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Topical Review

Electrophysiology of Turgor Regulation in Marine Siphonous Green Algae

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Abstract. We review electrophysiological measures of turgor regulation in some siphonous green algae, primarily the giant-celled marine algae, *Valonia* and *Ventricaria*, with particular comparison to the well studied charophyte algae *Chara* and *Lamprothamnium*. The siphonous green algae have a less negative plasma membrane potential, and are unlikely to have a proton-based chemiosmotic transport system, dominated by active electrogenic K^+ uptake. We also make note of the unusual cellular structure of the siphonous green algae. Hypertonic stress, due to increased external osmotic pressure, is accompanied by positive-going potential difference (*PD*), increase in conductance, and slow turgor regulation. The relationship between these is not yet resolved, but may involve changes in K^+ conductance (G_K) or active K^+ transport at both membranes. Hypotonic turgor regulation, in response to decreased external osmotic pressure, is $\sim$3 times faster than hypertonic turgor regulation. It is accompanied by a negative-going *PD*, although conductance also increases. The conductance increase and the magnitude of the *PD* change are strongly correlated with the magnitude of hypotonic stress.

Key words: Turgor — Electrophysiology of plants — Cell signaling — Transport physiology — Ion and water transport in plants — Cell Physiology — Green algae — Osmotic stress — Cell structure

Introduction

In this review we will describe electrophysiological measures of turgor regulation in some siphonous green algae, primarily the giant-celled marine alga, *Valonia*, and *Ventricaria* (Fig. 1*a*), its close relative (Olsen & West, 1988). We will compare the behavior of these and other giant-celled chlorophyte algae with that of the Charophytes *Chara* and *Lamprothamnium*, and discuss the differences with respect to algal phylogeny.

A cell must be able to detect turgor changes in order to regulate its turgor in response to environmental challenges. The mechanism by which a cell measures its turgor is not known and numerous hypotheses have been proposed (Coster & Zimmermann, 1976; Gutknecht et al., 1978; Pickard & Ding, 1993; Bisson & Kirst, 1995; Heidecker et al., 1999; Shepherd et al., 2002; Kacperska, 2004). These hypotheses generally invoke physical differences that occur as a result of pressure on or differential pressure across the membrane, such as proximity between elements of the membrane and cell wall, curvature or compression of the membrane, and tension within the membrane. Communication of these changes to the protoplast may occur by alteration in the activity of membrane proteins, particularly ion transporters, and hence may have electrophysiological consequences. For instance, stretch-activated or -inactivated channels within the membrane may change membrane conductance (*G*) and electrical potential difference across the membrane (*PD*). In order to affect turgor, these changes need to result in an alteration of cytoplasmic osmotic pressure that reverses the movement of water and restores the optimal turgor. Insofar as these processes involve the transport of charged species, they have electrophysiological consequences.

Advantages and Limitations of Studying Giant-celled Algae

Osmotic relations of the intact cell are controlled by the vacuole, which is often regarded as a simple salt

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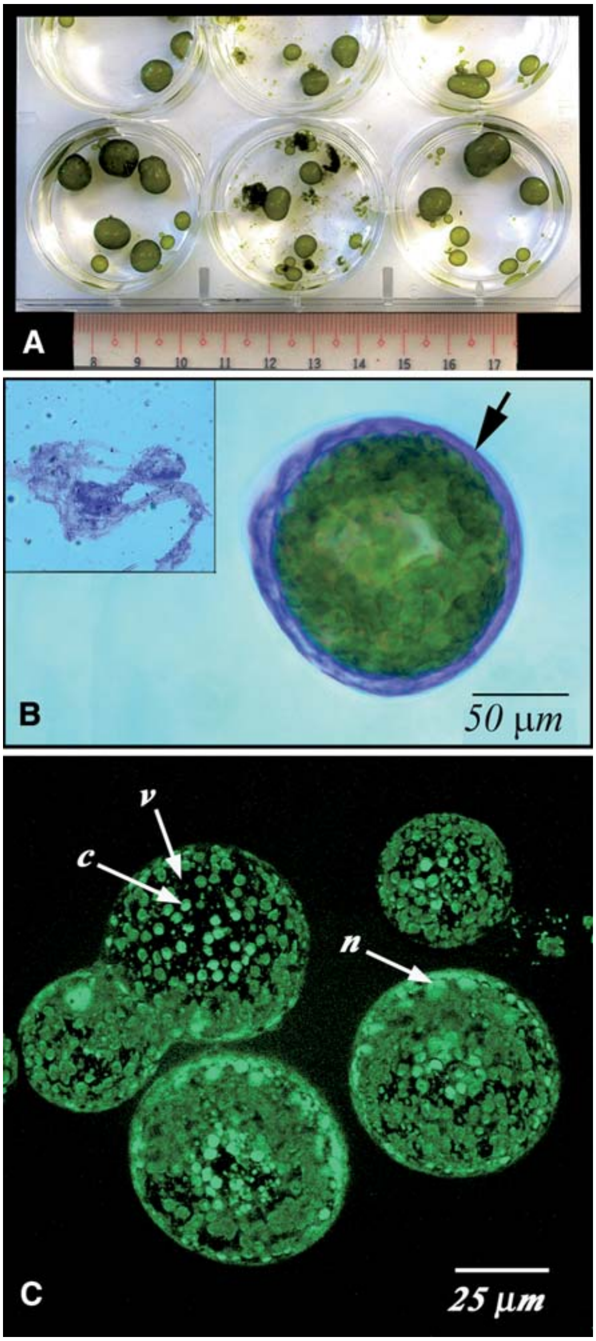

Fig. 1. (***A***) *Ventricaria ventricosa* cells as used in experiments on turgor-pressure regulation. The scale shows centimeters. The smaller cells (~ 3mm diameter) are used in voltage-clamping experiments. (***B***) An 8–hour old *Ventricaria* cell, stained with the cationic stain Toluidine Blue at low pH (0.5), demonstrating sulfated polysaccharides (*reddish-purple*). The exterior of the cell is coated in a thick layer of sulfated polysaccharides (*arrow*). The inset shows stained mucilage extruded from the interior of a crushed cell. Both the exterior of the cell and the interior ("*vacuole*") contain large amounts of sulfated polysaccharide mucilage. (***C***) Confocal microscopy of a cluster of five 18-hour old cells labeled with the fluorochrome 6-carboxyfluorescein. The fluorochrome labels only the cytoplasm, and does not enter the vacuolar compartment. This image shows a reconstruction of 12 optical sections made at 1.8 µm intervals. The cytoplasm shows the characteristic alveolate or sponge-like structure, with chloroplasts (*c*) and nuclei (*n*) fluorescing, whilst convoluted projections from the central vacuole (*v*) appear dark. The figure shows the topological complexity of the cytoplasm/vacuole interface. The cytoplasm is a mosaic of thicker and thinner regions. In dark-appearing regions, only the peripheral cytoplasm, which is very thin (~40 nm), separates the vacuole from the extracellular region.

solution enclosed by a tonoplast. Solute fluxes and electrophysiological responses can be measured and correlated with turgor, which was originally assumed to be explicable in terms of the osmotic contents. However, in the case of *Valonia* and *Ventricaria* (Heidecker et al., 2003a; Shepherd et al., 2004), colloidal substances are associated with cell walls and the vacuole (*see* Fig. 1*b*). Heidecker et al. (2003a) note that residual turgor remains after perfusing the vacuole of *Valonia* with medium isosmotic to seawater. It is not clear whether this effect is due to non-ideal (leaky) membranes, inability to completely perfuse out the hyperosmotic vacuolar contents, or matric potential of mucopolysaccharide remaining in the vacuole. When *Valonia* or *Ventricaria* sporulate, which involves pulling the cytoplasm away from the cell wall (Kopac, 1933; La Claire, 1982; Nawata et al., 1993; Shepherd et al., 2004), the remaining mother cell wall often retains a degree of turgidity. The most likely explanation is that the matric potential of the colloidal mucopolysaccharides left behind (Shepherd et al., 2004) reduces the water potential inside the empty cell wall to below that of seawater, and hence draws water in. This contribution of matric potential can be further studied, because of the comparative simplicity and accessibility of the giant cell system.

The giant cells can be perfused, that is, the internal contents can be removed and replaced with a chosen solution (Gutknecht, 1967). The experimenter can then control parameters when trying to understand the causes and significance of electrophysiological changes. Even turgor can be controlled in a perfused cell (Hastings & Gutknecht, 1974; Hastings & Gutknecht, 1976; Heidecker et al., 2003b). Advances in technology have enabled measurements on smaller cells using patch-clamp techniques, which require an intimate interaction between the membrane and the patch pipet, and consequently the removal of the cell wall. However, turgor is eliminated, and the use of fungal enzymes induces anti-pathogen responses with their own electrophysiological consequences (Gelli et al., 1997). While such techniques can provide useful information about the activity of particular transport systems, processes that involve complex relationships between different systems will inevitably be disrupted. Marked differences have been noted between the activity of individual transporters, as determined by patch-clamp techniques, and the integrated electrophysiological characteristics of intact cells (Tyerman & Skerrett, 1999).

Since wall-less cells have no turgor, impalement of intact, walled cells is the only way to assess electrophysiological parameters related to turgor regulation processes. The giant cells tolerate multiple impalements, allowing simultaneous measurement of turgor and electrophysiological parameters. However, multiply impaled cells are less likely to regulate turgor than non-impaled cells (Heidecker et al., 2003b). They have lower turgor, lower *PD*, recover more slowly from impalement, have a lower rate of regulation, and take longer to regulate than singly impaled cells (Bisson & Beilby, 2002). The damage probably arises from the pressure probe micropipette, which must have a large aperture to avoid clogging with the mucilage that the cells produce. Since the *PD* is close to zero and there is no streaming to gauge health, it is sometimes difficult to distinguish between a decrease in turgor due to regulation and a decrease due to cell decline.

Algal Phylogeny

Algae with giant cells occur throughout the algal phyla, and presumably have evolved many times. They have a reputation of being "shade" plants, i.e., adapted to grow in low light (Raven & Smith, 1977; Raven et al., 1979; Kirst & Wichmann, 1987). We have collected *Ventricaria* from reef regions with very low light—either deep (20 m) or underneath coral boulders on the reef flat. When collected from regions of higher light intensities it is apt to be covered by Aufwuchs (epiphytes). Growth at low light levels may enable the alga to avoid competition and overgrowth. In *Codium*, another siphonous green alga, parallel membranes such as the tonoplast and plasma membrane may act as "light pipes", akin to optic fibres (Ramus, 1978). Ramus suggested that trapped air could generate the necessary differences in refractive index, but Mandoli & Briggs (1982) found a similar effect in oats, and suggested that the membranes provide sufficient refractive difference from cytoplasm to generate the light pipe effect.

We focus here on green algae, which are more closely related to land plants than red or brown algae, and whose electrophysiology has been studied in greater detail. The brown algae are more closely related to the fungal group oomycetes (e.g., *Saprolegnia*, *Phytophthora*) than they are to the green or red algae (Kapraun, 2005). The closely related brown algae, *Fucus* and *Pelvetia*, have been extensively studied (Brownlee et al., 1998; Brownlee et al., 1999), but mainly from a developmental perspective. Of red algae, some work has been done on *Griffithsia monilis* (Bisson & Kirst, 1979) but little is known of its electrophysiology.

The explosion of molecular systematics over the past twenty years has led to substantial reclassifications of algal groups. The "green plants" or Viridiplantae are now viewed as containing two evolutionary lineages or clades, the Charophyta and the Chlorophyta (Fig. 2, Karol et al., 2001). The charophyte clade contains the charophyte algae and embryophytes (land plants), whilst the chlorophyte clade contains essentially the rest of the green algae. Extensive work has been done on Charophytes (reviewed elsewhere; Beilby et al., in press). The best studied Chlorophytes are *Valonia* and *Ventricaria*, in the Class Ulvales, Order Siphonocladales/Cladophorales complex (Fig. 2). Other taxa that we will briefly mention include *Chaetomorpha* in the same order, *Acetabularia* (Class Ulvales, Order Dasycladales) and *Codium* and *Derbesia* (Class Ulvales, Order Caulerpales) (Kapraun 2005).

Electrophysiology

Valonia and *Ventricaria* (Fig. 1*a*) were amongst the earliest subjects for electrophysiological study. Cable properties do not complicate conductance measurements in these roughly spherical cells. The *PD* between vacuole and external medium is ~ zero, usually slightly positive (Fig. 3*a*). It is difficult to measure the cytoplasmic *PD*, since the cytoplasm is very thin, with a convoluted, "sponge-like" topology (Shihira-Ishikawa & Nawata, 1992; Heidecker et al., 2003a; Shepherd et al., 2004; *see* also Fig. 1*c*). Davis (1981) reported relatively stable impalements of the cytoplasm, with *PD* of −70 mV, whilst the vacuole was 86 mV positive to the cytoplasm, giving the overall positive potential. The cytoplasmic *PD* depended on external K^+, hyperpolarizing by ~35 mV when K^+ decreased from 10 to 1 mM, and depolarizing by ~40 mV when K^+ increased from 10 to 100 mM. We did not find a similar stable negative cytoplasmic *PD* using Davis's technique. When we did obtain a transient negative potential, we were unable to voltage clamp it because of the very high conductance. This *PD* may be an artifact, or represent transient impalement of a cytoplasmic compartment with a very conductive plasma membrane. Nonetheless, the vacuolar potential shows a near Nernstian dependence on external K^+ (Davis, 1981; Beilby & Bisson, 1999), and so the potential across the plasmalemma is likely to be due primarily to K^+ diffusion.

Aplanospores, however, have a negative, K^+-sensitive *PD* (Damon, 1930; Gutknecht, 1966; Davis, 1981). Aplanospores are small cells that form by segregation of cytoplasmic domains followed by separation from neighboring domains, rounding up as independent cells, and synthesis of cell walls (Kopac, 1933; La Claire, 1982; Nawata et al,. 1993; Shepherd et al., 2004). Aplanospores were at one time

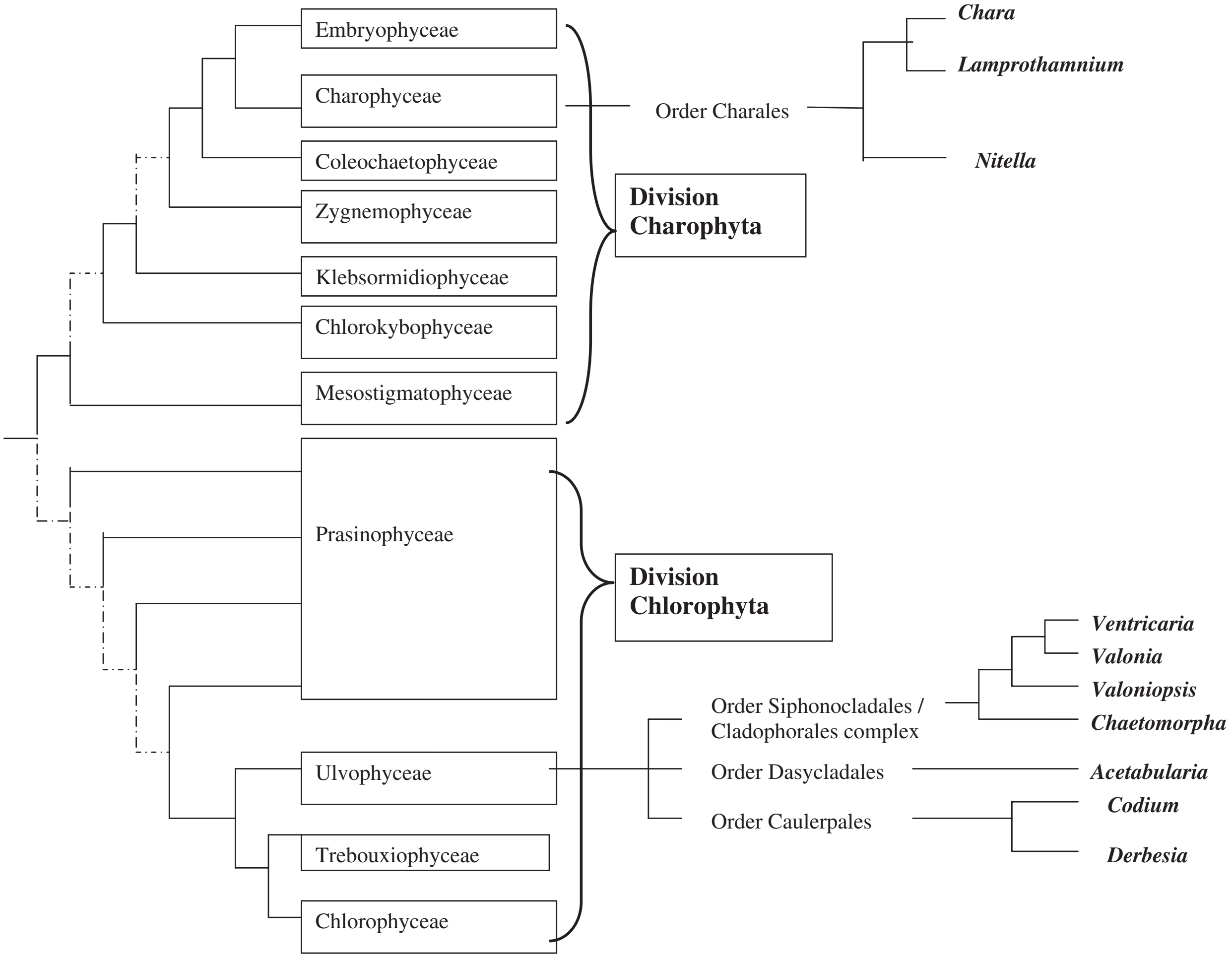

Fig. 2. A schematic diagram of the major relationships between the green algae, showing only the orders from which organisms have been selected for experiments into turgor-regulation. Mechanisms of turgor regulation differ in the two Divisions Charophyta and Chlorophyta, but turgor regulation within the Orders of these Divisions shows many similarities. Figure based on molecular systematic data (Fig 1, Karol et al., 2001; Fig. 1, Leliaert et al, 2003; Table 2 and Fig. 1*b*, Lewis & McCourt, 2004) and nuclear DNA data (Kapraun, 2005). The branch lengths are not quantitative. Note that the Class Prasinophyceae is currently undergoing revision; it may be polyphyletic, as indicated.

assumed to be avacuolate, but the vacuole forms more rapidly than previously thought (Shepherd et al., 2004). The negative potential of aplanospores suggests either that the vacuole, though present, is less likely to be impaled, or that the vacuolar *PD* differs from that in larger, mature cells. Thus the electrophysiological characteristics of the plasma membrane of *Valonia* and *Ventricaria* are more similar to animals than to freshwater algae, fungi, and terrestrial plants.

An indirect counterargument has been made (Wang et al., 1995) that the *PD* is generated by an electrogenic Cl^- pump, similar to *Acetabularia* (*see* below). Gutknecht (1966) found that the measured ratio of Cl^- influx to Cl^- efflux is consistent with passive transport. However, he also measured a net Cl^- influx under conditions when there is no driving force for passive movement (short circuit; *see* below). Together with the E_{Cl} being positive to the plasma membrane *PD*, some active Cl^- transport at the plasma membrane might occur.

The *PD* across the plasma membrane is less negative than in plant cells with an electrogenic H^+ pumping ATPase. Seawater has a pH of about 8.0, while soil and freshwater are often more acidic (pH 5–6). H^+ is therefore closer to equilibrium across the plasma membrane of marine siphonous algae. The Nernst potential for H^+, E_H, is −28 mV if cytoplasmic pH is 7.5. This indicates the necessity for active export of H^+, but $\Delta\bar{\mu}_H$ is small, and active transport by H^+ symports and antiports, as in the standard chemiosmotic model, is less favored, and has not been described. However, definitive experiments have not been performed.

How does the large positive *PD* across the tonoplast arise? Hastings and Gutknecht (1976) used the technique of short-circuit current to determine which ions were actively transported. In this

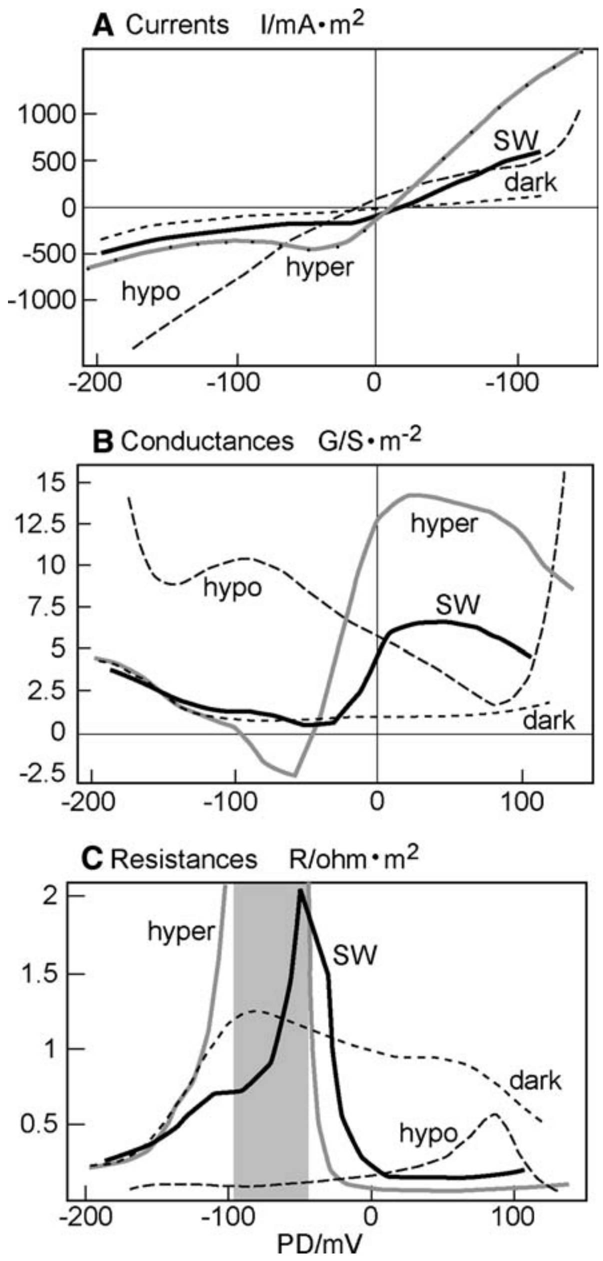

Fig. 3. The electrophysiological characteristics of *Ventricaria*. (*a*) The *I-V* curves: steady state in seawater (*SW*), two hours after impalement, illumination PAR 2.02 μmol.s^{-1}.m^{-2} (*thick black line*); a typical hypotonic profile (*Hypo*), 24 min after a drop of 80 mOsmol.kg^{-1} (*long-dashed line*); a typical hypertonic profile (*hyper*), 16 min after an increase of 100 mOsmol.kg^{-1} (*thick gray line*); profile in the dark, exposure for 22 min to PAR 0.5 μmol s^{-1}.m^{-2} (*short-dashed line*). (*b*) *G-V* characteristics calculated from the *I-V* curves by differentiation with respect to the membrane *PD*. Line types as in (*a*) (*c*) R-V characteristics calculated as reciprocals of *G-V* curves. Line types as in (*a*). The shaded region signifies undefined and negative resistance.

technique, the vacuole is perfused with a solution identical to the external medium, and the *PD* is clamped to zero. Thus there is no driving force for passive transport and any current carried (the short-circuit current) must be due to active transport. There is a 1:1 relationship between short-circuit current and K$^+$ fluxes, which suggests primary active transport of K$^+$, which would be electrogenic and could generate this positive potential. It is, however, difficult to distinguish between primary active inward transport of K$^+$ and an inward H$^+$ pump with tightly coupled K$^+$/H$^+$ antiport, as has been postulated for the chloroplast envelope (Song et al., 2004). The vacuolar pH is 5.8 in the light, producing a E_{H} across the tonoplast of -180 mV, assuming cytoplasmic pH is 7.5 (Davis, 1981). Transport into the vacuole is necessarily active, consistent with the decrease in vacuolar pH in the light. It is yet to be determined whether primary active H$^+$ transport takes place across the tonoplast, as it does in charophytes and higher plants (Shimmen & MacRobbie, 1987; Nelson & Klionsky, 1996; Lüttge & Ratajczak, 1997; Forgac, 1998; Nakanishi et al., 1999).

Thus we cannot make the simplifying assumption, often made for charophyte algae (Beilby, 1990; Shimmen et al., 1994), that the easily measured potential difference between the vacuole and the external medium (E_{VO}) can be primarily attributed to the plasma membrane. Instead, E_{VO} is the relatively small difference between two large numbers. This means that small changes in either membrane will contribute to changes in the overall *PD* in ways that are hard to disentangle.

The situation is further complicated by the fact that the overall conductance is also not dominated by one membrane. Davis (1981) placed current-injecting electrodes in the vacuole. Using relatively long single pulses, he monitored the change in membrane potential in the vacuole or cytoplasm, finding a plasma membrane conductance of 7.1 S m^{-2} and tonoplast conductance of 1.9 S m^{-2}. From the reciprocal sum of the series conductances:

$$1/G_{\text{total}} = 1/G_{\text{PL}} + 1/G_{\text{TP}}$$

Davis calculated overall conductance of 1.5 S m^{-2}. Heidecker et al. (2003) used a charge relaxation technique to separate conductance at the two membranes. In this technique, the membrane is subjected to a brief (1 μs) step charge pulse. After the pulse, the membrane *PD* decays with shape consistent with a dual exponential decay. The plasma membrane *PD* decayed more rapidly than the tonoplast *PD*. They found much higher conductances than Davis (1981), 103 S m^{-2} at the plasma membrane and 61 S m^{-2} at the tonoplast, suggesting the overall conductance is 38 S m^{-2}.

We (Beilby & Bisson, 1999) measured the conductance of the two membranes in series using two different techniques: Current-voltage *(I-V)* curves generated by voltage-clamping to a bipolar staircase (Fig. 3*b*), and injection of an alternating current (AC) and measurement of the voltage response. The latter technique gives higher values than *I-V* curves (11 S m^{-2} vs. 3 S m^{-2}), but both are closer to the values of Davis than of Heidecker et al.

Why are the measurements by the charge relaxation technique so much higher? Zimmermann et al. (1982) suggested that there are mobile charges in the membrane that move in response to voltage, and contribute to the relaxation after the charge pulse. If this is the case, this technique is not a good one for measuring conductance. These mobile charges, in the membrane or in the unstirred layers around the membrane, may also distort the results from injection of AC current, and explain why this technique gives higher values than the derivative of the *I-V* curves (Beilby & Bisson, 1999). Frequency dependence of this response could throw some light on the nature of these charges (Coster et al., 1996). In addition, curve-fitting techniques used to deconstruct data into multiple exponentials give apparently good fits even when data have been generated by other models. Therefore, the two techniques used for separation of the conductance at the two membranes—cytoplasmic impalement and charge relaxation—are both subject to criticism, the former because cytoplasmic impalement is questionable, and the latter because the technique is indirect and gives higher values than overall conductance (G_{total}) measured by vacuolar placement of electrodes.

Voltage clamping the vacuolar *PD* to a bipolar staircase protocol generates *I-V* characteristics (Beilby, 1984; Beilby, 1989; Beilby, & Bisson, 1999). The strength of *I-V* curve analysis is that the potential dependence of the conductance (or resistance) can give information on the nature of the ion transport systems responsible for the conductance/resistance. The resistance-voltage (*R-V*) analysis is instructive in this case, as the resistances in series are directly additive (Fig. 3*c*). The *I-V* curve for cells in seawater (SW) is roughly linear at positive potentials (Fig. 3*a*), but conductance sharply decreases at more negative potentials, with a minimum between −100 and −50 mV (Fig. 3*b*). Because the currents were obtained by clamping the *PD* of both plasma and vacuolar membranes, we cannot analyze in detail which transport systems at which membrane are responsible for different aspects of the *I-V* curve. Nonetheless, the steep, roughly linear portion of the *I-V* profile remains associated with the resting *PD* and both shift to more negative values at lower concentrations of K^+ in the medium (Fig. 1 of Beilby & Bisson, 1999). While this behavior implies a role for K^+ in the generation of this conductance, note that conductance rises as K^+ concentration in the medium falls. This is opposite to what one would predict if K^+ permeability were constant while K^+ decreased. Further, upon decrease of K^+ concentration, the new profile develops slowly, taking at least 20 min to stabilize. Slow changes in cytoplasmic K^+ could be affecting transport systems at the tonoplast. The *PD* oscillations that occur following a decrease in external K^+ concentration (Fig. 5 of Beilby & Bisson, 1999) also argue for feedback mechanisms controlling the *PD*, rather than simply slow diffusion through the wall and associated mucilage.

After studying the aplanospore formation and structure of mature cells, we (Shepherd et al., 2004) challenged the classic image of the plasma membrane and tonoplast as two independent membranes in series. Instead, the plasma membrane and tonoplast can be considered a "communal" membrane, in the sense that they do not have fixed identities. Membrane material can be transferred from plasmalemma to tonoplast through endo- and exocytosis during wound-induced cytoplasmic contraction and protoplast formation in a related alga, *Boergesenia* (O'Neill and La Claire, 1988). This blurs the distinction between membranes. Vesicle trafficking has the potential to modify membrane transport during turgor regulation in *Ventricaria*. We argued that the functions of the membranes may be polarized, and depend on the medium that each faces (whether sea water or "vacuolar sap"), or on insertion of specific proteins, rather like the epithelial cells of animals that are surrounded by a continuous membrane with distinct functions (Greger, 1988).

The mosaic of thicker and thinner regions of cytoplasm interpenetrated by a convoluted vacuole (Fig. 1*c*) may explain the wide range of *PD* measurements obtained by different researchers. We argued that wound-induced cytoplasmic contraction and aplanospore formation is enabled by a cytoplasm that is essentially a collective of separate cytoplasmic "domains", each consisting of a nucleus and chloroplasts, held together by extremely fine cytoplasmic strands containing microtubules. These domains are enclosed by outer ("plasmalemma") and inner ("tonoplast") aspects of the polarized communal membrane.

To be consistent with previous literature, we will continue to refer to these membranes as plasma membrane and tonoplast, and the interior milieu as a vacuole, but this may misrepresent the true nature of this organism.

Turgor Regulation

The solutes responsible for turgor regulation are primarily K^+ and Cl^- (Hastings & Gutknecht, 1976; Steudle et al., 1977). Na^+ is actively transported out of the cytoplasm at both membranes, but plays little part in adjusting turgor. K^+ is close to equilibrium at the plasma membrane, but must be actively transported into the vacuole. The evidence for active transport of Cl^- is ambiguous, as discussed above (Gutknecht, 1966; Wang et al., 1995).

Potassium fluxes are the key to turgor regulation. *Ventricaria* responds to hypertonic stress and decreased turgor with enhanced K^+ uptake. Flux measurements (Gutknecht, 1967; Hastings & Gutknecht, 1974; Hastings & Gutknecht, 1976; Steudle et al., 1977; Gutknecht et al., 1978) indicate that this is due to increased unidirectional (active) K^+ influx, which is minimal at normal turgor (~ 0.15 MPa). This K^+ influx does not further decline when hypotonic stress is imposed, and turgor increases. Rather, turgor is down-regulated by increased unidirectional K^+ efflux. It is unclear whether this is active or passive. What does electrophysiology tell us about these processes? A particularly powerful tool is the simultaneous measurement of turgor and electrophysiological parameters in a single cell during the course of regulation (Zimmermann & Steudle, 1974; Beilby & Bisson, 1999; Bisson & Beilby, 2002; Heidecker et al., 2003b).

HYPERTONIC STRESS

When turgor decreases, either through hyperosmotic stress or controlled by the turgor probe (Zimmermann & Steudle, 1974; Beilby & Bisson, 1999; Bisson & Beilby, 2002; Heidecker et al., 2003b), the membrane *PD* becomes more positive in most cells. The cell conductance also increases (Bisson & Beilby, 2002). Both the *PD* and conductance increases are consistent with stimulation of an electrogenic K^+ influx pump at the tonoplast. However, the conductance increase and the positive shift in potential are not always correlated (*see* Bisson & Beilby, 2002). The maxima in the conductance and *PD* increases are not simultaneous, and the conductance maximum follows the *PD* maximum (Bisson & Beilby, 2002). Interpretation is difficult because the measurements were made on two membranes in series, and these can have opposite effects on the membrane *PD* (*see* above). Hypertonic stress changes the *I-V* profile. The very high conductance near the resting potential falls more rapidly, even going negative, in regions of negative potential (Fig. 3*b*). The negative conductance indicates that the transporter is strongly *PD* dependent, as are large conductance K^+ channels in charophytes (Beilby, 1986).

On the other hand, Heidecker et al. (2003b) drew a different conclusion, using their charge-relaxation method to separate the behavior of the membranes. Although we point out (*see* above) that there are difficulties with interpretation with this technique, these studies are worth citing here, as they illustrate how useful this kind of information is, and perhaps they will stimulate the development of new techniques to further pursue these questions. They found that tonoplast conductance decreased at the most positive *PD*. This is opposite to what one predicts if an electrogenic pump at the tonoplast is responsible for the positive *PD* shift. They calculate that the plasma membrane further increases its already high conductance as the *PD* becomes more negative again. This is consistent with the Damon/Gutknecht/ Davis model, which predicts that an increase in passive K^+ permeability at the plasma membrane would drive the *PD* more negative.

Impalement of the *Ventricaria*/*Valonia* cells with a pressure probe as well as microelectrodes showed that the relationship between turgor regulation and the electrophysiological responses is also complex (Bisson & Beilby, 2002; Heidecker et al., 2003b). For instance, after a small (30 mOsmol kg^{-1}) osmotic stress, *PD* reached a positive maximum after ~ 160 min, then started to decline, while turgor, after the initial decrease, exhibited a steady recovery rate over 300 min (Fig. 1 of Bisson & Beilby, 2002). In other experiments a stronger positive-going *PD* did not necessarily equate with faster turgor regulation.

Heidecker et al. (2003b) reported similar behavior in *Valonia* subjected to larger (40 mOsmol kg^{-1}) hyperosmotic stress. Both membranes show initial decreases in conductance, followed by increases. Both increases remain elevated while pressure is regulated, and so they are associated with turgor regulation. The plasma membrane conductance increase is most strongly correlated with turgor regulation. Similar results were found when cells had turgor reduced either osmotically or with the turgor probe.

In summary, hypertonic stress is accompanied by positive-going *PD*, increase in conductance, and slow turgor-regulation in most, but not all cells. The relationship between these is not yet resolved, but may involve changes in K^+ conductance (G_K) or active K^+ transport at both membranes.

HYPOTONIC STRESS

Hypotonic stress of > 70 mOsmol kg $^{-1}$ results in a marked decrease in *PD*, often resulting in a negative *PD* (~ −30.5 mV; Bisson & Beilby, 2002). Negative *PD*s were not recorded with osmotic stress < 70 mOsmol kg $^{-1}$. At the onset of > 70 mOsmol kg $^{-1}$ hypotonic stress, a small positive *PD* shift is followed by a negative displacement, taking ~ 30 min to reach a peak negative *PD*, after which the resting value is recovered (after ~ 2 hours). The magnitude of the negative *PD* displacement is directly correlated with the magnitude of osmotic stress (Fig. 4). In their experiments, using a small osmotic stress (49 mOsmol kg^{-1}), Heidecker et al. (2003b) also reported a slight, transient positive shift in *PD* on hypotonic stress. The *PD* did not reach negative values, but remained similar to the control. Both groups found that the turgor regulation continued even though the *PD* recovered from the transients.

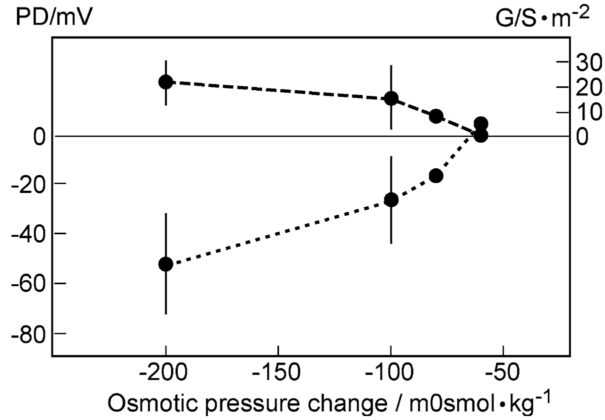

Fig. 4. Degree of depolarization and conductance increase with hypotonic shock in *Ventricaria*. Each point with an error bar is an average of three readings. Data from Bisson & Beilby (2002).

We (Bisson & Beilby, 2002) showed that conductance at the resting potential increased, reaching a maximum at the most negative *PD* (Fig. 3*b*). The increase in conductance was greater with greater osmotic stress (Fig. 4). With onset of hypotonic turgor regulation, the conductance increases in the negative region of *PD*, and decreases in the positive *PD* region (Fig. 3*b*, and Fig. 7 of Beilby & Bisson, 2002). A resting *PD* in the negative region coincides with a high conductance, whereas in the positive region, it coincides with a low conductance.

Using their charge-pulse technique, Heidecker et al. (2003b) found that the tonoplast conductance (G_{TP}) dropped sharply, with little change in the plasma membrane conductance (G_{PL}). Since this technique is done at the resting potential, which remained positive in their cells, this is consistent with our finding of a conductance decrease at positive potentials. Again, they found similar results whether turgor was decreased osmotically or with the pressure probe.

Hypotonic turgor regulation is ~3 times faster than hypertonic turgor regulation (Bisson & Beilby, 2002). In contrast to hypertonic stress, hypotonic stress is accompanied by a negative-going *PD*. However, conductance increases with both hyper- and hypotonic stress. The conductance increase and the magnitude of the *PD* change are strongly correlated with the magnitude of hypotonic stress. This appears not to be the case with hypertonic stress.

ACUTE HYPOTONIC STRESS

Acute hypotonic stress, resulting in dangerously high turgor, elicits an "emergency escape" mode of turgor regulation (Guggino & Gutknecht, 1982). The cell wall ruptures, and a small amount of cell sap is lost. This decrease in volume results in an immediate turgor loss. However, the cell contents remain hyperosmotic to the external medium. As the membrane heals over, water re-enters and turgor increases. This is therefore not strictly turgor regulation, but a temporary, transient reprieve from high turgor whilst the cell reduces the vacuolar osmotic pressure. Because ruptures occur in the cell, wound healing (cf. La Claire, 1982) must occur simultaneously with turgor regulation.

MODELS OF ION TRANSPORT

The *I-V* curves of *Ventricaria* cannot be interpreted with the same precision as those of charophytes (*see*, for instance, Beilby & Walker, 1996). Neither membrane consistently dominates the overall electrophysiology, nor can their activities be separated with confidence. We have interpreted the *I-V* characteristics primarily in terms of K^+ transport. The *I-V* curves during hypo- and hypertonic stress are mirrored across a vertical axis passing close to zero mV (Fig. 3*b*). An *I-V* curve with high conductance at positive potentials and high resistance at negative potentials is associated with activation of the K^+ pump (Fig. 3*b*, and Beilby & Bisson, 1999; Bisson & Beilby, 2002). Pump activation is correlated with positive *PD*. The pump profile is more conductive in the light, which is reasonable for a process that requires energy in a photosynthetic organism. The high resistance at negative potentials arises from pump inactivation (Beilby & Bisson, 1999). The pump profile is a feature of the hypertonic stress response, which stimulates active K^+ influx (Fig. 3).

During hypotonic stress, the conductance increases at negative potentials, whilst resistance increases at positive potentials (Fig. 3*b, c*). Early models of *Valonia*/*Ventricaria* transport found no evidence for active transport at the plasma membrane (Gutknecht, 1966). However, given our analysis of the "tonoplast" as a form of plasma membrane (Shepherd et al., 2004), it is possible that a similar pump operates under some circumstances in the plasma membrane. The high conductance at negative membrane *PD* could represent activation of a plasma membrane K^+ pump, moving K^+ into the external milieu. Alternatively this conductance increase could be passive K^+ transport. With the tonoplast K^+ pump inactivated at the time of hypotonic stress, K^+ would move passively from vacuole to cytoplasm. An increased cytoplasmic K^+ concentration should result in passive K^+ loss. Experiments are being designed to demonstrate whether the increase in unidirectional K^+ efflux under hypotonic stress is passive or active.

We (Beilby & Bisson, 1999) mathematically tested the transport model with passive efflux only at the plasma membrane. We replicated the measured *I-V* characteristics using reasonable values for the reversal potential of a tonoplast K^+ pump. This model predicts that metabolic inhibitors would inhibit the electrogenic active K^+ pump at the tonoplast, decreasing the tonoplast conductance and thereby making the overall membrane *PD* less positive, flattening the *I-V* curve at positive potentials, and preventing turgor regulation in response to hyper-, but not necessarily hypotonic stress. In support of this idea, darkness reduces conductance and flattens the *I-V* curve. However, the resting *PD* does not become less positive in some cells (Fig. 3*a, b*). Furthermore, Heidecker et al. (2003b) found that CN^- drives membrane *PD* to 0 mV, diminishes the tonoplast conductance, $G_{TP,}$ by more than 50% and inhibits turgor regulation under hypertonic but not hypotonic stress. However, G_{TP} is inhibited by similar amounts under hyper- and hypotonic stress, whereas our model predicts it would be inhibited under hypertonic stress only, when the K^+ pump is activated. CN^- also inhibits plasma membrane conductance, G_{PL}, more under hypertonic than hypotonic stress, providing some support for the hypothesis that K^+ export at the plasma membrane is active, although more inhibition under hypotonic regulation would be expected in this case. Alternatively, ATP or other metabolic activity could also affect passive permeability (Katsuhara & Tazawa, 1990; Wu & Assmann, 1995; Shyng & Nichols, 1998) and thus respond to the effect of inhibitors.

We would also predict that inhibitors of passive K^+ transport should decrease conductance at the plasma membrane but not the tonoplast. It is not clear what effect this change would have on the total membrane *PD*. Since K^+ must cross the plasma membrane under both regulatory regimes, inhibitors of its transport should inhibit both hyper- and hypotonic regulation. Heidecker et al. (2003b) showed that Ba^{2+}, a common K^+ channel inhibitor, decreases G_{PL} in control cells by ~50%, and the change in *PD*, if significant, is negative. G_{TP} is inhibited to a smaller extent under hypertonic, but not hypotonic stress. As expected, the hypertonic turgor regulation is also inhibited while the hypotonic regulation is unchanged. The inhibition of G_{TP} could be due to movement of Ba^{2+} through the plasma membrane or responses of the tonoplast conductance to changes in cytoplasmic K^+ concentration, but the response seems too rapid for that. These results depend on the accuracy of the technique for separating the two conductances.

Cl^- transport always accompanies K^+ transport during regulation and therefore Cl^- must cross the plasma membrane at the time of hyper- and hypotonic regulation. We predict that inhibiting passive Cl^- movement would decrease conductance and inhibit regulation under both types of stress. Since in our model K^+ has a bigger conductance and a major impact on the plasma membrane *PD*, Cl^- transport inhibition would have a smaller effect on both conductance and *PD* than K^+ transport inhibition. Heidecker et al. (2003b) showed that DIDS, a Cl^- channel inhibitor, inhibits G_{PL} by slightly less than Ba^{2+}, ~40%, and has no significant effect on *PD*. There is no effect on G_{TP}. However, like Ba^{2+}, it inhibits turgor regulation under hyper- but not hypotonic stress. This result is puzzling. It also suggests that Cl^- crosses the membrane by different mechanisms under hypo- and hypertonic stress conditions. Patch-clamp experiments (Heidecker et al., 1999) provide evidence that DIDS is a Cl^- channel inhibitor in this organism, although the relationship between the channel measured in protoplasts and activities in intact, turgid cells is unclear. Since protoplasts have no turgor, they would be expected to be responding as though to hypertonic stress, if turgor or pressure is the signal that initiates turgor regulation.

Ba^{2+} and DIDS have additive effects on conductance (Heidecker et al., 2003b), as would be expected if they were inhibiting different channels. There was residual conductance when both inhibitors were present; this could be due to a "leak" conductance, as seen in charophytes (Beilby 1989; Beilby and Shepherd, 2001; Beilby et al. in press). Infusion of the inhibitors into the vacuole has no effect on G_{TP}, but it is not clear whether this is because the tonoplast channels are not sensitive to them, or because the inhibitor exposure was too short for it to diffuse through the vacuolar mucilage. Little is known about the turgor-sensing and signal-transduction mechanisms in *Valonia* and *Ventricaria*. Calcium is likely to be involved, as in many organisms, but this has not been researched. There is no consensus on the location of the turgor sensor. Gutknecht and co-workers put it at the plasma membrane/cell wall interface (Hastings & Gutknecht, 1974, 1976; Gutknecht et al., 1978), based on the simple but compelling observation that cells do not show regulatory responses when turgor is normal, but the cell as a whole experiences an increase or decrease in pressure. Thus the turgor sensor is not measuring absolute pressure, but the pressure difference between the cell interior and the external medium. Since this differential occurs at the interface between the plasma membrane and the cell wall, the sensor must be operating there. Zimmermann and co-workers argued that the sensor involves pressure-induced changes in membrane thickness, which could occur at any membrane (Coster & Zimmermann, 1976; Zimmermann et al., 1982). Since changes in response to turgor are often seen first at the vacuolar membrane (Heidecker et al., 2003b), they suggest that the turgor sensor is there. These arguments are based on indirect measurements such as membrane breakdown and voltage relaxation, and the molecular mechanisms for these electrical phenomena are not well understood. The electrical responses seen at the tonoplast could be the response to turgor change, and not to its initial detection.

Patch-clamp experiments (Heidecker et al., 1999) identified a stretch-activated channel in the plasma membrane, and this could well play the role of a turgor sensor (Bisson & Gutknecht, 1980; Pickard & Ding, 1992).

Other Marine Chlorophytes

There have been a number of other algae, some with giant cells, whose turgor regulation has been studied, but in general relatively little is known of their electrophysiological behavior. *Valoniopsis*, also in the Siphonocladales/Cladophorales complex of the Ulvophyceae, is closely allied to *Valonia* and *Ventricaria*. It also has positive E_{VO} and high tonoplast resistance (Findlay et al., 1978), as well as structural similarities, including stationary cytoplasm and regularly spaced nuclei (Shepherd et al., 2004). *Chaetomorpha*, also in the Siphonocladales/Cladophorales complex (Fig. 2), has a plasma membrane *PD* of −72 mV. This depolarizes with increasing external [K^+], indicating that it is largely due to a K^+ diffusion potential, as in *Valonia* and *Ventricaria*. E_{VO} ranges from −29 mV to +5 mV. The more positive state is associated with a higher conductance at the tonoplast, consistent with activation of a vacuolar electrogenic pump, similar to that of *Valonia*. Alternatively, the difference may be due to Cl^- fluxes at the tonoplast, which flux ratio analysis indicates cannot be all passive (Findlay et al., 1971). K^+ may also be of primary importance in turgor regulation in this alga (Zimmermann & Steudle, 1971).

Codium and *Derbesia* (sometimes known as *Halicystis)* are in the order Caulerpales within the Ulvophyceae (Fig. 2). They show more typically negative potentials. Studies of turgor regulation in these algae have focused more on Cl^-. In *Codium* (Bisson & Gutknecht, 1975), Cl^- concentrations paralleled changes in turgor during regulation, while K^+ and Na^+ were more variable. This implies that Cl^- fluxes were regulated by turgor, whilst counter ion fluxes were determined by other factors, such as available energy. Cl^- also plays a major role in *Derbesia*. Active Cl^- transport carries 90% of the short-circuit current, and short-circuit current varies with turgor (Graves & Gutknecht, 1976).

After *Valonia* and *Ventricaria*, the best studied chlorophyte cells are the large cells of the Mermaid's Wineglass, *Acetabularia*, in the Order Dasycladales, also Class Ulvophyceae. Its electrophysiological characteristics are unique. *Acetabularia* has very negative membrane potential, which is due to an electrogenic Cl^--translocating ATPase (Gradmann, 1989). It experiences a constant increase in turgor pressure, even when the osmolarity of the medium is maintained constant (Wendler et al., 1983). Turgor pressure regulation is accompanied by action potentials, or by bursts of Cl^- efflux that do not affect the cytoplasmic *PD* (Gradmann, 1976; Gradmann & Mummert, 1980; Wendler et al., 1983).

Spontaneous action potentials become more frequent when cell turgor pressure increases (Wendler et al., 1983). Action potentials, which are very slow (~100 s; Mummert & Gradmann, 1991), result in a large Cl^- efflux (Gradmann et al., 1973), followed by a large K^+ efflux, (Gradmann, 1976). Action potentials are regarded as the main mechanism of turgor regulation (Mummert & Gradmann, 1991). Indeed, Gradmann & Mummert (1980) suggest that osmoregulation was the primary physiological role of action potentials in the evolutionary past.

The action potentials are accompanied by transient increases in cytoplasmic Na^+ (Amtmann & Gradmann, 1994). The proposal that *Acetabularia* maintains low internal Na^+ in a medium of high Na^+ via vesicular Na^+ transport (*see* Gradmann & Mummert, 1984) was disputed (Wendler et al., 1983). However, using Na^+-sensitive micro-electrodes, Amtmann & Gradmann (1994) demonstrated that Na^+ exchange is electrically silent, being unaffected by changes in membrane voltage, electrochemical gradients of H^+ or Cl^- across the plasma membrane, or by cytoplasmic ATP. They proposed that a V-type ATPase maintains a large pH gradient across the tonoplast, and that vesicles with vacuole-like properties accumulate Na^+ (and Cl^-) and release it from the cell via exocytosis. The vesicular compartment thus communicates with both the cytoplasm and the external medium.

Comparisons between Salt-tolerant Charophytes and *Valonia*/*Ventricaria*

The differences between these two groups of algae are summarized in Table 1. They differ strongly in both their fundamental electrophysiology and in their responses to salt stress.

Charophyte algae, some fungi, and land plants are characterized by a very negative membrane potential (−150 to −250 mV), due to a vanadate-sensitive H^+-pumping ATPase (Spanswick, 1972; Smith & Raven, 1979; Spanswick, 1980; Spanswick, 1981; Sze et al., 1999). The electrical characteristics are dominated by the plasma membrane, which is more negative and has a lower conductance than the tonoplast (Spanswick & Williams, 1964; Beilby & Shepherd, 1989). The strongly negative membrane potential affects the driving force for any passive ion movement. Moreover, many active transport systems are due to symport or antiport with protons, and are therefore dependent on the electrochemical driving force for H^+ ($\Delta\bar{\mu}_H$) generated by this pump (the so-called "chemiosmotic model"). Charophytes have both a "K state", in which the *PD* is dominated by

Table 1. Summary of the differences between Charophytes and *Valonia*/*Ventricaria*

	Lamprothamnium/*Chara*	*Valonia*/*Ventricaria*
PD across outer membrane	−80 to −220 mV	−70 mV
Source of PD	Electrogenic: H^+ ATPase, K^+ diffusion in K^+ state, H^+ diffusion in high pH state	K^+ diffusion ??
PD across inner membrane	+ 20 mV	+ 80 mV
Source of PD	H^+ ATPase and pyrophosphatase	K^+ ATPase ??
Ratio of membrane conductances	Conductance of outer membrane < inner membrane	Conductance of outer membrane > inner membrane
Hypotonic stress response		
PD	Less negative	More negative
Conductance	Increase over much of range	Increase at negative PD Decrease at positive PD
Transport systems	Increase in Cl^- and K^+ conductances, G_{Cl}, G_K (max around −50 mV); G_{bkg}	Increase in G_K? outward K^+ pump?
Signal transduction	Increased cytoplasmic Ca^{2+}	Sensor at plasma membrane
Hypertonic stress response		
PD	More negative	More positive
Conductance	No consistent change	Increase at positive PD Decrease at negative PD
Transport systems	Increase in proton pump (after initial decrease)	Increase in inward K^+ pump at tonoplast

See text for details.

E_K, and a hyperpolarized state dominated by the electrogenic H^+ pump (Spanswick et al., 1967; Bisson & Walker,1982; Beilby, 1986; Beilby, 1989). In *Valonia* and *Ventricaria*, the plasma membrane *PD* appears to be in a persistent K state. However, the tonoplast has a large positive *PD*, putatively due to active electrogenic K^+ uptake, and this renders the overall cell *PD* positive.

Turgor regulation is also very different in Charophytes and *Valonia*/*Ventricaria*. (*See* Beilby et al. (*in press*) for a review of turgor regulation in Charophytes.) In the canonical fast response to hypotonic stress, cells of Charophytes such as *Lamprothamnium* and *Chara longifolia* become less negative than E_K, and G_K and G_{Cl} both increase over a large voltage range, allowing passive loss of both ions from the cell (Bisson & Kirst, 1980; Okazaki et al., 1984; Hoffmann & Bisson, 1987; Beilby et al., 1999). The response requires a minimum concentration of external Ca^{2+} and an increase of cytoplasmic Ca^{2+} (Okazaki & Tazawa, 1990; Stento et al., 2000). There is another, slower response, which does not require membrane depolarization or Ca^{2+} entry (Bisson et al., 1995; Beilby et al., 1999; Stento et al., 2000). In contrast, *Valonia* and *Ventricaria* become more negative on hypotonic stress, due to an increase in either passive or active K^+ export at the plasma membrane, and inhibition of active K^+ uptake at the tonoplast. The conductance increases at negative potentials and decreases at positive (*see* Fig. 3*a* – *c*). Cl^- loss is likely to be passive.

In response to hypertonic stress, the *PD* of *Lamprothamnium* and *Chara longifolia* cells in general changes little or hyperpolarizes (Bisson & Kirst, 1980; Okazaki et al., 1984; Reid et al., 1984; Hoffmann & Bisson, 1987). There is an increase in H^+-pumping, which drives increased passive K^+ uptake and Cl^- uptake by H^+ symport (Beilby & Shepherd, 2001). *Valonia* and *Ventricaria PD*s become more positive, possibly due to an increase in active K^+ uptake at the tonoplast. Conductance increases at positive potentials and decreases at negative (Fig. 3 *a*–*c*).

The electrophysiological turgor regulatory responses of salt-tolerant charophytes and *Ventricaria* are very different. The end result, regulatory movement of K^+ and Cl^-, is the same.

Summary

The Charophyte algae have long been used as an organism to model processes shared by land plants, and have been useful in generating models to explain the electrophysiology and transport in these organisms. The very different electrophysiological processes in chlorophyte algae demonstrate the variety of possible responses to similar problems. By presenting alternative solutions, they can increase the breadth of tools available to those interested in engineering into economically important plants different solutions to stresses that decrease productivity.

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