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The electrophysiology of salt tolerance in charophytes

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Abstract — *Lamprothamnium succinctum* is a salt-tolerant charophyte that inhabits non-marine saline environments. *Chara australis* is a salt-sensitive charophyte that inhabits fresh waters. Electrophysiological responses of internodal cells from these species to increased Na^+ concentration in the medium are modeled and compared. A greater background conductance in high Na^+ media is found in both charophytes. *Lamprothamnium* is able to maintain a highly negative resting potential difference (PD) by increasing the rate of proton pumping. *Chara* cells, faced with high Na^+ medium, respond in more varied ways. However, even when it occurs, the increased pumping fails to prevent depolarization of the membrane PD from –243±15 mV (5 cells), to –188 ±15 mV (5 cells), over an average 65 min high Na^+ medium exposure. Furthermore, the proton pump becomes very sensitive to both depolarization and hyperpolarization imposed on the membrane by voltage clamp. Such pump inhibition impedes recovery of the negative resting PD after voltage or a spontaneous action potential. The significance of the small differences in one transporter rendering the plant salt-tolerant is discussed in the light of evolutionary adaptations.

charophytes / salt-tolerance / electrophysiology / current-voltage modeling / evolution

Résumé — **Electrophysiologie de la tolérance au sel des charophytes.** *Lamprothamnium succinctum*, charophyte tolérante au seul, croît dans des environnement salins non marins. *Chara australis,* charophyte sensible au sel, vit en eau douce. Les réponses électro-physiologiques des cellules inter nodales de ces espèces à une augmentation de la teneur en Na^+ de leur milieu sont modélisées et comparées. Les deux charophytes présentent une plus forte conductance de base dans les milieux riches en Na^+. *Lamprothamnium* est capable de maintenir une différence de potentiel (PD) au repos hautement négative par augmentation du taux de protons pompés. Les cellules de *Chara*, dans un milieu à forte teneur en Na^+ répondent de façons plus variées. Cependant, même quant elle se produit, l'augmentation du pompage ne prévient pas la dépolarisation de la PD membranaire de – 243 ± 15 mV (5 cellules) à – 188 ± 15 mV (5 cellules), au delà d'un temps d'exposition moyen de 64 mn dans un milieu à forte teneur en Na^+. De plus, la pompe à protons devient très sensible à la fois à la dépolarisation et à l'hyperpolarisation imposées à la membrane par la pression du voltage. Une telle inhibition de la pompe entrave le rétablissement de la PD négative au repos après l'application d'une tension ou d'un potentiel d'action spontanée. La signification des petites différences dans un transporteur rendant la plante tolérante au sel est discutée à la lumière des adaptations évolutives.

Charophytes / électrophysiologie / évolution / modélisation du couple intensité-tension / tolérance au sel

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INTRODUCTION

For over a hundred years morphological studies have suggested that there is a close evolutionary relationship between land plants, the Charales, and Coleochaetales. An explosion in molecular systematics over the past twenty years or so has now situated land plants phylogenetically within the Charophyta (Karol *et al.*, 2001). The mitochondrial genome of *Chara vulgaris* is strikingly similar to that of the liverwort *Marchantia* (Turmel *et al.*, 2003), and the living Charales are regarded as sisters to land plants (Lewis & McCourt, 2004; McCourt *et al.*, 2004; Turmel *et al.*, 2003).

The salt-tolerant *Lamprothamnium* and salt-sensitive *Chara* have a similar underlying electrophysiological "machinery": ion pumps, symporters, voltage-gated as well as calcium-activated ion channels (for review see Findlay, 2001), and stretch-activated (SA) channels. In *Lamprothamnium* the SA channels are thought to initiate the turgor response to osmotic pressures established during hypotonic challenge (for review see Shepherd *et al.*, 2002). The large size of internodal cells of the Charales makes them ideal material for electrophysiological experiments, and they have played a pivotal role in establishing the fundamentals of membrane electrophysiology of higher plants. Furthermore, they are the only experimentally accessible system where the ion transport behaviour of otherwise similar naturally salt-tolerant (*Lamprothamnium succinctum*) and naturally salt-sensitive (*Chara australis*) single cells can be contrasted. A comparison of the electrophysiological response of the *Lamprothamnium* and the *Chara* H^+ pump to increased salinity could shed some light on the question of whether extant salt-tolerant Charales originated in a freshwater environment.

Terms and concepts in charophyte electrophysiology

Many transport processes across the cell membrane involve movement of charge and can be modeled by electrical circuits (see Fig. 1a). Such models are useful for understanding the systems biology: interaction of various transporters enabling cells to grow, reproduce and overcome environmental challenges such as increase in salinity. The circuit in Fig. 1a illustrates only a small fraction of the charophyte plasmalemma transporters, but these contribute most of the total membrane conductance at the time of salinity increase. In the large charophyte cell the trans-membrane potential difference (PD), V, can be easily controlled by the voltage clamp method.

The transporters in Fig. 1a are of two kinds, ion channels and a pump (ATP-ase). In the case of most of channels, the motive force, ΔV_X, is the difference between the membrane PD (either free-running or controlled by voltage clamp) and E_X. E_X is the Nernst potential for the ion X transported by the channel. If V is more negative than E_X and the channel is open, positive ions (K^+, H^+) will be driven into the cell, which results in a negative current. No net current will flow if $V = E_X$. When V is more positive than E_X, positive ions will be driven out of the cell, which results in a positive current. The potential energy driving the currents originates from both the unequal concentrations of the ions on each side of the membrane, and from the imposed membrane PD. The conductances of the various transporters respond to the PD change in complex ways. These can be modeled by the Goldman-Hodgkin-Katz (GHK) equation, supplemented by Boltzmann statistics (see Materials and Methods).

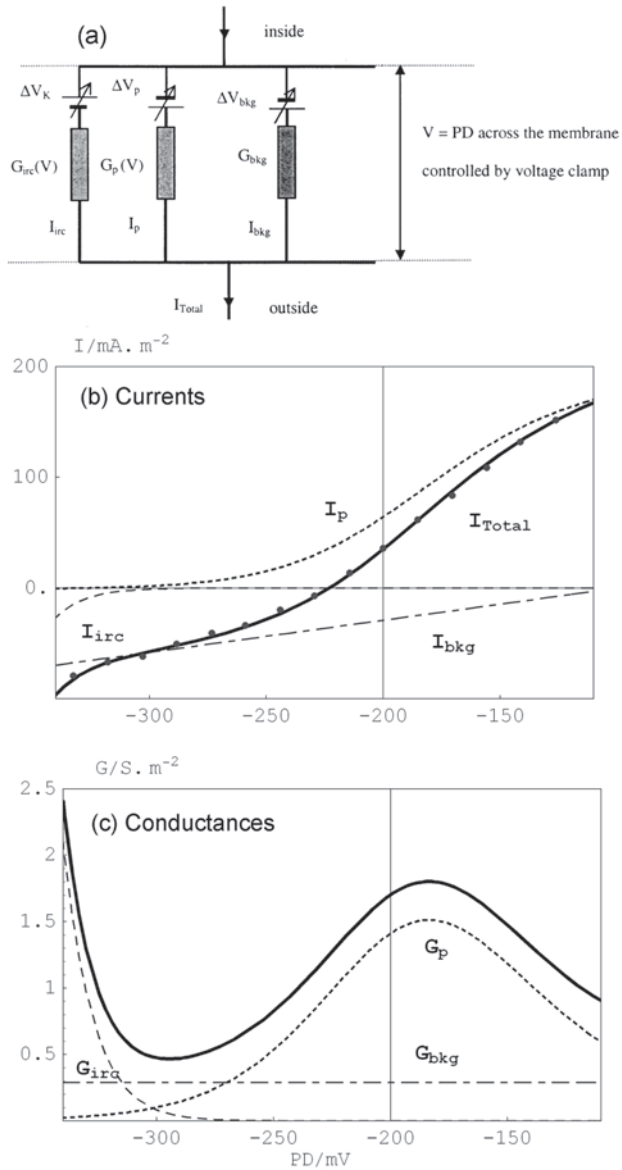

Fig. 1. (**a**) The membrane circuit model. Main conductances affected by increased medium salinity and their major ion carriers: inward rectifier,G_{irc} (V), (K^+); background conductance, G_{bkg}, (K^+, Na^+, Cl^-, H^+, ?); proton pump, G_p(V), (H^+). Note that only G_{bkg} is PD independent. G_{bkg} also depends on the turgor pressure and the ionic strength of the medium (Beilby & Shepherd, 2001; Shepherd *et al.*, 2002). See text for definitions of the motive forces, ΔV. The total current I_{Total} and I_{bkg} can flow either way under voltage clamp or in free running PD conditions. It is not known if the proton pump can generate an inward current. I_{irc} is predominantly inward (as expected). At the cell resting PD, the outward and inward currents cancel. (**b**) Application of the model to low salinity (0.15 SW) *Lamprothamnium* data (points). In this case the total current I_{Total} consists of I_p, I_{irc} and I_{bkg}. (**c**) The conductance-voltage (G/V) profiles of (b).

The nature of the motive force ΔV_p is of a different kind, and comes from the hydrolysis of ATP. In the free running state, the proton pump moves protons against their electrochemical gradient and hyperpolarizes the membrane PD by more than 100 mV. The pump current, I_p, dependency on membrane PD has been fitted by the HGSS (Hansen, Gradmann, Sanders & Slayman model, see Beilby, 1984, Blatt *et al.*, 1990 and references therein).

The current-voltage (I/V) characteristics for each type of transporter are very distinct. The free-running membrane PD is kept negative by the action of the proton pump current I_p (dotted line, Fig. 1b). The inward rectifier channels are activated as V approaches a PD more negative than ~–250 mV. The inward rectifier current, I_{irc}, is shown as long dashed line in Fig 1b. The ubiquitous background current, I_{bkg}, (unequally dashed line, Fig. 1b) is revealed when the cell is subjected to a metabolic blockade. The nature of the background conductance, G_{bkg}, is not clear at present. G_{bkg} is independent of PD. The reversal PD, E_{bkg}, is found at ~–100 mV in both *Chara and Lamprothamnium*. It cannot be attributed to the Nernst potential of any particular ion that is abundant in the cells and in their habitat. However, we have evidence that at least some of the channels contributing to the background conductance are mechanosensory, or stretch-activated, channels (SA channels: Shepherd *et al.*, 2002). Other types of transporters, such as Ca^{++}-activated Cl^- channels, large conductance K^+ channels and H^+ channels have been modeled (see Beilby, 1989, for review), but these transporters are not directly involved in the response to increased salinity, and were not included in the circuit in Fig. 1a.

In this paper we utilize the circuit model to resolve and to contrast the responses of the proton pump, the background conductance and the inward rectifier in *Lamprothamnium* and *Chara* at the time of salt stress.

MATERIALS AND METHODS

Plant Material and Media

Lamprothamnium succinctum was collected from a ditch leading to Lake Budgewoi in the Tuggerah Lakes system, Central Coast, NSW. The plants have been classified as *papulosum* previously (Bisson & Kirst, 1980), but the classification has been updated recently using a new technique on plant sexual organs (García & Chivas, 2004). The salinity of the ditchwater was measured using a hand-held refractometer, and was usually close to ~0.5 seawater (SW). The batch of cells used for the present experiments was collected after heavy rain, when the ditch contained ~0.24 SW, at pH 8.0. Plants were transferred to an "artificial ditchwater medium", containing, in mM: 72.5 NaCl, 2 KCl, 8.8 $MgSO_4$, 1.5 $CaCl_2$, 4.5 $NaHCO_3$ and 2 Na-HEPES, pH 7.6 (~0.15 SW) for at least 3 weeks prior to experiments. Internodal cells ~7 mm long were cut from the plants and allowed 1 week to recover before experiments. The solution used in the hypertonic treatment contained twice the above concentrations (~0.3 SW).

Chara australis Brown (García & Chivas, this volume, consider dioecious *C. australis* as a separate species *C. corallina* Klein. *ex* Willd.) was collected from a golf course lake in Little Bay, Sydney and planted in aquaria containing autoclaved garden soil and an aged mixture of rainwater, rotting leaves, and

distilled water. Internodal cells ~7 mm long were pretreated in artificial pond water (APW) containing, in mM: 1.0 NaCl, 0.1 KCl, 0.1 $CaCl_2$, 5.0 HEPES-Tris, pH 7.0. Two solutions were used for hypertonic treatment. Since the cells are fragile in solutions containing a high Na^+ concentration, the cell turgor pressure was first reduced for one hour, in a medium containing APW to which sorbitol was added, at a concentration of 90 mM (sorbitol APW). The hypertonic "high sodium" solution was osmotically balanced with this solution, and contained APW with increased calcium (1.0 mM $CaCl_2$) and sodium (50 mM NaCl) concentrations (high Na^+ APW).

Electrophysiology

The electrophysiological techniques were described previously (Beilby & Shepherd, 1996; Beilby, 1990; Beilby & Beilby, 1983). Briefly, the cells were mounted in a three-compartment chamber. The compartments were isolated using silicon grease (see Fig. 1 of Beilby *et al.*, 1997). The chamber was lit from below with a fiber-optic light source, and cells were observed using a dissecting microscope with maximum magnification of 120x. Microelectrodes were filled with 0.5 M KCl. The placement of the inner electrode was regarded as vacuolar, considering the very tough *Lamprothamnium* cell wall and the large part of the cell occupied by the vacuole (Beilby & Shepherd, 1996). However, Beilby (1990) found that at most times the series combination of the plasmalemma and tonoplast is dominated by the plasmalemma characteristics. Steady PDs were obtained after 30-40 min when the medium was refreshed by hand at regular intervals. The solutions were introduced by hand-held pipette, after emptying all three chambers by suction. Cells were compartment voltage-clamped (Beilby *et al.*, 1997). The I/V characteristics were obtained by voltage clamping the PD across the cell membrane to a bipolar staircase command generated by a LSI 11/73 computer, with pulse-widths of 60 ms separated by 250 ms at the resting PD. The I/V profiles were generated by averaging the last ten points of each current and PD pulse. The prolonged clamping protocol consisted of clamping the cell at the resting PD level for 2 s, then to a chosen PD level for 12 s and returning to the pre-clamp resting PD for another 2 s (Beilby & Beilby, 1993).

The model

The total clamp current, I_{Total}, passes through the cell membrane via different types of transporters (see Fig. 1). The background current, I_{bkg}, with PD–independent conductance, G_{bkg}, and reversal PD, E_{bkg}, near –100 mV is always present. The background current is fitted by an empirical equation

$$I_{bkg} = G_{bkg}\,(V - E_{bkg}) \tag{1}$$

The identity of the I_{bkg} carriers is not clear at present, but there is an experimental evidence for such a linear profile "background state" in both *Chara* and *Lamprothamnium* (Beilby, 1985; Beilby & Shepherd, 2001).

The pump current, I_p, dependency on membrane PD has been fitted by the HGSS (Hansen, Gradmann, Sanders & Slayman model, see Beilby, 1984; Blatt *et al.*, 1990 and references therein).

$$I_p = zFN\frac{k_{io}\kappa_{oi} - k_{oi}\kappa_{io}}{k_{io} + k_{oi} + \kappa_{io} + \kappa_{oi}} \tag{2}$$

$$k_{io} = k^{o}_{io}e^{\frac{zFV}{2RT}} \tag{2a}$$

$$k_{oi} = k^{o}_{oi} e^{-\frac{zFV}{2RT}} \tag{2b}$$

F, R, T are the Faraday constant, the Gas constant and temperature in degree Kelvin, z is the pump stoichiometry, which has been set to 1. *N* is a scaling factor (2×10^{-8}) and *V* is the PD across the membrane or membranes. The number of carrier states was reduced to two with a pair of PD — dependent constants, k_{io} and $k_{oi,}$ with a symmetric Eyring barrier; and PD — independent rate constants, κ_{io} and κ_{oi}.

The inward rectifier current, $I_{irc,}$ is carried by K^+ in both *Lamprothamnium* and in *Chara* and was modeled by Goldmann-Hodgkin-Katz (GHK) equation (3) multiplied by Boltzmann distribution of open probability $P_{o,irc}$ (eqn. 4) to simulate the channel closure, as the PD is clamped positive of the closing threshold (Amtmann & Sanders, 1999, Beilby & Walker, 1996).

$$I_{irc} = \frac{P_{o,irc} N_K P_K (z_K F)^2 V([K^+]_i - [K^-]_o e^{-\frac{z_K FV}{RT}})}{RT\,(1 - e^{-\frac{z_K FV}{RT}})} \tag{3}$$

$$P_{o,irc} = 1 - \frac{1}{1 + e^{\frac{z_g F(V - V_{50})}{RT}}} \tag{4}$$

Where z_K is the valence of the potassium ion, $[K^+]_o$ and $[K^+]_i$ are the concentrations in the medium and the cytoplasm respectively. $N_K P_K$ stands for the number of inward rectifier channels and their permeability and is treated as a single parameter, z_g is the number of gating charges, V_{50} is the half activation potential of channel closure

The conductances are calculated by differentiation of the currents with respect to membrane PD (see Fig. 1c). The G/V (conductance-voltage) characteristics are instructive in considering the whole system, as the parallel conductances of various types of channels are directly additive. The modeling of the data has been performed on Compaq Armada E500 notebook computer, using Mathematica 3.0. The goodness of fit was judged by eye.

RESULTS

Hypertonic response in Lamprothamnium

Three cells, acclimated to 0.15 SW, were exposed to the hypertonic medium (0.3 SW). The cells were initially in the electrophysiological pump state (see Fig. 1 of Beilby & Shepherd, 2001). A typical I/V sequence at the time of hypertonic regulation is shown in Fig. 2a. The points are experimental data. Each I/V profile was fitted with a current consisting of background current I_{bkg}, pump current I_p and inward rectifier I_{irc}. The model parameters are shown in Table 1. The effect on these transporters of increasing Na^+ concentration from 72.5 to 150 mM was monitored. The resting PD did not change for several minutes after exposure to 0.3 SW. The pump current diminished after 5 min (curve 2, dotted line, Fig. 2a, b and c). The pumping rate increased considerably after 41 min (curve 3, long dashed line, Fig. 2a, b and c). The maximum rate was observed at 2 hours 34 min

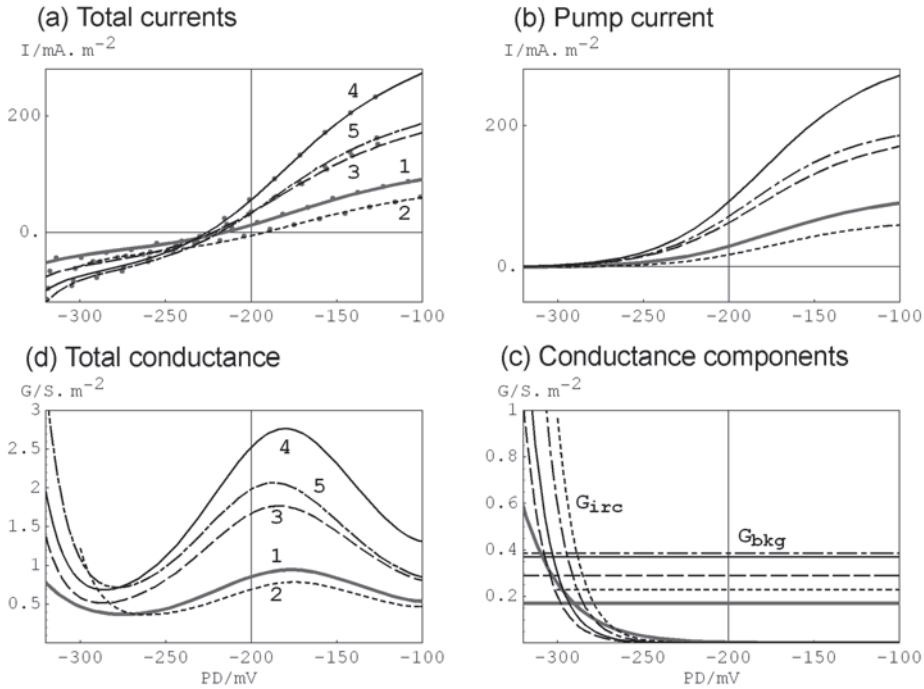

Fig. 2. The response of a *Lamprothamnium* cell, acclimated to 0.15 SW, to hypertonic challenge of 0.3 SW. (**a**) Data (points) and fitted I/V curves in steady state (curve 1, heavy line); 5 min 0.3 SW (curve 2, dotted line); 41 min 0.3 SW (curve 3, long dashed line); 2 hr 34 min 0.3 SW (curve 4, thin continuous line); 3 hr 30 min 0.3 SW (curve 5, unequally dashed line). (**b**) The modeled I_p for each I/V profile from (a) using the same types of lines. (**c**) The modeled G_{irc} and G_{bkg} for each I/V profile from (a) using the same types of lines. (**d**) Total conductances, using the same types of lines and same numbering system as in (a). See Table 1 for parameters.

(curve 4, thin continuous line, Fig 2a, b and c). The pumping rate started to decline at 3 hours 30 min (curve 5, unequally dashed line, Fig. 2a, b and c).

The increase in the pump current was reflected by increases in parameters k^0_{io},k^0_{oi}, and κ_{oi} (see Tab. 1). The resting PD became more negative (Fig. 2a and Tab. 1). G_{bkg} increased in the hypertonic medium, as observed previously (Beilby & Shepherd, 2001). Note that this increase was gradual over 3 hrs (Fig. 2c, Tab. 1). E_{bkg} remained at –100 mV throughout the hypertonic challenge.

The inward rectifier current, I_{irc}, was affected by the hypertonic treatment. The half activation PD, V_{50}, became more positive in the first 5 min following hypertonic treatment (Fig. 2c, Table 1). However, this shift is not as prominent as initially fitted (Beilby & Shepherd, 2001). The data have been refitted, as we became aware of the baseline shift caused by the voltage clamp to the negative levels (see Fig. 3).

Hypertonic response in Chara

Five *Chara* cells were exposed to both sorbitol and high Na APW. Several responses to the latter were encountered. Fig. 4a-d shows the average data from five cells. The differences in I/V and G/V profiles in APW and sorbitol APW

Table 1. Model parameters for the hypertonic challenge in *Lamprothamnium*. $[K^+]_i$ = 100 mM, $[K^+]_e$ = 2.0 mM in 0.15 SW, 4.1 mM, in 0.3 SW. V_p. is the pump model reversal PD, V_R is the resting PD.

Time in Hyper medium	I_p				I_{irc}			G_{bkg}	V_p	V_R
	k^0_{io}	k^0_{oi}	κ_{io}	κ_{oi}	$N_K P_K$	V_{50}	z_g			
hr and min	*sec*$^{-1}$				*m.s*$^{-1}\times10^{-7}$	*mV*		*S.m*$^{-2}$	*mV*	*mV*
steady state 0.15 SW heavy line 1	2800	1.5	0.5	55	2.0	–409	1	0.17	–308	–215
5 min 0.3 SW dotted line 2	2700	2.0	0.5	35	2.0	–355	2	0.23	–288	–192
41 min 0.3 SW dashed line 3	7000	3.2	0.5	100	2.0	–375	2	0.29	–327	–222
2 hr 34 min 0.3 SW thin line 4	11000	6.0	0.5	160	2.0	–370	2	0.37	–334	–225
3 hr 30 min 0.3 SW unequally dashed line 5	10800	5.0	0.5	106	1.8	–360	2	0.385	–328	–218

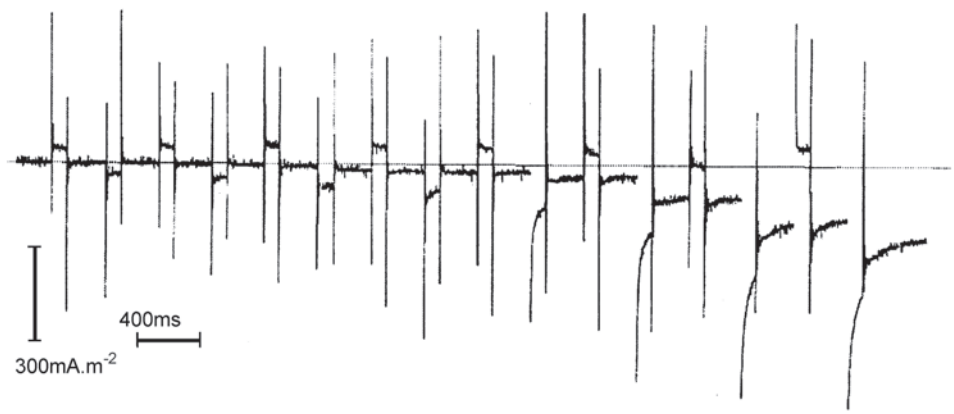

Fig. 3. The shift in the current baseline as seen in the raw data, from which the *Lamprothamnium* I/V curve no. 2 (Fig. 2a, dotted line) was constructed. First three negative pulses and first four positive pulses are not shown. Note that as the staircase pulses become more negative the current does not return to zero, when the clamp level returns to pre-clamp resting PD. The data from such pulses are not included in the construction of the I/V characteristics.

were not statistically significant. The total conductance increased in high Na^+ APW (Fig. 4d), mainly due to G_{bkg}, which rose by about a factor of four (Fig. 4c, Tab. 2). While small changes in the rate of proton pumping did occur, these failed

to prevent the cell membrane from depolarizing. The resting PDs were, respectively, –243 ± 15 mV in APW, –237 ± 10 mV in sorbitol APW and –188 ± 15 mV in high Na^+ APW.

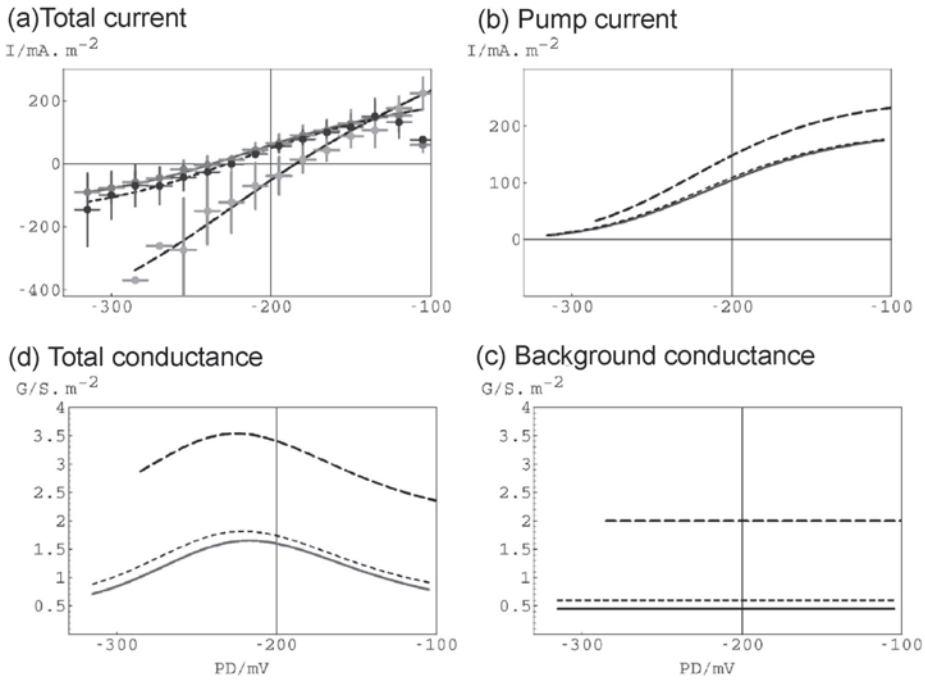

Fig. 4. The response of 5 *Chara* cells to hypertonic challenge. The data have been gathered into 15 mV slots (horizontal error bars). The standard errors are shown as the vertical bars. The continuous I/V profile was measured in APW, the short-dashed profile was measured in sorbitol APW (average exposure 96 min) and the long dashed profile in high Na^+ APW (average exposure 65 min). The data were fitted by I_p and I_{bkg} only. See Table 2 for parameter values.

Table 2. Model parameters for the hypertonic challenge in *Chara.* Statistics for five cells shown in Fig. 4. $V_{p.}$ is the pump model reversal PD, V_R is the resting PD.

medium and time	I_p				G_{bkg}	V_p	V_R
	k^0_{io}	k^0_{oi}	κ_{io}	κ_{oi}			
min	*sec*$^{-1}$				*S.m*$^{-2}$	*mV*	*mV*
APW steady state	8000	0.5	0.5	100	0.45	–377	–236
sorbitol APW Average time 96 min in	8800	0.5	0.5	100	0.6	–379	–227
high Na^+ APW Average time 65 min in	12000	0.5	0.5	130	2.0	–394	–184

When the modeling was applied individually to each cell, a range of pump responses to high Na^+ APW was revealed. In one cell the proton pump exhibited a slight increase of k^0_{io} and κ_{oi}, in another both k^0_{io} and k^0_{oi} increased, while κ_{oi} decreased. Only in one cell the proton pump response closely resembled that of *Lamprothamnium* by increasing k^0_{io}, k^0_{oi} and κ_{oi} (see Fig. 5b and Tab. 3 for parameter values).

It was not possible to voltage-clamp *Chara* cells in high Na^+ APW to PDs sufficiently negative to activate the inward rectifier current I_{irc}. All the *Chara* I/V profiles were fitted by I_p and I_{bkg} only. The voltage clamp to negative PDs resulted in a persistent post-clamp depolarization. This effect is demonstrated in Fig. 6, where the membrane PD is clamped to the resting PD of –142 mV for 2 sec, subsequently to –320 mV for 12 sec (first arrow), and then back to –142 mV for 2 secs (second arrow). Note the large negative current after the cell was clamped to the pre-clamp resting PD. A separate investigation (Beilby and Westermann, unpublished results) found that the proton pump became temporarily inhibited by clamp PDs more negative than –400 mV, when cells were in APW. Increased medium salinity brought the onset of this effect to more positive PDs.

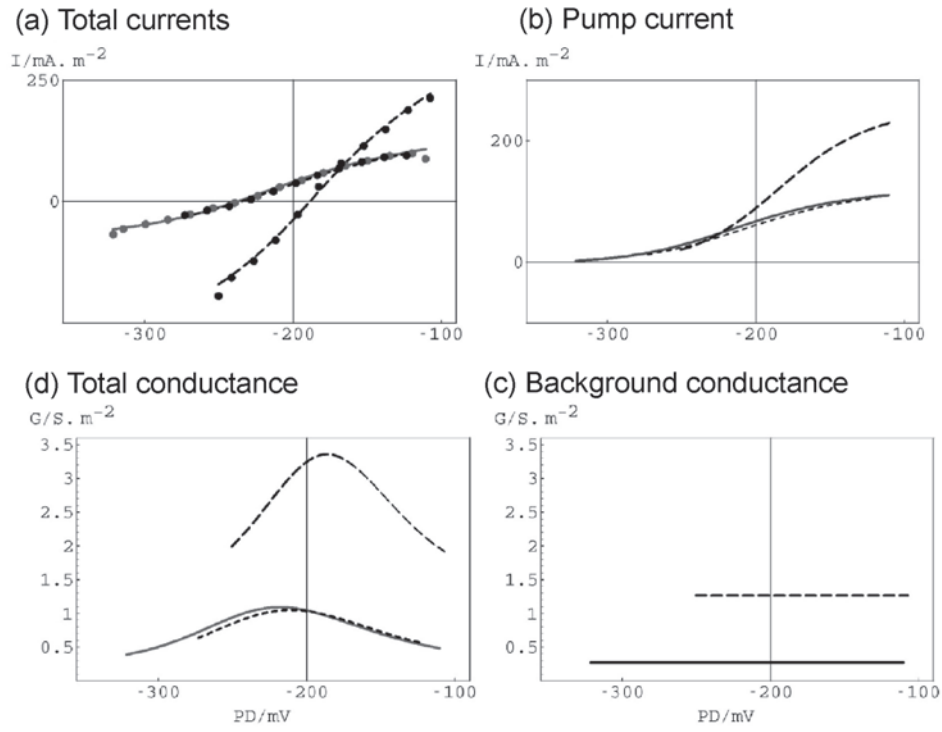

Fig. 5. The *Lamprothamnium*-like response of a single *Chara* cell to hypertonic challenge. The data are shown as points. The continuous I/V profile was measured in APW; the short-dashed profile was measured in sorbitol APW (71 min) and the long-dashed profile in high Na^+ APW. The data were fitted by I_p and I_{bkg} only. The G_{bkg} in APW and sorbitol APW overlap (Fig. 5c). See Table 3 for parameter values.

Table 3. Model parameters for the hypertonic challenge in single *Chara* cell with similar pump response to high salt as that in *Lamprothamnium* (Fig. 5).
$V_{p.}$ is the pump model reversal PD, V_R is the resting PD.

medium and time	I_p				G_{bkg}	V_p	V_R
	k^0_{io}	k^0_{oi}	κ_{io}	κ_{oi}			
min	*sec*$^{-1}$				*S.m*$^{-2}$	*mV*	*mV*
APW steady state	6000	0.5	0.5	63	0.27	–358	–238
sorbitol APW 71 min	4900	0.5	0.5	63	0.25	–353	–235
high Na^+ APW 85 min	12000	5.5	0.5	135	1.27	–334	–189

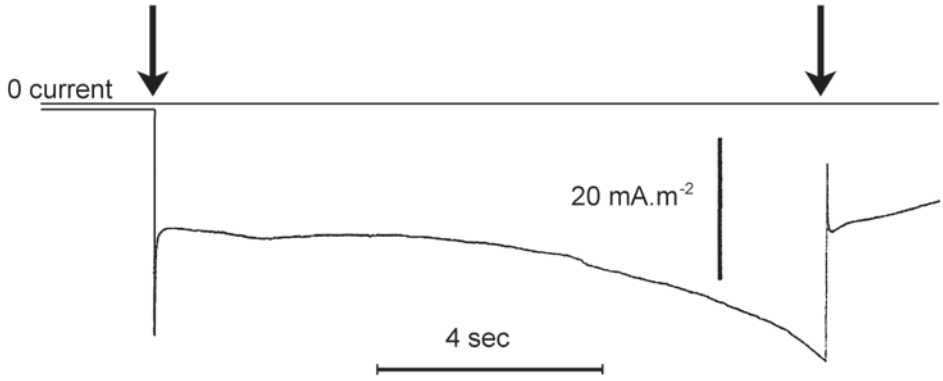

Fig. 6. The current response to prolonged voltage-clamping protocol at 2 s at resting PD of –142 mV, then for 12 s at –320 mV (first arrow) and finally 2 s at pre-clamp resting PD of –142 mV (second arrow). The cell was exposed to high Na APW for 1 hr 41 min. Note that the current does not return to zero, when the clamp PD is returned to pre-clamp resting level.

DISCUSSION

What does it take to be salt-tolerant?

Exposure of cells to a more saline medium generally causes water outflow, cell shrinkage and a decrease in turgor pressure. In both *Chara* and *Lamprothamnium* the background conductance (G_{bkg}) increases in more saline media. A rise in pump activity counteracts this increase in G_{bkg} in *Lamprothamnium*, and the resting PD remains negative. Okazaki *et al.* (1984) and Okazaki & Tazawa, 1990 also observed that *Lamprothamnium* cells hyperpolarize at the time of exposure to more saline media. Another salt-tolerant charophyte, *Chara longifolia* (formerly *buckellii*) exhibits transient hyperpolarization upon

exposure to more saline media (Hoffmann & Bisson, 1990). The cells of salt-tolerant charophytes are able to regulate their turgor pressure mainly by importing K^+, Cl^- and Na^+ over many hours (Bisson & Kirst, 1980; Hoffmann & Bisson, 1990).

What is the transporter machinery instrumental in the turgor regulation? The K^+ is imported through the inward rectifier (I_{irc} in Fig. 1). Cl^- is imported through the $2H^+/Cl^-$ symporter, yet to be incorporated into the model in Fig. 1, but described in *Chara* (Beilby & Walker, 1981). I_{irc} is activated only at negative membrane PDs, and the symporter is powered by the H^+ trans-membrane electrochemical gradient. The H^+ pump activity is crucial to provide both the pH gradient and the negative potential that enable K^+ and Cl^- import. The pathway for Na^+ into the vacuole is unknown, but I_{bkg} is implicated (Davenport & Tester, 2000). Davenport *et al.* (1996) have found that Na^+ influx increases in less turgid *Chara* cells. Therefore, the ability to regulate turgor is an important part of salt-tolerance.

The response of the *Chara* proton pump to increased salinity contrasts with that of *Lamprothamnium* in surprisingly small, but complex ways. First, the resting PD in *Chara* is always more positive in more saline media. This depolarization is due to the increase of Na^+ concentration, rather than a decrease in turgor pressure, since the cell PD and conductance hardly change in the presence of sorbitol (see Figs 4 and 5). Whilst the *Lamprothamnium* proton pump invariably increases its rate in more saline media, the *Chara* proton pump has more variable responses. Only in some cells the same pump rate constants increase similarly to those in *Lamprothamnium* (see Fig. 5 and Tab. 3). However, in none of the cells was an increase of the pumping rate sufficient to prevent membrane depolarization. The differences between *Chara* and *Lamprothamnium* proton pumps are subtle, but may be highly significant for the salt-tolerance of the cell.

There is another subtle difference between the *Lamprothamnium* and *Chara* proton pump. Note that the I/V curves of *Lamprothamnium* (Fig. 2) all span the same PD window, while curves from *Chara* in high Na^+ medium (Figs. 4 and 5) span a smaller PD window. The reason for this is the "hyperpolarising effect" (Beilby & Westermann, unpublished observations). When the *Chara* cell is in APW (containing only 1 mM NaCl), the proton pump is inhibited by voltage clamping the PD to levels more negative than –400 mV. The degree of pump inhibition increases with more negative PD, and the H^+ channels are transiently activated. This effect is not unique to charophytes, and was also observed in wheat root protoplasts (Tyerman *et al.*, 2001). The onset of the "hyperpolarising effect" occurs at more positive PDs in more saline media (see Fig. 6). The effect is proportional to Na^+ concentration, and it becomes impossible to voltage-clamp a *Chara* cell in 100 mM NaCl.

The pump instability follows both hyperpolarization (via voltage clamp) and depolarization (spontaneous action potentials). The cells exhibit spontaneous action potentials (AP) in high salt, probably initiated by hyperpolarization-activated Ca^{++} channels (Beilby & Westermann, unpublished observations). This effect also contributes to the depolarization of the *Chara* membrane in the high salt. The *Lamprothamnium* cells also exhibit the "hyperpolarizing effect" (see Fig. 3), but the onset does not change with salinity.

Yao *et al.* (1992) compared the electrophysiology of another salt tolerant charophyte, *Chara longifolia*, to *Chara australis*. They found that both the background conductance and the pump rate increased in *C. longifolia* in more saline media. They also found that the alkaline bands cover more of the cell surface in

C. longifolia, and proposed that the large G_H (proton conductance) in the alkaline bands is responsible for the greater background conductance in saline media (Yao & Bisson, 1993; Yao *et al.*, 1992). The contribution of the passive proton conductance to G_{bkg} will be investigated in future experiments. However, other ions, including Na^+ are likely to be involved, considering the E_{bkg} of –100 mV.

Future directions of research

The Na^+/ Ca^{++} concentration ratio in the medium may well be as important to *Chara* as the Na^+ concentration *per se*. Hoffmann *et al.* (1989) found that more Na^+ entered *Chara* cells in high Na^+ medium (70 mM) when external Ca^{++} was low (an order of magnitude less than we used in our experiments; 0.1 as opposed to 1 mM;). However the Na^+ influx decreased in the presence of very high external Ca^{++} concentration (7 mM). The cells were exposed to the solutions for 6 days, which was sufficient time for Na^+/ Ca^{++} cation exchange processes in the cell wall to equilibrate.

Both marine and dilute marine waters may have a different chemical composition, in terms of ion ratios, to most fresh waters. Ionic ratios in marine and brackish waters, where *Lamprothamnium* grows, are conservative. However, ion ratios in fresh waters and saline lakes can be much more diverse, and this diversity impacts upon the local organisms. For example, entirely different ostracod species inhabit waters that have evolved along different chemical pathways as marine and meteoric waters are mixed (Radke *et al.*, 2003). This may be because the different Na^+/ Ca^{++}, Na^+/ H^+, and alkalinity/Cl^- ratios in these waters govern acid-base balance and Na^+ and Ca^{++} regulation (Radke *et al.*, 2003). In short, it is very difficult to make an exact comparison experimentally between marine/brackish and freshwater charophytes, because they inhabit waters that differ not only in ion concentration, but also in ionic ratios.

The cell wall/extracellular matrix first encounters the marine/brackish or freshwater environment. *Chara* cells die in a wide range of ionic solutions, but their survival is enhanced by increased Ca^{++} or Sr^{++} in the medium, and this is related to ion exchange properties of the cell wall (Kiyosawa & Adachi, 1990). In contrast, *Lamprothamnium* cells secrete extracellular sulphated polysaccharide mucilage, which reduces hydraulic conductivity and, we hypothesise, acts as a Na^+/ Ca^{++} exchanger (Shepherd & Beilby, 1999; Shepherd *et al.*, 1999). Modifications of the cell wall/extracellular matrix may contribute to the efficiency of the *Lamprothamnium* proton pump and the vulnerability of the *Chara* proton pump in a saline environment. On the other hand, we have in this paper identified some differences between the *Lamprothamnium* and *Chara* proton pumps that do appear to contribute to *Lamprothamnium*'s ability to survive in a huge range of salinities, from freshwater to about twice seawater.

The present research sets the stage for testing three hypotheses: (i) the salt tolerant plants have unique genes; (ii) the salt-tolerant plants have similar genes (similar transporters) as salt-sensitive plants, but express more of the relevant transporters at the time of stress; (iii) the biochemistry, the mechanosensitivity and/or the transporter interaction is different in salt-tolerant and salt-sensitive plants. The cDNA sequence for the vacuolar H^+ pump in *Chara* was 71% similar to that of land plants (Nakanishi *et al.*, 1999). Molecular sequencing of the plasmalemma H^+ pump in salt-tolerant and salt-sensitive charophyte genera will determine if there are significant genetic differences in this transporter.

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