and change in g_M were caused primarily by alteration of $[K^+]$ outside the membrane, this would imply that i_c and g_M were sensitive to $[K^+]_o$. This was not observed; without ATP, g_M and i_c are much less sensitive to $[K^+]_o$ at -180 mV than at -125 mV. This observation suggests that hyperpolarization reduces g_K.

Second, if the passage of current does significantly alter $[K^+]$ in the cell wall and/or the inner cytoplasm, the i/ψ curves should not intersect at a constant ψ_K, since ψ_K would depend on ψ_c. However, if the PD had a major effect only on g_K and did not alter ψ_K, the i/ψ curves would all intersect at the same PD. Figure 5*A* shows intersection at -138 mV, while the the average intersection for 83 cells was -137.5 mV. Both are so close to ψ_K (-140 mV) as to make it clear that the effect of ψ_c on the i/ψ curve is produced by a change in g_K while $[K^+]_o$ remains essentially constant.

It is notable that the i/ψ curves for high and low g_K do not intersect on the current axis, but on average at -6.4 mA m^{-2}; this current requires explanation.

An equivalent statement is that the resting PD of the perfused preparation (without ATP) is, at −123 mV, some 17 mV below ψ_K, at −140 mV. In the external medium are Na^+ and H^+, which might enter by uniport, providing some or all of this depolarization. Current changes caused by a change of external concentration can be used to calculate permeability ratios, if we assume that:

$$\Delta i_c \propto P_j \Delta c_j \quad (1)$$

where the change in i_c (Δi_c) is produced by the change in concentration (Δc_j) of ion j. On this basis we find $P_{Na}/P_K(\alpha)$ at −128 mV to be 0.18 and $P_H/P_K(\beta)$ at −123 mV to be 72. These values can be used to predict a resting PD of −132 mV with the following equation (Hodgkin & Katz, 1949):

$$F\psi = RT \ln\{([K^+]_o + \alpha[Na^+]_o + \beta[H^+]_o)/ ([K^+]_i + \alpha[Na^+]_i + \beta[H^+]_i)\}. \quad (2)$$

Thus a further depolarization of 9 mV requires explanation; this is equivalent to an unidentified current of ca. 3–4 mA m^{-2} at ψ_K. It is possible that this represents an outward current of MES^- anion, which would mean a value of $P_{MES}/P_K(\gamma)$ of 1.2×10^{-3}, if we calculate γ from the Goldman equation:

$$F\psi = RT \ln\{([K^+]_o + \alpha[Na^+]_o + \beta[H^+]_o + \gamma[MES^-]_i)/ ([K^+]_i + \alpha[Na^+]_i + \beta[H^+]_i)\}. \quad (3)$$

We cannot at present attribute this current to MES^- with any certainty; efflux of $EGTA^{2-}$ or SO_4^{2-}, or influx of Ca^{2+}, may also play some part in the depolarization. The fact that Eq. (2) generally fits the resting potential of intact (depolarized) cells suggests that the unidentified current may be peculiar to perfused preparations: clearly both MES^- and $EGTA^{2-}$ are still possible candidates.

The values found here for α and β can be compared with earlier values: $\alpha = 0.06$ (Hope & Walker, 1961); $\beta = 25$ (Richards & Hope, 1974). If these lower values are inserted into Eq. (2), the predicted resting PD is −136.6 mV and the unidentified current is rather larger.

The dependence of g_K on ψ_M is consistent with, and helps to explain, the results of Hope (1965) on *Chara* and of Findlay (*see* Walker, 1980) on *Hydrodictyon*. The implication is that the permeability to K^+ is reduced as the cell hyperpolarizes to the PD characteristic of the proton pump, and increases as the cell depolarizes towards ψ_K when the pump is inhibited. It is not, however, consistent with the results of voltage-clamp studies of K^+ fluxes by Walker and Hope (1969) on *Chara* but is consistent with those of Richards and Hope (1974) on *Chara* and Kitasato (1968) on *Nitella*.

The perfusion of ATP at −123 mV increased the dependence of i_c and g_M on pH_o and at the same time reduced the dependence on $[K^+]_o$. Under these conditions g_M also consistently decreased by ca. 0.15 S m^{-2} upon perfusion with ATP (Fig. 12) although a current of 17 mA m^{-2} was required to maintain ψ_M at the resting PD. (Figure 7 is an example of a cell at −180 mV that showed a similar decrease in g_M upon perfusion with ATP.) The situation with ATP present is less easy to understand, but the result that the dependence of the resting PD on $[K^+]_o$ is decreased by the perfusion of ATP suggests that either the activity of the electrogenic mechanism, or ATP itself, influenced g_K.

Effects of Adenylates on the Putative Proton Pump

The effects of pH on ψ_M in whole cells have been taken as evidence that the electrogenic mechanism is a proton pump (Kitasato, 1968; Spanswick, 1972; Saito & Senda, 1974). This is supported by the fact that measured fluxes of other ions are not of a sufficient order of magnitude to account for the current attributed to the electrogenic mechanism (Richards & Hope, 1974; Spanswick, 1972, 1974). Our result that the ATP-induced current is strongly pH dependent supports this hypothesis.

The specificity of the proton pump for ATP is shown by the small effect of the other nucleotides UTP, GTP and CTP on i_c and g_M. Shimmen and Tazawa (1977) have also reported that an ATP analog, AMP-PNP did not cause hyperpolarization.

We have shown that low concentrations of ATP are sufficient to drive the proton pump at a reduced rate. Shimmen and Tazawa (1977) found that an estimated ATP concentration of 40 to 50 μM was sufficient to hyperpolarize the membrane.

The inhibition by ADP of the effects of ATP on the proton pump (Figs. 7, 8, 9, 11) has not been previously shown. The way ADP does this is not yet clear. It may be by competition for the ATP binding site, by decreasing the free energy available from ATP hydrolysis or by an allosteric effect of ADP on the proton pump. It does suggest that the relative concentrations of ATP and ADP in *Chara* cells *in vivo* may control the activity of the proton pump and consequently ψ_M. This is discussed further in *Stoichiometry of the Proton Pump*.

The Conductance of the Proton Pump

Changes in g_M caused by ATP are not simply related to the corresponding changes in i_c. Figure 13 shows

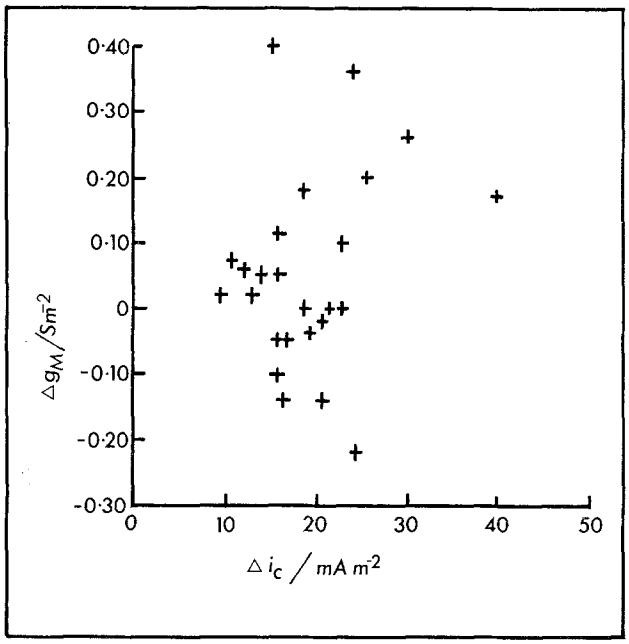

Fig. 13. The change in g_M (Δg_M) and in i_c (Δi_c) caused by perfusion with AS. The cells were clamped at −180 mV

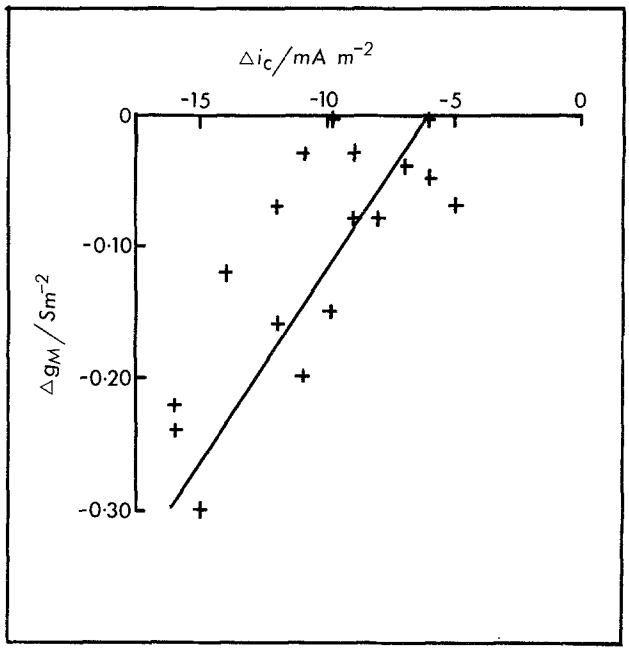

Fig. 14. The change in g_M (Δg_M) and in i_c (Δi_c) caused by adding 2 mM ADP to cells perfused with AS

that the perfusion of AS through cells clamped at −180 mV could cause an increase in g_M of up to 0.40 S m^{-2} or a decrease of up to 0.22 S m^{-2} for the same change in i_c (+20 mA m^{-2}). ATP may cause changes in g_M by two mechanisms: an addition of a pump conductance and a fall in a passive conductance. The overall effect on g_M may then be either an increase or decrease, depending on the relative magnitudes of each change.

For the same cells as those that contribute to Fig. 13, there is a strong connection between changes in g_M and in i_c caused by the addition of 2.0 mM ADP to the ATP-containing PM (Fig. 14); the regression coefficient is 0.82. Thus a relationship exists between the change in g_M and the change in i_c when the proton pump is inhibited by ADP. The decrease in pump conductance caused by ADP is interpreted as larger than any corresponding increase in passive conductance.

Richards and Hope (1974) reported that experiments with inhibitors, pH_o and low temperature do not suggest that the proton pump makes a major contribution to total g_M in *Chara*, although they agree with Kitasato (1968) that in *Nitella* proton conductance may account for most of g_M. Keifer and Spanswick (1978), in another study of inhibitors, interpreted the large decreases in g_M caused by CCCP, DCCD, DES and DNP as reductions of the proton pump conductance.

Shimmen and Tazawa (1977) found that replacement of their perfusion medium containing Mg-ATP with ATP-depleted medium caused no concurrent change in g_M with ψ_M. It has also been reported (Fujii et al., 1979) that decreasing the ATP concentration to 1 μM, by perfusion with hexokinase, instantaneously depolarizes the membrane but does not initially alter g_M. With time g_M was found to decrease to ca. 50% of the initial value. They interpreted this as the turning off of a pump with a small conductance. But since the cells were not clamped, the conclusions drawn from these results, and the results for the effects of inhibitors described above, are affected by voltage-dependent g_M changes and changes in g_M which may depend on pump activity.

Stoichiometry of the Proton Pump

The ψ_M at which the proton pump would stall (ψ_p) is given by:

$$F\psi_p = \Delta G_{ATP}/n + RT \ln ([H^+]_o/[H^+]_i \quad (4)$$

where n is the number of protons pumped outwards per ATP hydrolyzed (Spanswick, 1974).

Equation (4) allows us to calculate the values of ψ_p, using the values of Alberty (1972) for ΔG_{ATP} (−53.4 kJ mol^{-1} with AS and −47.8 kJ mol^{-1} with AS +2.0 mM ADP) (Table 6).

If the pump current can be shown to go to zero, and reverse, as ψ_m goes to a particular value ψ_o, and beyond, then we could identify ψ_o with ψ_p in Eq.

Table 6. Calculated values of ψ_p for different values of pH_o and [ADP]

	pH_o 5.5		pH_o 6.5	
	0.2 mM ADP	2.0 mM ADP	0.2 mM ADP	2.0 mM ADP
$n=1$	−414 mV	−356 mV	−471 mV	−412 mV
$n=2$	−137 mV	−105 mV	−196 mV	−164 mV

(4), and use our knowledge of the other variables to find n. A real reversal of pump current will always indicate a thermodynamic stalling, independently of possible allosteric regulating effects of ATP (Spanswick, 1980) or ADP; but an observation of low pump current may result from such a kinetic effect and should be viewed with caution. As we shall see, the interpretation of actual intersections of current-voltage curves in terms of pump reversal is made more difficult by the possibility of allosteric effects.

Originally it was hoped to obtain clear evidence of ψ_p from i/ψ curves obtained with and without ATP; Curves *2* and *3* of Figs. 7, 8 and 9 would intersect at ψ_p if the only effect of ATP were to allow the proton pump to operate, whether or not it also effects allosteric regulation. However we have both direct and indirect evidence that ATP reduces the passive conductance of the membrane (including g_K), so that the intersection of Curves *2* and *3* is hard to interpret. It is in any case very variable.

We can, however, get some information from the i/ψ Curves *3* and *4* in Figs. 7, 8 and 9; they represent the conditions of ADP=0.2 mM and 2.0 mM, respectively, and have a lower slope at the higher ADP concentration. Intersection points between Curves *3* and *4* occur at −269 mV with pH_o 6.5 and −228 with pH_o 5.5. If the only important change caused by the increase in ADP concentration is on the operation of the pump whether this effect is thermodynamic or allosteric, we can discuss this result in terms of what it means for the i/ψ curves of the proton pump. Such hypothetical curves are shown in Fig. 15*A*. They were constructed using values of the current caused by ATP at −123 mV, the value of ψ_p for $n=1$, and the experimentally determined intersection point for OM (pH 6.5). It can be seen that, as the i/ψ curves are continued to their respective values of ψ_p, they must intersect again at another ψ_M. Such a result would imply an allosteric rather than a thermodynamic effect of ADP. However, it seems unlikely since the i/ψ curves with adenylates are usually linear, and since it implies that in some range of ψ_M the proton current would be greater for the cell with the higher concentration of ADP. The double intersection of the curves for [ADP] 0.2 and 2.0 mM need not be postulated if $n=2$, because in this case the calculated values of ψ_p are more positive than the observed inter-

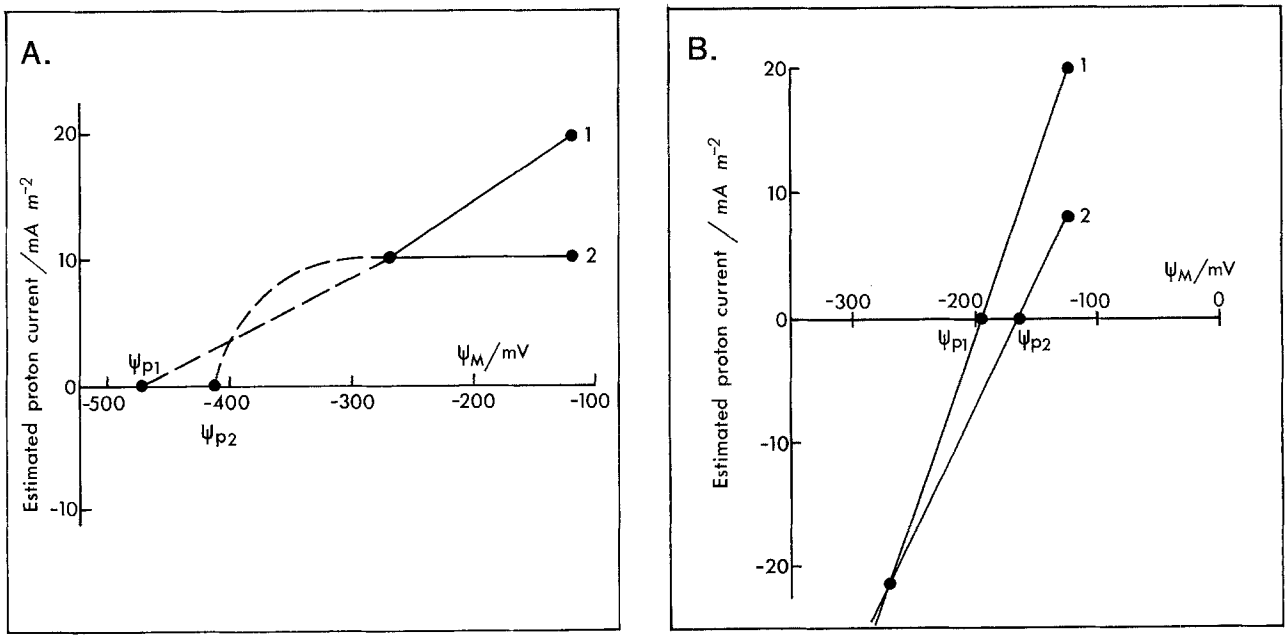

Fig. 15. The estimated proton current of perfused cells in OM (pH 6.5) derived from the change in i_c caused by ATP, plotted against ψ_M. The imposed condition is that curve *1* (+AS) and curve *2* (+AS+2.0 mM ADP) intersect at −269 mV. For a proton pump with $n=1$ (15*A*) curve *1* crosses the ψ_M axis at −471 mV (ψ_{P_1}) and curve *2* crosses at −412 mV (ψ_{P_2}). For a proton pump with $n=2$ (15*B*) curve *1* crosses the ψ_M axis at −196 mV (ψ_{P_1}) and curve *2* crosses at −164 mV (ψ_{P_2})

Table 7. Experimentally determined limits on ψ_p and n

pH_o	Limits on ψ_p/mV		Limits on n	
	Upper	Lower	Upper	Lower
5.5	−170	−228	1.8	1.5
6.5	−178	−269	2.1	1.6

section point (Fig. 15*B*). This argument means that it is much easier to accept values of ψ_p that are more positive than −269 mV at pH_o 6.5; at pH_o 5.5 the corresponding limit on ψ_p is −228 mV.

We have also the observations of clamp current at $\psi_c = -180$ mV, from which approximate resting PD's can be calculated (Table 3). The approximate values are −178 mV at pH_o 6.5 and −170 mV at pH_o 5.5. In each case the resting PD is expected to lie between ψ_K (−140 mV) and ψ_p, so that the value of ψ_p should be more negative than the resting PD.

Our observations can thus set plausible limits on ψ_p and hence on n, as shown in Table 7.

Although substantial unstirred layers must exist in the experiments described here, both the flux of H^+ and the rates of consumption and production of ATP and ADP should be small near ψ_p: we do not believe that these effects substantially affect our values of ψ_p or consequently our estimation of n. Nor, as has been pointed out above, do we think the estimates of ψ_p or of n are affected by allosteric regulation. We thus regard our experiment as indicating that the value of n lies between 1.5 and 2.0, where clearly these limits are not very precisely known.

For intact *Chara* cells, Walker and Smith (1975) found that ψ_M is close to ψ_p for a two-proton pump, between pH_o 4.5 and 7.5. From this they suggested that there is a proton pump with a stoichiometry of two, running close to equilibrium. Given that we found that the i/ψ curve for the intact cell usually had a higher slope than that of the perfused preparation, it is not impossible – if a fractional value of n is admitted – that in the whole cell it is closer to 2.0 than in perfused one. Such a difference in slope could be explained on this basis.

Slayman and Gradmann (1975) postulated that the proton pump of the fungus *Neurospora* behaves as an ideal current source, providing a constant H^+ efflux at all values of ψ_M in the range −300 to −50 mV. Later studies of i/ψ curves (Gradmann et al., 1978; Warncke & Slayman, 1980) indicate that the *Neurospora* proton pump usually has a stoichiometric ratio close to one, but under certain conditions, such as severe energy restriction, the pump stoichiometry changes to the more efficient value of 2 H^+ transported per ATP hydrolyzed.

Conclusions

The perfused plasmalemma of *Chara*, in the absence of ATP, supports a membrane PD and conductance similar to those of depolarized intact cells; this PD results from the passive diffusion of K^+, Na^+ and H^+ with permeabilities in the ratios 1:0.18:72, together with at least one other ion. The conductance due to K^+ depends on membrane PD and time, the steady value increasing as ψ_M becomes more positive, in the range −160 to −120 mV (for $[K^+]_o$ equal to 0.25 mM).

The perfusion of the plasmalemma with a solution containing ATP causes a hyperpolarizing current, presumed to be produced by a proton pump. The membrane PD is then similar to that of an intact cell, but the conductance is usually much lower. This effect of ATP is inhibited by 50% by 2 mM ADP.

The stoichiometry of the proton pump is difficult to determine at present from i/ψ curves because of the apparent effects of ATP or pump activity on passive permeabilities; the present results lead to values of n between about 2.0 and 1.5.

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