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Electrical Characteristics of the Tonoplast of *Chara corallina*: A Study Using Permeabilised Cells

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Use of permeabilised cells of *Chara corallina* provides a unique opportunity to study the electrical characteristics of the tonoplast whilst being able to control ionic conditions on the outside of the membrane. Current-voltage (I/V) analysis over wide voltage spans, and admittance measurements at 5 Hz showed that many permeabilised cells had a similar conductance and capacitance to the tonoplast of intact cells. Cells developed two regions of negative-slope conductance upon addition of external Cl^-, which suggests the existence of potential-dependent Cl^- channels in the *Chara* tonoplast. With Cl^- concentrations similar to those expected in vivo, the resting potential was more sensitive to changes in external K^+ than Cl^-; however, a decrease in external K^+ did not significantly alter the shape of the I/V relation.

Key words: Admittance — Cl^- channel — Charophyte — Current-voltage (I/V) — Membrane capacitance — Voltage clamp.

Ion movements across the tonoplast are important for several reasons, such as the maintenance of cytoplasmic homeostasis and the movement of storage molecules (e.g. MacRobbie 1979). However, there has been very little study made on the movement of ions across the tonoplast, due largely to its inaccessibility and the difficulty with which changes in ionic composition can be made on each side of the membrane.

There has been some direct study on the electrical properties of the tonoplast of giant algal cells (mainly charophytes) by the insertion of micropipettes into the cytoplasm and vacuole of intact cells. The conductance of the tonoplast has been measured in giant algal cells by many workers (Table 1), and is usually higher than that of the plasmalemma, but the degree of this difference varies greatly. The tonoplast capacitance has only been occasionally determined (Table 2, which includes measurements from higher plants), but also seems to be higher than that of the plasmalemma, by a factor of about three to five.

A small number of I/V curves of the tonoplast have been published, but most curves have been linear (Coster 1969, Findlay et al. 1969, Findlay 1970, Bentrup et al. 1985, 1986). All but one (Bentrup et al. 1985, 1986) of these studies have been obtained using a current rather than a voltage clamp; this is unfortunate, as use of the former method means that much information on channel opening an closing is lost (e.g. Hille 1984). Hedrich et al. (1986) have also published an I/V curve (by voltage clamping) of the tonoplast of isolated barley mesophyll vacuoles, which

Abbreviations: APW, artificial pond water; C, capacitance; EGTA, ethylene glycol-bis(2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; G, conductance; I/V, current-voltage; PD, potential difference; TEA, tetraethylammonium.

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shows strong inward rectification, and a very low G around the reversal potential. However, they expose both surfaces of the tonoplast to unusual ionic conditions, including 1 mM Ca^{2+} (normally submicromolar in the cytoplasm). Similarly, Bentrup et al. (1985, 1986), who clamped the tonoplast PD of isolated vacuoles of *Chenopodium rubrum* included 30 μm EGTA in their patch pipette which was exposed to the inside of the vacuole; this would reduce greatly the Ca^{2+} concentration in the vacuole, where several mM Ca^{2+} is usual (e.g. Hoagland and Davis 1929, Macklon 1984); this could well cause unusual results. Voltages spanned for all curves have been small (i.e. across less than 100 mV), and this also reduces the usefulness of the curves. The narrow voltage spans have been at least partly due to so-called "punchthrough" (Coster 1969), where the application of higher currents cannot further polarise the PD of the cell; this has been described at both positive and negative potentials (Coster 1969, Findlay et al. 1969).

Table 1 Previously measured tonoplast conductances and resting potentials in giant celled algae

Species	G ($S \cdot m^{-2}$)	Resting P. D. (mV)	Reference
Chara corallina (syn. *C. australis*)	10	+10 to +20	Findlay and Hope 1964
	1.5	—	Skierczynska 1968
	approx. 11	+10	Coster 1969
	5.5 to 7	+10 to +20	Coster and Smith 1977
	6.5	—	Smith 1983
	3	−4	Moriyasu et al. 1984b
(cytoplasmic droplets[a])	14.4	−4	Homble 1987
Chara inflata	2.8 to 6.5	—	Tyerman et al. 1986
Chara vulgaris and *C. rudis* (cytoplasmic droplets)	6.3 to 9.1	+5 to +20	Prishchepov et al. 1981
Nitella sp.	at least 3	—	Walker 1960
(cytoplasmic droplets)	4.4 to 15.6	—	Inoue et al. 1973
	3 to 10	—	Kobatake et al. 1975
N. flexilis	3.3	+50	Yurin et al. 1976
	2.1	+11	Yurin et al. 1979a
	2.7	+16	Yurin et al. 1979b
	1 to 2	+10 to +25	Bobrov et al. 1980
(cytoplasmic droplets)	11 to 25	+20 to +50	Inoue et al. 1971, Takenaka et al. 1975
N. translucens	1.2	—	Spanswick and Costerton 1967
	1.7	+22	Spanswick 1970
Nitellopsis obtusa	1.0	+19	Findlay 1970
	40	—	Bernhardt and Pauly 1974
	435[b]	—	Skierczynska et al. 1977
Chaetomorpha darwinii	1.4 to 2.0	+43 to +77	Findlay et al. 1971
Griffithsia spp.	1.4 to 2.5	+20 to +60	Findlay et al. 1969

[a] The membrane of cytoplasmic droplets is believed to consist primarily of tonoplast (Sakano and Tazawa 1986, Luhring 1986).

[b] But 0.94 without the "corrections" (see Smith 1983) they apply.

Table 2 Previously measured tonoplast capacitances

Species	C ($mF \cdot m^{-2}$)	Method of measurement	Reference
Chara corallina	29 to 54	Sine waves of frequency 1 to 100 Hz	Coster and Smith 1977
	60	5 Hz sine waves	Smith 1983
Nitella sp. (cytoplasmic droplets)	12.5	Sine waves of frequency 60 to 10^5 Hz	Miyake et al. 1973
Nitella flexilis (cytoplasmic droplets)	10	—	Inoue et al. 1971, Takenaka et al. 1975
Nitellopsis obtusa	28	Current pulse analysis	Findlay 1970
	21	Maxwell-Wagner analysis of tonoplast and plasmalemma in series	Bernhardt and Pauly 1974
Beta vulgaris	17	Current pulse analysis	Korzun et al. 1984
Chenopodium rubrum	8	Current pulse analysis	Bentrup et al. 1986

There has also been some work on the I/V characteristics of characean cytoplasmic droplets, the membrane of which is believed to be primarily tonoplast (Sakano and Tazawa 1986, Luhring 1986). Essentially linear I/V relations have also ben described across this membrane in studies with current clamps causing only small voltage shifts (Inoue et al. 1973, Takenaka et al. 1975). However, Prishchepov et al. (1984) clamped the PD between +20 and −220 mV, and made numerous solution changes outside and inside the droplet. Steady-state I/V curves had three regions of negative-slope G, which were attributed to gating characteristics of Cl^- and K^+ channels. At rest, G was predominantly due to K^+, and was inhibited by TEA, a result also confirmed in recent work by Homble (1987). In the work of Prishchepov et al., however, most solution changes were made on the vacuolar side of the tonoplast. A major advantage of using the permeabilised cells described in this paper is that ionic and osmotic conditions can easily be controlled on the cytoplasmic side of the tonoplast, which is probably where the cell exerts more control in vivo.

Access to the tonoplast of the characean cells is gained using a technique developed recently by Shimmen and Tazawa (1983), where the plasmalemma of the cells is made irreversibly and highly permeable to small molecules and ions. The technique has been mainly used to study the control of cytoplasmic streaming (Shimmen and Tazawa 1983, Tominaga et al. 1983, 1985a, b), but it has not been used previously to study the electrophysiology of the tonoplast. Briefly, permeabilisation is effected by removing Ca^{2+} from outside the plasmamembrane of a lightly plasmolysed cell at low temperature. Meanwhile, the tonoplast, which is normally exposed to zero turgor and submicromolar concentrations of Ca^{2+}, remains semi-permeable and the vacuole can retain neutral red for several hours. The tonoplast retains the characteristic H^+-pumping Mg^{2+}-ATPase and the H^+-pumping Mg^{2+}-PPiase activities (Shimmen and MacRobbie 1987a, b); the usefulness of the system for transport studies has been assessed elsewhere (Tester et al. in press). The capacitance was measured using computerised techniques, and a refined I/V analysis was used to investigate transport characteristics of the tonoplast (Beilby and Beilby 1983); the effects of K^+ and Cl^- concentrations on the I/V characteristics are described.

Materials and Methods

Permeabilisation—Young leaf cells (3 to 5 mm long) of *Chara corallina* were cut and then allowed

Table 3 Final concentrations (mM) of chemicals used for permeabilising cells

	"Low Cl^-"	"High Cl^-"	"Low K^+"	"Low pH"
EGTA	0.1	0.1	0.1	0.1
HEPES	30	30	40	30
MES	90	30	0	118
KOH	105	55	22	105
$MgCl_2$	2	2	2	2
KCl	8	58	8	8
NaCl	2	2	2	2
pH	7.6	7.6	7.6	6.6

to stand overnight in APW (1 mM NaCl, 0.1 mM KCl, 0.5 mM $CaCl_2$, 2 mM HEPES/NaOH, pH 7.5). Old cells could not be permeabilised using this method. Cells were permeabilised in ice-cooled solutions containing either 14 mM Cl^- ("low Cl^-") or 64 mM Cl^- ("high Cl^-": see Table 3). Changes upon addition of Cl^- were not due to a 'salt effect' (i.e. a combined effect of both the added anion and cation), as solutions were made by replacing KMES with KCl, with no consequent change in the concentration of K^+. Similarly, solutions of lower K^+ or lower pH were also made (Table 3). In interpretation of results, it is assumed that there is no significant effect of MES^- on membrane conductance.

Usually, 90 mM sorbitol was added to the low and high Cl^- solutions, and 160 mM sorbitol to the low K^+ solution; these values were varied, however, depending upon the age and condition of the culture. If the osmolarity was too high, cells showed large splits in the lines of chloroplasts and the tonoplast appeared to become leaky; with too low an osmolarity, cells did not

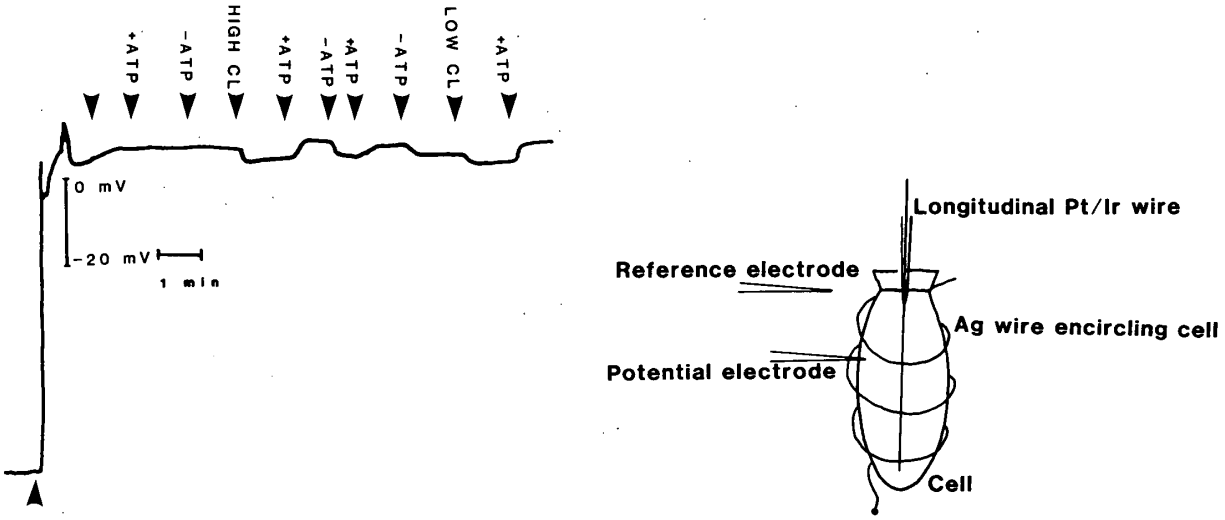

Fig. 1*a* The depolarisation of a cell of *Chara corallina* in APW upon addition of ice-cold low Cl^- permeabilising medium, added at the first arrow; cell was then returned to room temperature (second arrow) and solutions changed as indicated, showing PD changes upon addition and removal of ATP, and upon changes in Cl^- concentration. Vertical bar refers to potentials to right of bar; to left, bar length is equivalent to 50 mV. Horizontal bar represents one minute, with time moving from left to right. *b* Diagram showing the arrangement used for electrical measurements.

plasmolyse and hence did not permeabilise. Cells of *C. corallina* did not require pretreatment to be successfully permeabilised, unlike the cells of *Nitella* spp used by Shimmen and Tazawa (1983).

Upon addition of permeabilisation medium, the cells depolarised rapidly, often having an action potential (see Fig. 1a). Cytoplasmic streaming stopped (Shimmen and Tazawa 1983), and the regular arrangement of chloroplasts became disrupted (see Shimmen and Tazawa, 1983). After checking for cessation of streaming, either 1.0 mM K_2ATP or 0.2 mM Na_2PP_i was added; if the solutions were at room temperature, streaming recovered.

All cells were illuminated by a fibre optics source (Intralux H150 Volpi) at a light intensity of 50–100 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Solutions were flowed at a constant rate during an experiment, at approximately 10 $ml \cdot min^{-1}$; the volume of the chamber was about 1 ml.

Electrical measurements—Cells were space-clamped by the insertion of a Pt/Ir wire along the axis of the intact cell; the PD measuring electrode was inserted into the vacuole (see Fig. 1b for geometry). Electrodes were inserted when the cell was in APW, and the cell was permeabilised after the membrane potential had recovered to at least −180 mV. As the permeabilised cell is turgorless, manipulations and electrode insertions had to be done with the intact cell. The space clamp was essential as the distortion due to the cable properties of the cell tends to linearise the I/V curves (Smith 1984), especially when clamping the high G tonoplast, whose cable length would be much shorter than that of the plasmalemma.

Most experiments were done with the cell kept in ice-cooled solutions (Fig. 2 and 4 to 7), but in later experiments the temperature of solutions was increased to room temperature after permeabilisation (Fig. 1a and 3). As described in Beilby and Beilby (1983), the voltage clamp was controlled by a Minc 11 computer and the data logging provided a time resolution of 2 ms. The I/V curves were obtained from a bipolar staircase of voltage clamp commands, with

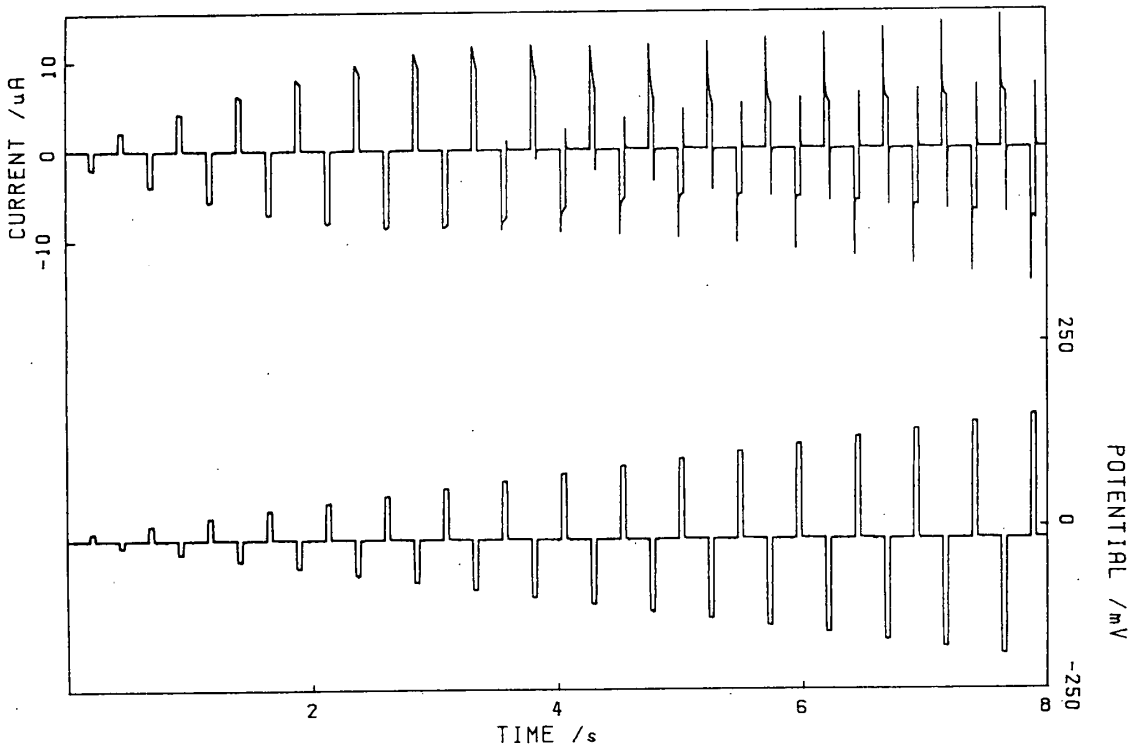

Fig. 2 A typical bipolar staircase of voltage clamp commands (lower trace), with resulting current (upper trace). The cell is in ice cold high Cl^- permeabilising solution without ATP or PPi; cell surface area 18 mm^2. Note the inductive behaviour at low potential displacements, and capacitive later on.

pulsewidths of 40 ms, and 200 ms between pulses (Fig. 2). For small departures of potential from the resting PD, the current reached a pseudosteady-state level, so the calculation of G at the reversal potential by differentiation of the I/V curve would have been accurate. However, for larger potential shifts, the current was still changing at the end of the pulse (Fig. 2), so the I/V curve, while still providing a valid qualitative description of channel kinetics, will not be quantitatively exact.

The tonoplast was clamped up to spans from −200 to +200 mV (e.g. Fig. 5 and 7); beyond these voltages, sharp inward and outward rectifications respectively were seen. Conductance was estimated by several methods: from the slopes at I=0 of I/V curves; by differentiation of polynomial-fitted I/V curves; or from admittance measurements at 5 Hz (Beilby and Beilby 1983). Capacitance at 5 Hz was obtained as described in Beilby and Beilby (1983); the frequency was the same as that used by Smith (1983). Averaged values are followed by the standard error of the mean and the number of replicates.

It was also possible to obtain an I/V curve of the tonoplast in the intact cell by placing one glass electrode in the cytoplasm and the other in the vacuole with the current-injecting electrode, although, of course, the ionic and osmotic conditions on the cytoplasmic side of the tonoplast could not be controlled.

Results

I/V curves of intact cells—The running of an I/V curve on the tonoplast in the intact cell was usually fatal. However, a few curves were obtained (e.g. Fig. 3), and these were generally quite linear; some showed inward rectification at negative potentials- at around −60 mV in the example presented. The conductance varied between cells, from 5 to 20 $S \cdot m^{-2}$, and the resting PD from +20 to +40 mV.

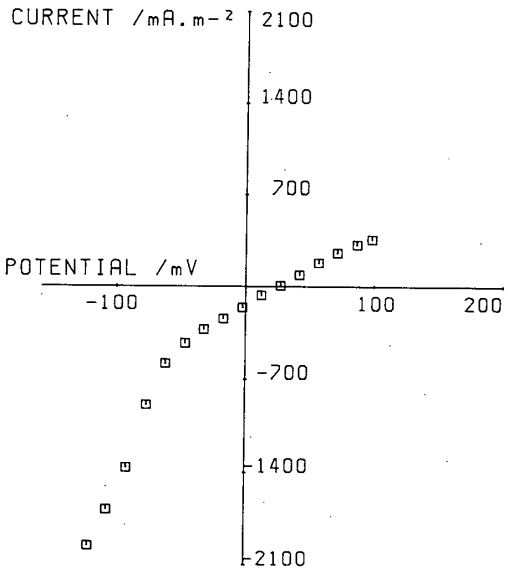

Fig. 3

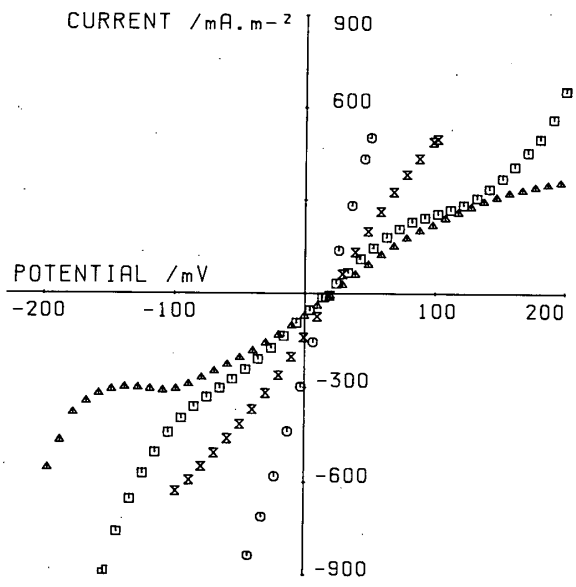

Fig. 4

Fig. 3 I/V curve of the tonoplast of an intact cell of *Chara corallina* in APW at room temperature. G=5.1 $S \cdot m^{-2}$; reversal potential=+20 mV.

Fig. 4 The time dependence of I/V profiles of a permeabilised cell in ice cold low Cl^- solution. I/V curves of a cell of *Chara corallina* in low Cl^- permeabilising medium 6 (○; G=15 $S \cdot m^{-2}$), 15 (⧖; G=6.8 $S \cdot m^{-2}$), 20 (△; G=3.2 $S \cdot m^{-2}$) and 40 (□; G=4.1 $S \cdot m^{-2}$) minutes after permeabilisation. Reversal potential=+18 mV for all curves.

Resting potential difference of permeabilised cells—After permeabilisation, the PD of the vacuole with respect to the external solution was always near 0 mV, with most cells having a small positive PD (e.g. Fig. 1a). The resting PD was equal to the reversal potential on each I/V curve (Fig. 4 to 8); these values are between −2 and +18 mV (average of +7.0±3.2 mV, n=11). The PD tended to decay towards 0 mV over several minutes, as would be expected if the PD was due to electrogenic pumps and the ATP and PP_i were washed away upon permeabilisation. Decay of the PD towards zero could be slowed if ATP was applied immediately after permeabilisation. However, addition of ATP did not always cause a hyperpolarisation of membrane PD, even in cells returned to room temperature after permeabilisation. In Fig. 1a is a trace from one cell which showed a clear change in PD, of approximately 4 mV upon addition and removal of ATP, although not for a few minutes immediately after permeabilisation when the cell was very conductive (see below).

Conductance of permeabilised cells—Conductance was estimated from the slopes at I=0 of 11 I/V curves (from the highest and lowest G from four cells with variable G; and the average G from three cells with a relatively constant, higher G), and from admittance measurements on five cells. Conductance of the permeabilised cells was quite variable, ranging from 1 to 49 $S \cdot m^{-2}$. Although this variability was often due to intrinsic and as yet unknown properties of individual cells, a wide range of conductances in the same cell were also found, depending on the time after permeabilisation and ionic conditions in the external medium. Immediately after permeabilisation, most cells were very conductive (G about 30 $S \cdot m^{-2}$). In at least half the cells, G stayed high, and these cells were discarded; in other cells G spontaneously decreased within a couple of minutes (Fig. 4), and responded to changes in the external medium. After some time,

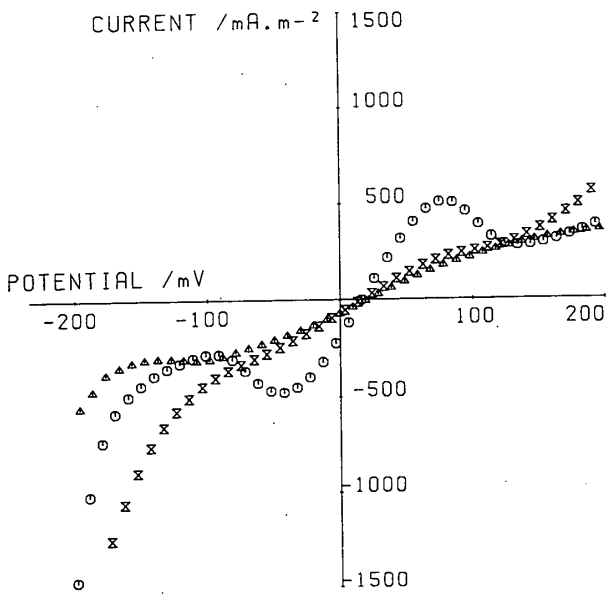

Fig. 5

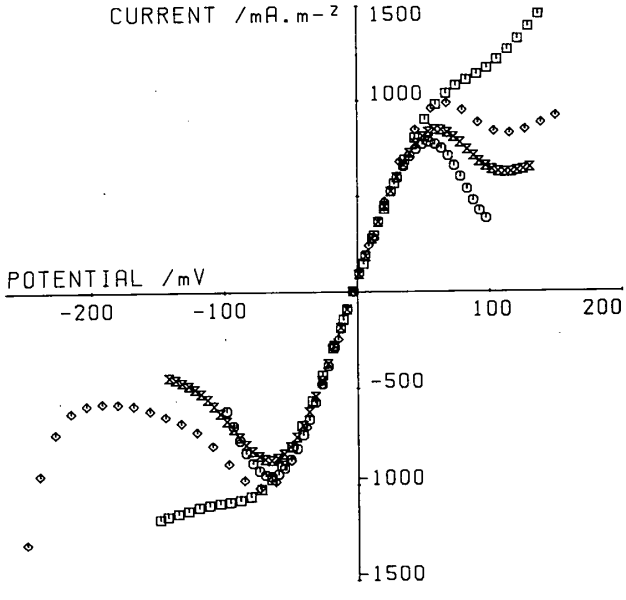

Fig. 6

Fig. 5 I/V curves of a permeabilised cell of *Chara corallina* in high Cl^- (64 mM: ○, G=20 $S \cdot m^{-2}$) and low Cl^- (14 mM: before ⧖, G=4.0 $S \cdot m^{-2}$ and after △, G=3.1 $S \cdot m^{-2}$ the high Cl^- treatment) permeabilising solutions. All were ice cold solutions. Reversal potential=+16 mV for all curves.

Fig. 6 The time dependence of I/V profiles of a permeabilised cell in high Cl^-. I/V curves of a permeabilised cell of *Chara corallina* in ice cold high Cl^- permeabilising medium 17 (○), 24 (⧖), 31 (◇) and 49 (□) minutes after permeabilisation; G=19 $S \cdot m^{-2}$, reversal potential=−2 mV. First curve in presence of ATP, and last three in the absence of ATP; ATP did not appear to affect significantly the curves, and it is not required for the regions of negative-slope conductances to be formed. The effect of ATP on the rate of change of these regions is unknown.

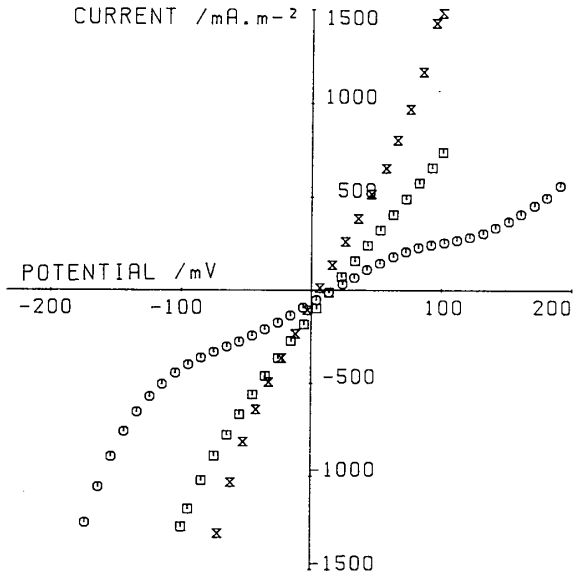

Fig. 7 I/V curves of a permeabilised cell of *Chara corallina* in low K^+ (30 mM: ⧖, $G=13\ S\cdot m^{-2}$, reversal potential $=+4$ mV) and high K^+ (113 mM: ○, before, $G=3.7\ S\cdot m^{-2}$, and □, after the low K^+ treatment, $G=8.8\ S\cdot m^{-2}$, both reversal potentials $=+14$ mV) permeabilising solutions. All curves with cell in ice-cold solutions with 14 mM Cl^-.

depending upon the cell and the size of currents passed across the tonoplast, G increased again towards its initial values, and the cell was discarded. Other changes of G with time are discussed below. A continued high G in many cells is clearly a problem, and the reason for this is unknown.

Nevertheless, in cells in which the G did decrease, the conductance was similar to that of an intact cell, especially when the concentration of ions in the external medium approached that of the cytoplasm. When the concentration of Cl^- was 14 mM, in all low conductance cells G was between 1 and 15 $S\cdot m^{-2}$, and in over half these, G was between 1 to 8 $S\cdot m^{-2}$. This was lower than G at 64 mM Cl^- (see below).

Addition of ATP or PP_i appeared to have little effect on the I/V relations of the permeabilised cell. No hyperpolarisation has yet been observed upon addition of PP_i, although this has only been done to date at low temperature. Reducing pH by one unit (Table 3) had little effect on PD, perhaps depolarising the tonoplast by about 0.5 mV (results not shown).

Shapes of I/V curves of permeabilised cells—Changing the concentration of Cl^- in the permeabilising medium significantly altered the characteristics of the I/V curve. When the cells were in the low Cl^- solution, the I/V profile was essentially linear, at least between -150 and $+150$ mV (Fig. 5), but when in the high Cl^- solution, an I/V curve with two regions of negative-slope G was found (Fig. 5 and 6). The conductance of the tonoplast increased when the solution was changed from low to high Cl^-, and the membrane usually hyperpolarised by a couple of mV. Interestingly, if the solution was changed immediately after permeabilisation, the reverse change in PD was seen (Fig. 1a). All the Cl^- effects were reversible (Fig. 3 and 5). The effect of high Cl^- on the I/V relation changed with time, the slope of the negative-slope G region decreasing with time; however, the voltage over which the negative-slope G occurs does not appear to change (Fig. 6). The average voltage spans over which the negative-slope G are found are -95 to -45 mV and $+80$ to $+135$ mV. The significant time factors make the averaging of curves difficult, so only example curves are presented for the moment.

Reduction in the concentration of K^+, from 113 to 30 mM whilst maintaining the Cl^- concentration at 14 mM depolarised the tonoplast by about 10 mV. The conductance increased with reduced K^+ (Fig. 7).

Action potential—When cells in low Cl^- solutions were clamped for eight seconds to potentials up to ± 50 mV, there was little change in G with time, and no indication of excitability.

Capacitance—The capacitance at 5 Hz of the tonoplast varied between 3.6 and 109 $mF\cdot m^{-2}$;

the average C was $46.2 \pm 5.9\ mF \cdot m^{-2}$ (n=13). This is similar to a value made by us on a tonoplast of an intact cell (the same cell from which the I/V curve in Fig. 3 was obtained), of $36\ mF \cdot m^{-2}$. Most measurements on permeabilised cells were done on high G cells (av. $G = 35\ S \cdot m^{-2}$), and more measurements need to be done with low G cells. Nevertheless, these results suggest that, even in these high G cells, there is a membrane whose capacitive properties do not appear to be significantly different from those of the tonoplast of the intact cell.

Capacitive spikes of current were usually seen on the staircases run for I/V curves, but in some staircases, either all or some of the current responses were inductive (e.g. Fig. 2).

Discussion

I/V curves of intact cells—It was possible to obtain an I/V curve of the tonoplast in the intact cell, but the clamping regime was usually fatal. This may be the consequence of the highly conductive tonoplast, where the ionic currents may alter the composition of the cytoplasm (see also Beilby and Blatt 1986), or it may be because of an electrical breakdown of the plasmalemma due to a large current flow across this membrane (Lunevsky et al. 1983); however, quite large currents can be passed across the plasmalemma of single membrane samples of *Chara corallina* (Beilby and Blatt 1986). The most likely reason for cell death could be because, in the intact cells, the plasmalemma PD is not clamped (only the tonoplast is; there is one electrode in the vacuole and one in the cytoplasm), and the high currents imposed will polarise the plasmalemma to well over 1 V, a PD certainly large enough to break down the membrane.

In any case, a much wider voltage span for clamping was possible with the permeabilised system than in the intact cell; the voltage range found consistently possible with the cells used in these experiments was from -200 mV to $+200$ mV. Beyond these voltages, sharp rectifications were observed; Korzun et al. (1984) described a membrane breakdown of isolated beet vacuoles with voltage displacements of 150 to 200 mV, Luhring (1986) and Homble et al. (1987) also have described irreversible breakdown of patches of cytoplasmic droplet membranes with voltage displacements of up to 100 mV.

Resting potential difference of permeabilised cells—The average resting PD of the permeabilised cells was small and positive, as has often been found across the tonoplast of intact cells (e.g. Table 1). The PD was generally smaller than that measured by us across the tonoplast of intact cells (cf Fig. 3), but the significance of this difference is not known.

Only small hyperpolarisations to a more positive PD were observed upon addition of ATP to the permeabilised cell. However, this would be expected, due to the high G of the tonoplast. Although activation of the H^+-ATPase leads to an increased membrane current of approximately $100\ mA \cdot m^{-2}$ at the plasmalemma (e.g. Beilby 1984, Takeuchi et al. 1985), the pump current for the tonoplast may be only about $20\ mA \cdot m^{-2}$ (Bentrup et al. 1986, Moriyasu et al. 1984a). This would produce a hyperpolarisation of 2 mV with a total membrane G of $10\ S \cdot m^{-2}$, and of only 0.7 mV with $G = 30\ S \cdot m^{-2}$.

Conductance and I/V curves of permeabilised cells—The average G of permeabilised cells measured in this study are slightly higher than those measured for the tonoplast by other authors (Table 1), but the values obtained by us were certainly not unreasonable. It is clear that the tonoplast of the permeabilised cell can be clamped over a wide voltage range with the techniques described here, and that I/V curves can provide some features in common with those of cytoplasmic droplets (Prishchepov et al. 1984). In this preliminary report, several featues of the I/V curves have been described. The most interesting is the response to changes in the concentration of Cl^- in the permeabilising medium. An increase in the concentration of Cl^- induces the appearance, or at least large stimulation of, regions of negative-slope conductance. A similar shaped relation has been found for the K^+ channel of the *Chara* plasmalemma (Beilby 1986). In both systems, the

regions of negative-slope conductance are not due to membrane excitation, as the baseline current between voltage pulses always returns to zero (see Fig. 2).

If both regions of negative-slope G in the tonoplast I/V curve are due to the gating characteristics of the same channel (i.e. opening, then closing), then a simple consequence of Ohm's Law is that the reversal potential of the major permeant ion to the channel should be between the two regions of negative-slope G (e.g. Finkelstein and Mauro 1977). The voltage range between the two regions of negative-slope G appears to be from around -95 mV to $+135$ mV. This is consistent with the movement of Cl^-, K^+ or possibly Na^+, depending on the concentration of vacuolar Na^+ (e.g. compare Sakano and Tazawa 1984 with Tazawa et al. 1974). Due to the large Cl^- effect, it seems likely that the channnels observed are Cl^- channels. Other workers have proposed these for the tonoplast of charophytes (Berestovskii et al. 1976, Lunevsky et al. 1983), gating during the tonoplast action potential; also, Kikuyama and Tazawa (1976) found a large effect of vacuolar Cl^- concentration on resting PD between the vacuole and outside, suggesting the existence of open Cl^- channels at rest.

However, Prishchepov et al. (1984) and Reeves et al. (1985) found the resting PD across cytoplasmic droplet membranes responded more to changes in K^+ than Cl^- on the vacuolar side of the membrane, as we have found in this work with solution changes on the cytoplasmic side of the membrane. Prishchepov et al. (1984) found three areas of negative-slope G in the I/V relation across the membrane around a cytoplasmic droplet, between -45 to -66 mV, -100 to -118 mV and $+46$ to $+69$ mV. Of these, only the first overlaps with those presented here, and they found that was inhibited by TEA, which, however, was applied at high concentrations (40 mM; the accompanying anion is unknown). Their second area of negative-slope G, at more negative potentials than the one found in this study, was inhibited by reduced concentrations of Cl^- on the vacuolar side of the tonoplast (i.e. 3 mM rather than 133 mM). This is similar to the Cl^- effect described here. Both K^+ and Cl^- channels have also been tentatively identified in patch clamp studies of cytoplasmic droplets (Tyerman et al. in press).

It is possible that when the cell is in low Cl^- solutions, K^+ conductance dominates the tonoplast G, and when Cl^- is increased in the permeabilising solution, the potential dependent Cl^- channels dominate the tonoplast G. It should be noted here that the so-called "low Cl^-" solutions contain 14 mM Cl^-, which is probably slightly higher than the Cl^- activity in the cytoplasm of the normal intact *Chara* cell (e.g. Coster 1966, Jones and Walker 1980, Coleman 1986). So, in vivo, K^+ could dominate the tonoplast conductance. This is borne out by the sensitivity of the PD of the tonoplast of permeabilised cells to changes in external K^+ (but compare the results of Kikuyama and Tazawa 1976).

There remains the problem of why the shape of the tonoplast I/V curve did not change very much with changes in external K^+. It is possible that the K^+ channels are not potential dependent, but this would be unlikely if the outer membrane of cytoplasmic droplets do originate from the tonoplast, as patch clamp studies show PD dependence of the K^+ channels in the droplet membrane (Luhring 1986, Homble et al. 1987, Laver and Walker 1987). It is possible that the change in K^+ concentration did not cause a large enough change in the K^+ reversal potential for regions of negative-slope G to develop (see, for example, Finkelstein and Mauro 1977). More extensive studies clearly need to be done.

The patch clamp study by Laver on cytoplasmic droplets shows that K^+ channels have a maximum G near $+100$ mV ("vacuole" with respect to cytoplasm), and close at negative potentials and potentials more positive than $+150$ mV. This suggests more ready movement of K^+ from the vacuole to the cytoplasm, and could, incidentally, provide a mechanism for depolarisation back to the resting potential during the action potential. Luhring (1986) and Homble et al. (1987) described similar rectification for high unitary conductance K^+ channels in the membrane of cytoplasmic droplets. This does not correlate to the behaviour of the I/V curves

described here, but explains remarkably well the whole cell I/V curves of K^+ channels in the plasmalemma (Beilby 1985, 1986). It is possible that the cytoplasmic droplet contains significant amounts of plasmalemma, as suggested by Andrianov et al. (1983); or perhaps the K^+ channels in both the plasmalemma and tonoplast have similar gating characteristics. Kolb et al. (1987) found strong inward rectification (i.e. K^+ flow mainly from the cytoplasm to the vacuole) of K^+ channels in isolated barley vacuoles, which is opposite behaviour to that found in cytoplasmic droplets.

Action potential—Although the tonoplast of intact cells can be stimulated by small potential shifts (Lunevsky et al. 1983), in the permeabilised cells there was no sign of excitability, at least with the techniques described here. This may be due to a change in the properties of the tonoplast during permeabilisation, or may indicate that a rise in cytoplasmic Ca^{2+} is required for excitation at the tonoplast (cf. Lunevsky et al. 1983, Kikuyama 1986), which may not occur fluxes in the permeabilised system due to the large volume of the bathing solution and Ca^{2+} buffering capacity by EGTA.

Capacitance—The average C of 45 $mF \cdot m^{-2}$ is similar to values measured by other workers (Table 2). Even in the high G cells used in these experiments, there is a membrane whose capacitive properties do not appear to be significantly different from those of the tonoplast of the intact cell. The appearance of an inductive response during the running of a voltage-clamp staircase is similar to that occurring in the plasmalemma of cells in the "K-state" (Beilby 1985). However, interpretation of such a phenomenon must await further refinements of the techniques so the opening and closing kinetics of the channels can be investigated.

Conclusions—The permeabilised cell is a useful system to study tonoplast transport, as membrane identification is much more certain than that of vesicles or cytoplasmic droplets, and the tonoplast is maintained in a more natural environment than with isolated vacuoles, as most cytoplasmic molecules would be retained by the cell wall (in contrast to turgid cells of *Chara* (Kiyosawa 1975), the walls of plasmolysed cells of various higher plants are reported to be permeable to molecules up to only about 1,500 Da: Carpita et al. 1979). It is hoped to combine the technique of permeabilisation with that of vacuolar perfusion to allow control of ionic activities on both sides of the tonoplast (Shimmen and MacRobbie 1987a, b), and hence study in more detail ion movements across the tonoplast.

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