J. Membrane Biol. 213, 47–63 (2006)
DOI: 10.1007/s00232-006-0047-9

The Journal of
Membrane
Biology

Elucidation of the Mechanisms Underlying Hypo-osmotically Induced Turgor Pressure Regulation in the Marine Alga *Valonia utricularis*

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Received: 24 June 2006/Revised: 9 October 2006

Abstract. Exposure of the giant marine alga *Valonia utricularis* to acute hypo-osmotic shocks induces a transient increase in turgor pressure and subsequent back-regulation. Separate recording of the electrical properties of tonoplast and plasmalemma together with turgor pressure was performed by using a vacuolar perfusion assembly. Hypo-osmotic turgor pressure regulation was inhibited by external addition of 300 μM of the membrane-permeable ion channel blocker 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB). In the presence of 100 μM NPPB, regulation could only be inhibited by simultaneous external addition of 200 μM 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), a membrane-impermeable inhibitor of Cl^- transport. At concentrations of about 100 μM, NPPB seems to selectively inhibit Cl^- transporters in the tonoplast and K^+ transporters in the plasmalemma, whereas 300 μM NPPB inhibits K^+ and Cl^- transporters in both membranes. Evidence was achieved by measuring the tonoplast and plasmalemma conductances (G_t and G_p) in low-Cl^- and K^+-free artificial seawater. Inhibition of turgor pressure regulation by 300 μM NPPB was accompanied by about 85% reduction of G_t and G_p. Vacuolar addition of sulfate, an inhibitor of tonoplast Cl^- transporters, together with external addition of DIDS and Ba^{2+} (an inhibitor of K^+ transporters) also strongly reduced G_p and G_t but did not affect hypo-osmotic turgor pressure regulation. These and many other findings suggest that KCl efflux partly occurs via electrically silent transport systems. Candidates are vacuolar entities that are disconnected from the huge and many-folded central vacuole or that become disconnected upon disproportionate swelling of originally interconnected vacuolar entities upon acute hypo-osmotic challenge.

Key words: *Valonia utricularis* — Charge-pulse relaxation — Tonoplast conductance — 5-Nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) — Turgor pressure regulation — Vesicular K^+ transport

Introduction

Adjustment of turgor pressure in response to changes in the osmolality of the surrounding environment is a key requirement for plant survival and growth (Green, Erickson & Buggy, 1971; Cleland, 1971, 1977; Zimmermann, 1978; Bentrup, 1980; Findlay, 2001; Hedrich et al., 2001; Shabala & Lew, 2002; Zimmermann et al., 2004). Close control of turgor pressure over a large range of external osmolalities is particularly seen in marine algae inhabiting estuaries, coastal pools and surf zones (Kesseler, 1964, 1965; Steudle & Zimmermann, 1971; Zimmermann & Steudle, 1974; Bisson & Kirst, 1980; Kirst, 1990; Shepherd, Beilby & Heslop, 1999; Stento et al., 2000; Bisson & Beilby, 2002). In these species, excursions of turgor pressure from the steady-state value upon hypo-osmotic or hyper-osmotic challenge are reversed by release or uptake of K^+ and Cl^-. Progress in the elucidation of the mechanisms underlying turgor pressure sensing and transformation of turgor pressure signals into membrane transport has recently been achieved by recording simultaneously turgor pressure and the electrical parameters of the plasmalemma and tonoplast of osmotically stressed cells of the giant, multinucleated marine alga *Valonia utricularis* (Heidecker et al., 2003a, 2003b; Mimietz et al., 2003). These electrophysiological studies together with electron microscopic investigations of turgescent cells have given evidence that the tonoplast is multifolded,

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resulting in a sponge-like organization of the 5-μm-thick cytoplasm surrounding the central vacuole (occupying about 95% of the cell volume; Shihira-Ishikawa & Nawata, 1992; Mimietz et al., 2003; Shepherd, Beilby & Bisson, 2004). The many vacuolar entities encircled by cytoplasmic strands are interconnected between each other and the central vacuole. These structural peculiarities apparently give rise to extended unstirred layers. Unstirred layers together with a highly filamentary network of acid mucopolysaccharides within the central vacuole are apparently linked with the capability of the alga to regulate turgor pressure (Heidecker et al., 2003a; Mimietz et al., 2003). Vacuolar perfusion of cells under clamped turgescent conditions and concomitant electrical measurements of the individual conductances of the tonoplast and the plasmalemma (using integrated microelectrodes for generation of charge-pulse relaxations; Wang et al., 1997a) have given further clear-cut evidence that the onset of turgor pressure regulation by modulation of ion transport is triggered by turgor pressure but not by osmotic pressure gradients or water flow (Heidecker et al., 2003b). These studies have also demonstrated that changes in turgor pressure are most likely sensed by the multifolded tonoplast because the disturbance of the water equilibrium by hypo- or hyper-osmotic stress is reflected first in changes of the area-specific conductance of this membrane before conductance changes are considerably delayed in the plasmalemma. However, the signaling pathways from the tonoplast to the plasmalemma as well as the processes underlying the coordination between the ion transporters in both membranes at the onset of turgor pressure regulation are mainly unresolved. At present, we know from the work of Gutknecht (1968) and Zimmermann, Büchner & Benz (1982) that K^+ (and partly Cl^-) influx strongly decreases with an increase in turgor pressure. We know further (Heidecker et al., 2003b) that external addition of Ba^{2+} and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), i.e., membrane-impermeable K^+ and Cl^- transport inhibitors, prevents hyper-osmotic, but not hypo-osmotic, turgor pressure regulation. Heidecker et al. (2003b) have also shown that part of the ion transporters in the plasmalemma are adenosine triphosphate-dependent and that transporters are upregulated upon hyper-osmotic challenge, whereas K^+ and Cl^- efflux under hypo-osmotic conditions occurs most likely only in a passive way.

The modulation of ion transporters in the tonoplast under hypo- and hyper-osmotic turgor pressure regulation has yet to be explored. The problem is the failure up to the present to block these transporters by external or vacuolar addition of Ba^{2+} and DIDS. Addition of inhibitors or other vacuolar-extrinsic compounds to the artificial perfusion solutions also very frequently gives rise to cell damage. Thus, manipulations of the vacuolar sap designed to selectively inhibit ion transporters across the tonoplast (and the plasmalemma) are extremely limited and may demand different approaches for hyper- and hypo-osmotically induced turgor pressure regulations due to the disproportionate responses of the vacuolar entities upon turgor pressure increase (Mimietz et al., 2003).

In this report, we demonstrate that ion transporters in the tonoplast as well as in the plasmalemma of *V. utricularis* could reversibly be inhibited by 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB). This potent inhibitor of ion channels in animal and plant cells is membrane-permeable and can thus be added externally without deterioration of the cells (Wangemann et al., 1986; Schroeder, Schmidt & Sheaffer, 1993; Thomine, Guern & Barbier-Brygoo, 1997). Turgor pressure measurements in the presence of this inhibitor under various pressure clamp and media conditions revealed that hypo-osmotic turgor pressure regulation could be inhibited by 300 μM external NPPB or by addition of 100 μM NPPB and 200 μM DIDS to artificial seawater. Under these conditions, the conductances of the tonoplast and of the plasmalemma were reduced by >80% with respect to the initial values. A reduction of the conductances of the two membranes of the same order of magnitude was also observed when the Cl^- transporters in the tonoplast were inhibited by vacuolar (membrane-impermeable) sulfate in the presence of external Ba^{2+} and DIDS. However, under these conditions, hypo-osmotic turgor pressure regulation was not inhibited. These findings and many other results can only be explained at present if we assume that part of the KCl efflux during hypo-osmotic turgor pressure regulation is electrically silent (i.e., electroneutral). Candidates for salt vehicles are disconnected tiny vacuolar entities that are accessible by NPPB but not by sulfate, Ba^{2+} or DIDS because of the plasmalemma and tonoplast impermeability of these inhibitors.

Materials and Methods

Culture Conditions

Cells of *V. utricularis* (Cladophorales, Chlorophyceae) were collected at the coast of Ischia, in the gulf of Naples, Italy, and cultivated in 40-liter tanks of Mediterranean seawater (MSW, osmolality 1,127 mosmol · kg^{-1} and pH 8.1) under a 12 h light/dark regime (2 x 36 W Fluora lamps; Osram, Munich, Germany) at 16°C. The seawater was continuously aerated.

Experimental Conditions

For the experiments, mature and geometrically even cells were selected and fixed in a small Perspex™ (Röhm, Darmstadt, Germany) chamber perfused with artificial seawater (ASW)

containing 545 mM NaCl, 12 mM KCl, 11 mM $CaCl_2$ and 10 mM $MgCl_2$. Chemicals were purchased from Merck (Darmstadt, Germany) if not stated otherwise. The pH was adjusted to 8.1 by addition of 10 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid)/NaOH (HEPES/NaOH; Sigma, St. Louis, MO). For hypo-osmotic experiments, the osmolality of ASW was reduced by appropriate reduction of the NaCl concentration. K^+-free, iso-osmotic ASW was prepared by replacing KCl by NaCl. Lowering of Cl^- in iso-osmotic ASW to 20 mM was achieved by appropriate replacement of Cl^- with 2-(*N*-morpholino)ethanesulfonic acid (MES^-). By taking the reflection coefficient of MES^- into account (Heidecker et al., 2003a), the low-Cl^- solution contained 590 mM MES^-, 545 mM NaOH, 12 mM KOH, 11 mM $CaSO_4$, 10 mM $MgCl_2$ and 10 mM HEPES (low-Cl^- ASW). The pH was adjusted to 8.1 with 15 mM H_2SO_4. The osmolality of the various solutions was determined cryoscopically using the Osmomat 030-M (Gonotec, Berlin, Germany). The osmolality values of the solutions given below represent the mean of five independent measurements.

The experiments were performed at about 22°C. A fiber-optic light source (model KL 1500; Schott, Mainz, Germany) was used to illuminate the cells during the experiments.

Inhibitor

For evaluation of the contribution of the ion transporters in the tonoplast and plasmalemma to the turgor pressure regulation process, NPPB (Sigma) dissolved in dimethyl sulfoxide (DMSO) at a concentration of 3% (w/v) was used. Final concentrations of the inhibitor were adjusted to 50–300 μM. The corresponding final DMSO concentration did not exceed 0.3%. DMSO did not affect turgor pressure and the electrical properties of the cells, as verified by control experiments in which cells were treated with up to 0.5% DMSO. The NaCl concentration of media containing the inhibitor and DMSO was adjusted accordingly to maintain iso-osmotic conditions. Evaporation of DMSO and, in turn, changes in the osmolality of the media during the experimental procedure were avoided by closing the measuring chamber with a plastic cover sheet. In part of the experiments, plasmalemma K^+ transporters were blocked by $BaCl_2$ (Merck). DIDS (Sigma) was used to selectively inhibit plasmalemma Cl^- transporters. Usually, inital concentrations of $BaCl_2$ and DIDS were adjusted to 2 and 0.2 mM, respectively (Spiess et al., 1993; Heidecker et al., 2003b; Binder et al., 2003). In some experiments, 5 mM $BaCl_2$ and/or 0.4 mM DIDS were applied.

Vacuolar Perfusion Assembly

Turgor pressure measurements as well as vacuolar membrane potential measurements and separate determination of the RC (resistance/capacitance) properties of plasmalemma and tonoplast were performed by using the vacuolar perfusion assembly described in detail elsewhere (Wang et al., 1997a; Ryser et al., 1999; Heidecker et al., 2003b). Briefly, two borosilicate microcapillaries (tip diameter about 30 μm) were inserted into the central vacuole. If not stated otherwise, the microcapillaries were filled with artificial vacuolar sap (AVS) containing 420 mM KCl, 210 mM NaCl, 3 mM $CaCl_2$ and 3 mM $MgCl_2$ (osmolality 1,210 mosmol $\cdot$ kg^{-1}). The pH was adjusted to 6.3 with 3 mM phosphate buffer. Chemicals were purchased from Merck. The perfusion rate was adjusted to 15 μl $\cdot$ min^{-1}. After about 60 min, perfusion was stopped. This perfusion time was sufficient to exchange the vacuolar sap (50–160 μl) completely by AVS (Wang et al., 1997a). Integrated pressure transducers additionally allow measurements of turgor pressure and turgor pressure relaxations. Pressure regulation valves were used to change or to clamp turgor pressure away from the steady-state values.

The electrical parameters of the membranes were measured using the charge pulse technique (*for details*, *see* Zimmermann et al., 1982; Benz & Zimmermann, 1983). Briefly, charging of the membranes occurs by injection of current pulses of 1 μs duration and an amplitude of about 20 mA. Subsequent rapid relaxation of the induced voltage (within a few milliseconds) is mediated exclusively by charge movement through the membranes; thus, unstirred layer and concentration polarization effects do not play a role. The microelectrode for charge-pulse injection consisted of a 10-μm-thick platinum wire and was carefully moved through one of the microcapillaries deeply into the vacuole. The wire was connected to a fast, commercial pulse generator (model 214B; Hewlett-Packard, Palo Alto, CA) through a diode with a reverse resistance higher than 10^{10} Ω. The reference current-electrode in the bathing ASW consisted of a coiled wire. The microelectrode for recording the vacuolar membrane potential and voltage relaxation was filled with 3 M KCl and contained an Ag/AgCl wire. The microelectrode was integrated into the other microcapillary as described elsewhere (Wang et al., 1997a; Ryser et al., 1999). In order to test for rectifying properties of the membranes, charge pulses of both positive and negative polarity were injected.

Vacuolar Perfusion Solutions

The dependence of tonoplast conductance (G_t) on the vacuolar K^+ concentration was investigated by perfusing the cells with a modified AVS solution that contained 420 mM tetraethylammonium (TEA^+) instead of K^+. In low-Cl^- AVS medium, 630 mM Cl^- was replaced by 660 mM MES^- and by 60 mM H_2SO_4, keeping the effective osmotic pressure of the medium constant. The final Cl^- concentration was 12 mM. This high concentration of H_2SO_4 was required to adjust the pH of the AVS medium to the normal value of 6.3 at this extremely low Cl^- concentration. For control measurements and to examine the effect of SO_4^{2-} on the tonoplast membrane, AVS with 522 mM Cl^- and 60 mM SO_4^{2-} was used. Additionally, the sulfate concentration was varied (from 3, 6.5, 10, 40 to 60 mM) by adequate replacement of Cl^-, whereas all other components (including pH) remained unchanged. The final Cl^- concentrations were 636, 629, 622, 562 and 522 mM, respectively. To examine a possible influence of vacuolar Ca^{2+} on G_t, AVS medium containing 20 mM $CaCl_2$ and 10 mM K_2SO_4 was used as well as a Ca^{2+}-free AVS medium. The osmotic pressure of these solutions was adjusted by dilution with distilled water and by adding KCl, respectively. It should further be noted that liquid junction potentials induced at the potential-recording electrode during vacuolar perfusion with these media were corrected if necessary.

Analysis of Charge-Pulse Relaxation Spectra

The semilogarithmic plot of the voltage relaxation, V_g, *vs.* time, t, recorded on cells challenged with a charge pulse could be fitted by the sum of two exponential decays for a first approximation using a multiple exponential-fitting program (Benz & Zimmermann, 1983) and/or the Levenberg-Marquardt algorithm of nonlinear regression:

$$V_g(t) = V_1 \exp\left(\frac{-t}{\tau_1}\right) + V_2 \exp\left(\frac{-t}{\tau_2}\right) = V_0\left(a_1 \exp\left(\frac{-t}{\tau_1}\right) + a_2 \exp\left(\frac{-t}{\tau_2}\right)\right)$$

with

$$V_0 = V_1 + V_2$$

and

$$a_1 = \frac{V_1}{V_0}; a_2 = \frac{V_2}{V_0} \quad (1)$$

where $V_{1,2}$ are the initial absolute amplitudes of the rapidly and slowly decaying component of the voltage relaxation, respectively; $\tau_{1,2}$ are the corresponding relaxation time constants; and $a_{1,2}$ are the corresponding initial relative amplitudes (*for details, see* Wang et al., 1997a; Ryser et al., 1999). As shown previously (Wang et al., 1997a), the fast relaxation resulted from the electrical properties of the plasmalemma, whereas the slow one must be attributed to those of the tonoplast. Accordingly, the area-specific resistances and capacitances of the tonoplast and the plasmalemma are given by the following equations:

$$C_p = \frac{Q}{A_{Cell} V_1}; R_p = \frac{1}{G_p} = \frac{\tau_1}{C_p} \quad (2)$$

$$C_t = \frac{Q}{A_{Cell} V_2}; R_t = \frac{1}{G_t} = \frac{\tau_2}{C_t} \quad (3)$$

where Q is the injected charge; A_{cell} is the geometric surface area of the cell; and $C_{t,p}$, $G_{t,p}$ and $R_{t,p}$ are the area-specific capacitances, conductances and resistances of the tonoplast and plasmalemma, respectively.

Results

Turgor pressure, P, and the vacuolar membrane potential, V_m, usually assumed constant values ranging 0.050–0.200 MPa and + 4.0–+ 9.0 mV, respectively, when measured 30 min after insertion of the perfusion inlet and outlet microcapillaries into the vacuole of *V. utricularis*. These values were taken as evidence that leaks were completely resealed. In most of the experiments, the RC properties of the two membranes were determined by injection of positive charge pulses. However, in part of the experiments, sequences of positive and negative pulses were injected alternately into the vacuole. Voltage relaxation curves induced by charge pulses of positive and negative polarity yielded data for the capacitance and conductance of the tonoplast and the plasmalemma that differed by $< 10\%$ (for an exception, see further below). This finding indicates that tonoplast and plasmalemma have no rectifying properties and that inward and outward current obviously passed through the same membrane areas. Therefore, membrane conductance and capacitance data obtained by positive and negative charging of the membranes were pooled.

In the first set of experiments, the effects of vacuolar ions on turgor pressure and the electrical parameters of the two membranes were investigated.

Sulfate, an Inhibitor of the Cl^- Conductance of the Tonoplast

The response of the electrical parameters of the tonoplast and plasmalemma to external iso-osmotic replacement of Cl^- in ASW by sulfate was determined on cells which were not perfused after impalement of the microcapillaries. Sulfate concentrations of up to 50 mM did not induce changes in the membrane potential, V_m, or in the electrical conductances of the tonoplast, G_t, and the plasmalemma, G_p (*data not shown*). Obviously, sulfate did not interfere with the Cl^- conductance of the plasmalemma. Consistent with this, external sulfate had no effect on hypo-osmotic turgor pressure regulation (*data not shown*).

Dramatic changes of G_t, but not of G_p, V_m and P, were observed upon vacuolar application of sulfate. Interfering effects of Cl^- on the tonoplast conductance could be excluded by experiments in which the native vacuole sap (containing about 640 mM Cl^- and 3 mM sulfate; Spiess, 1996) was replaced by AVS containing 60 mM sulfate and 522 mM Cl^- followed by perfusion with low-Cl^- AVS (12 mM Cl^-, 660 mM MES^- and 60 mM sulfuric acid; for details, see Materials and Methods). Clear-cut changes in V_m were obviously only induced by changes of the vacuolar Cl^- concentration but not by sulfate. In the experiment shown in Figure 1A, the vacuolar membrane potential dropped from $V_{m1} = +5.4$ mV to $V_{m2} = -12.0$ mV (values mean ± standard deviation [SD]; $V_{m1} = +7.3 \pm 3.0$ *vs.* $V_{m2} = -11.7 \pm 0.5$ mV; $n = 3$) same notation throughout the text while clamping the pressure to $P_0 = 0.087$ MPa. The effect on V_m was reversible, as shown by perfusion of the vacuole with standard AVS again (Fig. 1A). It is evident from Figure 1B that G_p did not respond to perfusion with sulfate-containing high-Cl^- AVS, whereas G_t dropped immediately from $G_{t0} = 67$ S · m^{-2} to $G_{t1} = 21$ S · m^{-2} (mean values $G_{t0} = 61 \pm 9$ S · m^{-2} *vs.* $G_{t1} = 25 \pm 8$ S · m^{-2}; $n = 3$). No further change in G_t was observed upon perfusion with low-Cl^- AVS and subsequent replacement of the low-Cl^- AVS by (sulfate-free) standard AVS. The initial reduction of G_t by sulfate-containing medium was obviously not an artifact induced by perfusion because replacement of native vacuolar sap by standard AVS did not affect G_t. Hence, these data can only be interpreted in terms of an irreversible inhibition of the tonoplast Cl^- conductance by increasing the vacuolar sulfate concentration to 60 mM. [1]

The inhibitory effect of sulfate on G_t decreased with decreasing concentration. Inhibition was only reversible at concentrations lower than 40 mM, as shown by subsequent perfusion with standard AVS. Plot of the G_t values (normalized to the G_t values recorded with native vacuolar sap or after perfusion

[1] Unfortunately, for preparation of AVS media with a low Cl^- concentration, 60 mM sulfuric acid were required to adjust the pH to the usual value of 6.3. Hence, no experimental strategy was available to separate a possible effect of lowering the vacuolar Cl^- concentration on G_t from an effect induced by vacuolar sulfate.

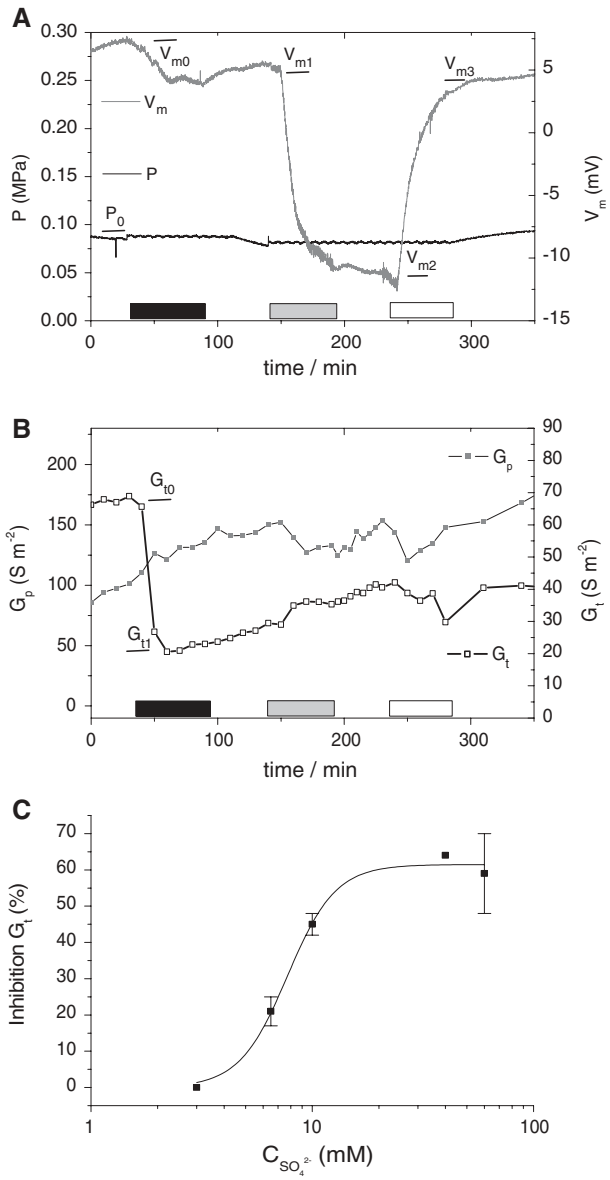

Fig. 1. Effect of vacuolar sulfate on the tonoplast conductance of *V. utricularis*. (**A**) Typical response of the vacuolar membrane potential, V_m (*gray trace*), and the turgor pressure, P (*black trace*), to vacuolar perfusion with AVS containing 60 mM sulfate (*black bar*, $[Cl^-]$ = 522 mM) and subsequent perfusion with low-Cl^- AVS containing 60 mM sulfate, 12 mM Cl^- and 630 mM MES^- (*gray bar*). The cell was bathed in ASW throughout the experiment; the turgor pressure remained almost constant at P_0 = 0.087 MPa and was clamped at that value during vacuolar perfusion. The first perfusion step led to a slight decrease of V_m from V_{m0} = +7.4 mV to V_{m1} = +5.4 mV. When the vacuole was subsequently perfused with low-Cl^- AVS, V_m responded by a strong hyperpolarization to V_{m2} = −12.0 mV within 80 min. Hyperpolarization was reversed by subsequent perfusion with standard AVS (*white bar*, V_{m3} = +4.8 mV). (**B**) Time courses of tonoplast conductance, G_t (□), and plasmalemma conductance, G_p (■), for the same experiment as shown in (*A*). Whereas G_p remained more or less constant during perfusion with different AVS media, G_t dropped from G_{t0} = 67 S · m^{-2} to G_{t1} = 21 S · m^{-2} during perfusion with AVS containing 60 mM sulfate and 522 mM Cl^- but remained unaffected by subsequent perfusion with low-Cl^- AVS and standard AVS, respectively. (**C**) Hill plot of the inhibition of tonoplast conductance, G_t, by vacuolar sulfate; G_t inhibition is normalized to the conductance of the tonoplast in the absence of sulfate. The following concentrations were tested (number of repetitions in parentheses): 3 (1), 6.5 (4), 10 (3), 40 (1) and 60 (3). Mean values and SD are given where appropriate. Fitting the Hill equation (*continuous line;* Weiss, 1997) to the data yielded 50% inhibition at a sulfate concentration of 7.7 mM and a Hill coefficient of 4.0.

with standard AVS) yielded a sigmoid curve, which could be fitted by the Hill equation (Weiss, 1997)[2]. Inspection of the curve in Figure 1C shows that maximum inhibition occurred at a concentration of 40 mM sulfate and half-maximal inhibition at a sulfate concentration of 7.7 mM. From the data, a Hill coefficient of 4.0 was calculated. Indirect effects of sulfate on G_t by binding of free Ca^{2+} in the vacuolar sap are unlikely, even though G_t decreased from 48 ± 9 S · m^{-2} to 32 ± 9 S · m^{-2} (i.e., by about 33%, n = 5) when AVS (containing 3 mM Ca^{2+}) was replaced by Ca^{2+}-free AVS. Calculations using the computer program Bound and Determined (Brooks & Storey, 1992) demonstrated that a concentration of 10 mM sulfate, which inhibits 90% of the sulfate-sensitive component of G_t (Fig. 1C), can only induce about 5% change in the free Ca^{2+} concentration. Moreover, 10 mM sulfate had an almost identical effect on G_t of an alga when the AVS contained 3 or 20 mM Ca^{2+}. On average, G_t was reduced by 47% (n = 3) and 40% (n = 2), respectively. This excluded the possibility that the effect was due to precipitation of Ca^{2+} by sulfate.

Cells bathed in ASW retained their capability for hypo-osmotic turgor regulation after perfusion with AVS containing 40 mM sulfate. This could be demonstrated by vacuolar perfusion with sulfate and a subsequent 80-min pressure clamp at a value of about 0.050 MPa above P_0. Upon release of the clamp, turgor pressure started to relax slowly until P_0 was almost reestablished (n = 9, *data not shown*). The time constant of turgor pressure relaxation was in agreement with previous results (Heidecker et al., 2003b).

In the presence of 60 mM sulfate and 522 mM Cl^- in the vacuolar sap, no further reduction in G_t occurred when the external Cl^- concentration was reduced from about 600 to 20 mM by a perfusion of the bath with low-Cl^- ASW (n = 2, *data not shown*). In agreement with previous results (Wang et al., 1991; see also below, Fig. 3), lowering of the external Cl^- resulted in a decrease of G_p by >60%. V_m decreased by about 4 mV. Consistently, G_t and V_m remained constant in three out of five experiments when sulfate

[2]This was justified by the finding that replacement of the vacuolar sap by AVS did not result in any changes of turgor pressure and the electrical parameters of the two membranes

(15 mM) was added to the AVS about 50 min after lowering the external Cl^- concentration to 20 mM. In two experiments, a further decrease in G_t was observed (by about 45% with respect to the initial value); V_m also dropped to more negative values. Similar results were obtained when 60 mM sulfate was added to the vacuolar sap of cells incubated in low-Cl^- ASW ($n = 2$). In these experiments, the pressure was clamped to the initial steady-state pressure, P_0.

Effect of Replacement of Vacuolar K^+ by TEA on Tonoplast Conductance

Perfusion of the central vacuole with AVS in which K^+ had been replaced by an equivalent concentration of TEA was performed on cells preperfused with AVS and on cells in which the vacuolar sap had not been replaced by AVS before treatment with TEA. During the experiments, the turgor pressure was clamped to its original value. Both experimental approaches yielded similar results. V_m decreased slightly from 4.0 ± 0.6 to 2.7 ± 0.4 mV ($n = 6$). G_p remained unaffected, whereas G_t showed a quite variable response. In three cells G_t increased by up to 37% at the end of perfusion, in two cells G_t remained unchanged and in one cell G_t decreased by 21%.

Effects of External NPPB on Turgor Pressure and the Electrical Membrane Properties under Iso-osmotic Conditions

As mentioned at the beginning, addition of extrinsic compounds to the vacuolar perfusion solution could result in membrane or cell damage. This was also observed very often when the membrane-permeable inhibitor NPPB was added to the perfusion solution at a concentration of 50 μM or more. Therefore, the effect of NPPB on the tonoplast and plasmalemma conductances was exclusively studied by external application. However, it should be noted that in experiments in which detrimental effects of vacuolar NPPB did not occur, similar results were obtained as in the case of external addition.

A typical experiment showing the effect of external 300 μM NPPB on P, V_m and the membrane conductances of a nonperfused *Valonia* cell is shown in Figure 2. The osmotic pressure of the ASW was kept at a constant value of 1,151 mosmol · kg^{-1} throughout the experiment. Addition of the inhibitor at $t = 90$ min resulted in a decrease of the vacuolar membrane potential from $V_{m1} = +8.3$ mV to $V_{m2} = +2.0$ mV and in a slow decrease of the turgor pressure from $P_0 = 0.181$ MPa to about 0.150 MPa (Fig. 2A). The plasmalemma conductance, G_p, and the tonoplast conductance, G_t, dropped from $G_{p1} = 108$ S · m^{-2} to $G_{p2} = 15$ S · m^{-2} (i.e., by 86%) and from $G_{t1} = 67$ S · m^{-2} to $G_{t2} = 10$ S · m^{-2} (i.e., by 85%) (Fig. 2B). After 63 min, the inhibitor was removed. This resulted in recovery of the electrical parameters. The initial values were (almost) reestablished after 207 min. P continued to decrease slowly to $P_1 = 0.134$ MPa after removal of the inhibitor but started to increase again about 200 min later.

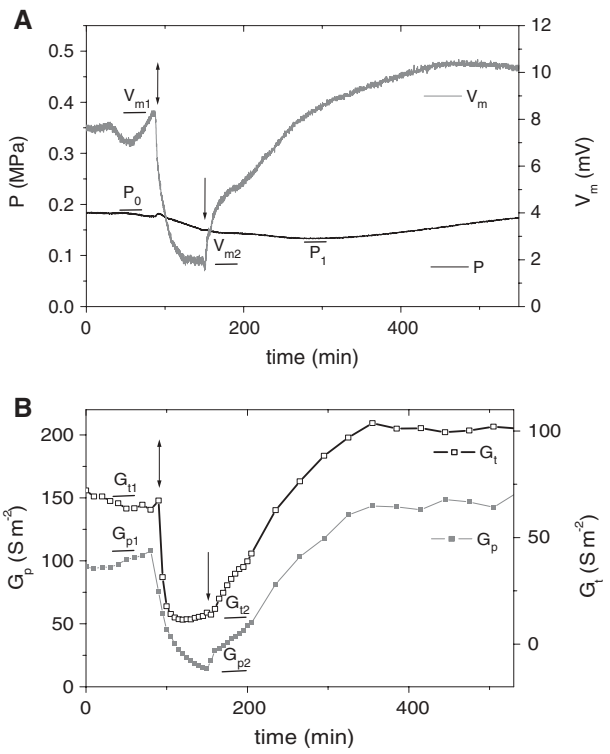

Fig. 2. Typical response of the turgor pressure (P), vacuolar membrane potential (V_m) and plasmalemma and tonoplast conductances (G_p and G_t, respectively) of a *V. utricularis* cell upon external addition of 300 μM NPPB to iso-osmotic ASW. (**A**) Effect of NPPB on V_m (*gray trace*) and P (*black trace*). Addition of the inhibitor (*double-headed arrow*) caused a drop in V_m from $V_{m1} = +8.3$ mV to $V_{m2} = +2.0$ mV within 30 min. After removal of the inhibitor 63 min later (*downward arrow*), V_m increased again and reached approximately the initial value. P decreased slowly in the presence of the inhibitor from $P_0 = 0.181$ to 0.155 MPa. After removal of NPPB, the decrease continued until about 200 min later a minimum value of $P_1 = 0.134$ MPa was attained and subsequently a slow increase occurred. (**B**) Response of G_p (■) and G_t (□). Addition of the inhibitor (*double-headed arrow*) resulted in a decrease of the tonoplast conductance from $G_{t1} = 67$ S · m^{-2} to $G_{t2} = 10$ S · m^{-2} and of the plasmalemma conductance from $G_{p1} = 108$ S · m^{-2} to $G_{p2} = 15$ S · m^{-2}. After removal of the inhibitor (*downward arrow*), the conductances slowly started to recover.

The impact of various concentrations of NPPB on the electrical properties of osmotically unstressed *Valonia* cells is summarized in Table 1. Note that in part of the experiments the pressure was clamped to P_0 before the blocker was added. This had no influence on the effect of NPPB on V_m, G_p and G_t, as shown by similar experiments on unclamped cells. Therefore, the data were pooled. It is clear from the

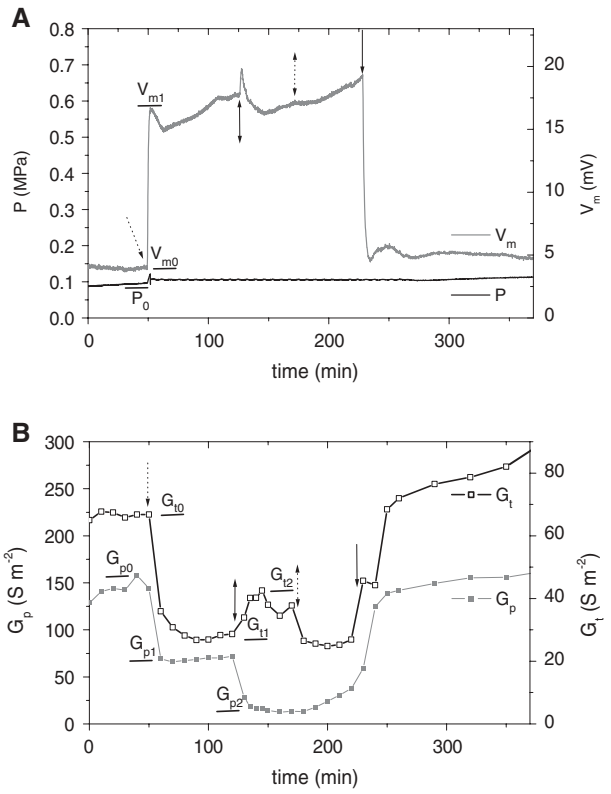

Fig. 3. Electrical response of a *V. utricularis* cell to low-Cl^- ASW and 100 μM NPPB. During the entire experiment, the turgor pressure was clamped at P_0 = 0.100 MPa (*black trace in A*). Pressure did not change upon release of the clamp at t = 260 min when the cell had been transferred into standard ASW. NPPB was added at t = 125 min to the low-Cl^- ASW (*double-headed solid arrow*) and removed after 49 min (*double-headed dotted arrow*). (**A**) After replacement of standard ASW medium by low-Cl^- ASW ([Cl^-] = 20 mM, *downward dotted arrow*), the vacuolar membrane potential, V_m, increased from V_{m0} = +4.0 mV to V_{m1} = +16.0 mV (*gray trace*). It returned to the original value upon transfer back into ASW (*downward solid arrow*). 100 μM, NPPB, had apparently no effect on V_m, except for a transient increase immediately after addition. (**B**) Response of plasmalemma and tonoplast conductance. G_p (■) decreased from G_{p0} = 144 S · m^{-2} to G_{p1} = 68 S · m^{-2} after perfusion with low-Cl^- ASW (*downward dotted arrow*) and further to G_{p2} = 13 S · m^{-2} upon addition of 100 μM NPPB (*double-headed solid arrow*). The tonoplast conductance, G_t (□), decreased upon reduction of the external Cl^- concentration from G_{t0} = 67 S · m^{-2} to G_{t1} = 27 S · m^{-2} but then increased slightly after addition of NPPB to G_{t2} = 37 S · m^{-2}. These effects were reversible, as demonstrated by removal of the inhibitor (*double-headed dotted arrow*) and perfusion with standard ASW (*downward solid arrow*).

data in Table 1 that the effect of the inhibitor on V_m, G_p and G_t was concentration-dependent. Maximum percentages of inhibition of G_p and G_t were achieved with 300 μM NPPB (on average 88% and 83%, respectively).

It should be noted that removal of the inhibitor after 60–130 min of exposure led always to recovery of the vacuolar membrane potential and the membrane conductances. It is also interesting to note that 100 μM NPPB had no effect on G_p when cells were pretreated with 2 mM Ba^{2+}, an inhibitor of plasmalemma K^+ transport, whereas G_t responded in the usual way (n = 2, *data not shown*). In contrast, when 60 mM sulfate was added to the perfusion solution, the effect of NPPB on G_t almost disappeared, as expected in light of the results obtained in the foregoing section. Only in two out of seven experiments was a slight reduction of G_t by about 5 S · m^{-2} observed.

Effect of NPPB on Turgor Pressure and Electrical Membrane Parameters in Low-Cl^- and K^+-Free Iso-osmotic ASW

A substantial reduction of the Cl^- concentration in ASW provided more detailed information on the effect of NPPB on transporters contributing to plasmalemma and tonoplast conductance under iso-osmotic conditions. Reduction of the external Cl^- concentration from 599 to 20 mM was achieved by iso-osmotic substitution with MES^- and 26 mM SO_4^{2-} (15 mM was added as sulfuric acid for pH adjustment to 8.1). Because cells were not able to maintain a constant turgor pressure in low-Cl^- ASW, the pressure was clamped at the original turgor pressure (P_0) in order to eliminate possible effects of pressure fluctuations on the electrical parameters of the alga during the course of the experiment. After release of the pressure clamp at t = 260 min when the alga had been transferred back into ASW, the original turgor pressure was recorded (Fig. 3A), indicating that the manipulations of the external Cl^- concentration during the experiment had no irreversible effects on the osmotic balance of the cell.

As demonstrated in Figure 3A, removal of external Cl^- under pressure clamp conditions induced a prompt increase in the vacuolar membrane potential from V_{m0} = +4.0 mV to V_{m1} = +16.0 mV; simultaneously, G_p was reduced from G_{p0} = 144 S · m^{-2} to G_{p1} = 68 S · m^{-2} (Fig. 3B). The tonoplast conductance also dropped immediately from G_{t0} = 67 S · m^{-2} in ASW to G_{t1} = 27 S · m^{-2} in low-Cl^- ASW. This response of G_t was somewhat unexpected since the tonoplast was not directly exposed to the external medium. However, this drop in G_t was also found in the case of 12 other cells (G_{t1} = 39 ± 14 S · m^{-2} *vs.* G_{t0} = 79 ± 27 S · m^{-2}, corresponding to a decrease of about 50%). Only in three cells did G_t remain constant. On average, G_p decreased from G_{p0} = 144 ± 32 S · m^{-2} to G_{p1} = 45 ± 23 S · m^{-2} and V_m increased from V_{m0} = +5.7 ± 2.0 mV to V_{m1} = +19.9 ± 4.4 mV upon exposure to low-Cl^- ASW (n = 16).

In contrast to high-Cl^- ASW (compare Table 1), addition of 100 μM NPPB to the low-Cl^- ASW at t = 126 min did not affect V_m significantly (Fig. 3A).

Table 1. Effects of external NPPB on V_m, G_p and G_t of *V. utricularis* cells recorded under iso-osmotic conditions*

C_{NPPB} (μM)	V_{m1} (m*V*)	V_{m2} (m*V*)	G_{p1} (S · m^{-2})	G_{p2} (S · m^{-2})	G_{t1} (S · m^{-2})	G_{t2} (S · m^{-2})
50 (n = 4)	+4.1 ± 2.8	+4.0 ± 2.6	179 ± 97	149 ± 95	59 ± 36	39 ± 23
100 (n = 8)	+5.0 ± 2.0	+2.6 ± 2.5	124 ± 35	58 ± 14	50 ± 24	17 ± 6
200 (n = 3)	+5.8 ± 1.0	+1.7 ± 1.3	155 ± 99	28 ± 9	70 ± 14	16 ± 5
300 (n = 5)	+7.3 ± 2.4	−0.4 ± 1.6	160 ± 52	20 ± 7	59 ± 27	10 ± 5

*Values were taken before (V_{m1}, G_{p1}, G_{t1}) and about 50 min after addition of the inhibitor (V_{m2}, G_{p2}, G_{t2}; compare Fig. 2). The data are mean values ± SD.

Table 2. Effects of a reduced external Cl^- concentration (from 599 to 20 mM) and additional further external application of 100 μM NPPB (n = 4)*

V_{m0} (m*V*)	V_{m1} (m*V*)	V_{m2} (m*V*)	G_{p0} (S · m^{-2})	G_{p1} (S · m^{-2})	G_{p2} (S · m^{-2})	G_{t0} (S · m^{-2})	G_{t1} (S · m^{-2})	G_{t2} (S · m^{-2})
± 4.0	− 19.6	− 20.8	− 129	− 45	− 12	− 79	− 35	− 55
±1.2	±5.1	±3.8	±15	±20	±2	±33	±15	±27

*Data were derived from four independent measurements, as shown in Figure 3. Values were taken before reduction of the external Cl^- concentration (V_{m0}, G_{p0}, G_{t0}), before application of 100 μM NPPB (V_{m1}, G_{p1}, G_{t1}) and about 50 min after addition of the inhibitor (V_{m2}, G_{p2}, G_{t2}). The data represent mean values ± SD.

Consistent with the finding in high-Cl^- ASW, a clear-cut decrease of the plasmalemma conductance from G_{p1} = 68 S · m^{-2} to G_{p2} = 13 S · m^{-2} was observed (Fig. 3B). Interestingly, this decrease in G_p was not associated with a corresponding decrease in the tonoplast conductance as observed in high-Cl^- ASW (Fig. 3B). The opposite effect, i.e., a slight increase in G_t, seemed to occur. However, this was most likely an artifact because the time constants of both relaxations were on the same order of magnitude under these conditions, thus preventing accurate separation of the tonoplast relaxation from the plasmalemma one (*for details, see* Heidecker et al., 2003b). A reduction in G_t can also be excluded because in such a case a resolution of the two relaxations would have been possible due to the increase of the tonoplast relaxation time, τ_2. Evidence for the assumption that G_t remained unaltered in the presence of NPPB also came from the finding observed upon removal of the inhibitor after a further 49 min. G_t assumed within about 5 min the original value, whereas the recovery of G_p was much slower. Subsequent exchange of low-Cl^- ASW against ASW after t = 229 min resulted in reestablishment of the original values of the membrane conductances. Similar results were obtained in three other independent experiments (*see* Table 2) and when 100 μM NPPB was added to the ASW before application of low-Cl^- ASW (n = 4, *data not shown*). Upon addition of NPPB, G_p as well as G_t decreased to the same extent as reported above for this treatment (Table 1); subsequent lowering of the Cl^- concentration resulted obviously in a further decrease of G_p but not of G_t, indicated by merging of the two relaxations of the tonoplast and the plasmalemma (see above).

Complete removal of external K^+ while keeping the Cl^- concentration of the ASW constant resulted in opposite effects of NPPB on the membrane conductances (Fig. 4). As in the case of low-Cl^- ASW, exchange of ASW against K^+-free ASW resulted in a slow decrease of turgor pressure. Therefore, the pressure was also clamped at P_0 during exposure to K^+-free ASW (Fig. 4A). Approximately 50% of the cells showed very rapidly indications of a beginning degradation (e.g., a restructuring of the cytoplasm and a drastic increase in the tonoplast capacitance, C_t). Thus, for the experiments only cells with an intact morphological appearance were used. Removal of K^+ resulted in a decrease of V_m, G_p and G_t. In the experiment shown in Figure 4, V_m decreased from V_{m0} = +6.5 m*V* to V_{m1} = –5.0 m*V* (Fig. 4A). G_p dropped from G_{p0} = 86 S · m^{-2} to G_{p1} = 34 S · m^{-2} and G_t decreased from G_{t0} = 82 S · m^{-2} to G_{t1} = 54 S · m^{-2} (Fig. 4B). On average, the following values were recorded: V_{m0} = +7.4 ± 2.1 m*V* to V_{m1} = −9.4 ± 4.6 m*V*, G_{t0} = 75 ± 26 S · m^{-2} to G_{t1} = 51 ± 21 S · m^{-2} (corresponding to a reduction of about 30%) and G_{p0} = 168 ± 94 S · m^{-2} to G_{p1} = 58 ± 39 S · m^{-2} (n = 7). [3]

[3]It should be noted that under these conditions the reduction of G_p was more pronounced when charge pulses of negative polarity were applied. In K^+-free ASW, G_p values obtained by injection of a negative charge pulse were about half those derived from experiments in which positive pulses were applied (n = 2 cells). The discrepancy between the values is probably due to the fact that the K^+ gradient imposed on the plasmalemma under these conditions is much steeper than in ASW (12 mM K^+). Reduction of G_t upon replacement of ASW by K^+-free ASW was also slightly stronger with negative pulses (about 42%) than with positive pulses (about 30%), probably because cytosolic K^+ was also affected by this treatment.

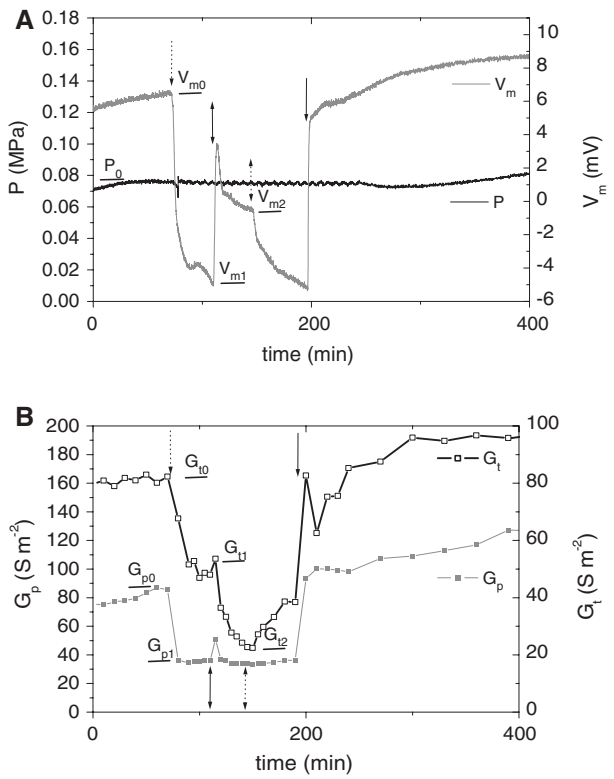

Fig. 4. Electrical response of a *V. utricularis* cell to K^+-free ASW and 200 μM NPPB. During the entire experiment, the turgor pressure was clamped at $P_0 = 0.080$ MPa (*black trace in A*). Pressure did not change upon release of the clamp at $t = 250$ min when the cell had been transferred into standard ASW. NPPB was added at $t = 110$ min to the K^+-free ASW (*double-headed solid arrow*) and removed after 33 min (*double-headed dotted arrow*). (**A**) Upon substituting K^+ in the bath for Na^+ (*downward dotted arrow*), the vacuolar membrane potential exhibited a decrease from $V_{m0} = +6.5$ m*V* to $V_{m1} = -5.0$ m*V* (*gray trace*). It increased to 3.4 m*V* after exposure to NPPB (*double-headed solid arrow*) in order to decrease then again to $V_{m2} = 0$ m*V*. The NPPB effect on V_m was reversible when NPPB was removed (*double-headed dotted arrow*) and assumed its initial value after transfer into ASW (*downward solid arrrow*). (**B**) After perfusion of the bath with K^+-free ASW (*downward dotted arrow*), plasmalemma conductance, G_p (■), declined from $G_{p0} = 86$ S · m^{-2} to $G_{p1} = 34$ S · m^{-2} and tonoplast conductance, G_t (□), from $G_{t0} = 82$ S · m^{-2} to $G_{t1} = 54$ S · m^{-2}. Addition of 200 μM NPPB to the bath (*double-headed solid arrow*) hardly affected G_p but resulted in a further decrease of G_t to $G_{t2} = 22$ S · m^{-2}. G_t recovered when NPPB was washed away (*double-headed dotted arrow*) and standard ASW was subsequently provided again (*downward solid arrrow*).

Addition of 100 μM NPPB to K^+-free ASW did not affect G_p but resulted in a decrease of the tonoplast conductance from $G_{t1} = 53 \pm 32$ S · m^{-2} to $G_{t2} = 31 \pm 17$ S · m^{-2} ($n = 4$). V_m increased from $V_{m1} = -8.3 \pm 4.0$ m*V* to $V_{m2} = -4.9 \pm 3.6$ m*V*. NPPB (200 μM) enhanced the reduction of G_t due to removal of external K^+ to a final value of $G_{t2} = 22$ S · m^{-2} (Fig. 4B). As shown in this figure, sometimes also a slight decrease of G_p could be recorded. V_m increased to a maximum value of 3.4 m*V* and subsequently relaxed back to $V_{m2} = -0.5$ m*V* (Fig. 4A). On average, G_t decreased from $G_{t1} = 41 \pm 14$ S · m^{-2} to $G_{t2} = 16 \pm 6$ S · m^{-2} and V_m increased from $V_{m1} = -8.3 \pm 3.2$ m*V* to $V_{m2} = -1.1 \pm 0.8$ m*V* ($n = 3$). A similar decrease of G_t was obtained by addition of 300 μM NPPB to K^+-free ASW; G_t dropped from $G_{t1} = 64 \pm 19$ S · m^{-2} to $G_{t2} = 25 \pm 7$ S · m^{-2} ($n = 4$). At this concentration, a clear-cut effect of the inhibitor was also observed on G_p, at least for the four cells investigated ($G_{p1} = 42 \pm 6$ S · m^{-2} *vs.* $G_{p2} = 27 \pm 8$ S · m^{-2}, $n = 4$). V_m increased on average from $V_{m1} = -5.5 \pm 1.6$ m*V* to $V_{m2} = +0.9 \pm 0.7$ m*V*.

Interestingly, when cells were first exposed to 100 or 200 μM NPPB, G_t remained unaffected by subsequent removal of K^+ from the bath or even increased slightly ($n = 9$, *data not shown*). G_p also did not respond to removal of K^+ when cells were treated with 200μM NPPB, whereas at a concentration of 100μM some additional reduction of G_p was induced by subsequent exposure to K^+-free ASW.

It should be noted that in all experiments the effect of NPPB on the electrical parameters was more or less reversible, as proved by transfer of the algal cells back into ASW.

Effect of NPPB on Hypo-osmotically Induced Turgor Pressure Regulation in ASW

In order to test whether hypo-osmotic turgor regulation was affected by NPPB, the inhibitor was added during the second phase of turgor pressure readjustment for a time interval of about 100 min (*see* Heidecker et al., 2003b). A typical experiment is shown in Figure 5A. The osmolality of the ASW in the bath was reduced from 1,144 mosmol/kg to 1,118 mosmol/kg by dilution with distilled water. The turgor pressure increased from $P_0 = 0.100$ MPa to a maximum value of $P_1 = 0.146$ MPa due to passive water inflow. One hundred and sixty minutes after acute hypo-osmotic challenge, 300 μM NPPB was added during downregulation of pressure. Sixty minutes later, turgor pressure relaxation ceased completely at a value of 0.120 MPa. The effect of NPPB on turgor pressure regulation was completely reversible, as shown by exchange of the inhibitor-containing medium against ASW after 137 min. Turgor pressure decrease continued until a new steady-state value of about 0.075 MPa was reached.

Upon hypo-osmotic challenge of the cell, the vacuolar membrane potential decreased transiently from $V_{m0} = +7.0$ m*V* to a minimum value of +3.5 m*V* at the end of the initial water influx phase. During the following turgor pressure regulation phase, V_m partly recovered. Upon addition of 300 μM NPPB, V_m decreased rapidly within 5 min from $V_{m1} = +5.5$ m*V* to a final value of $V_{m2} = -0.5$ m*V*.

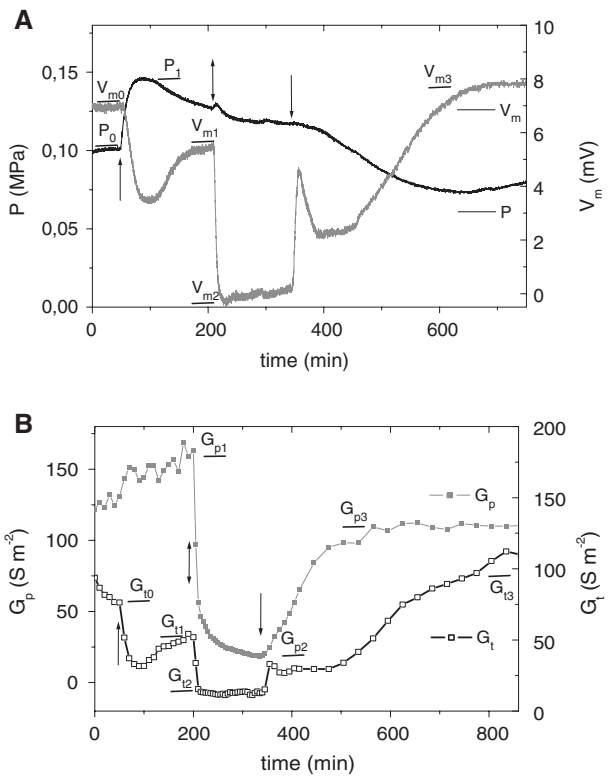

Fig. 5. Effect of 300 μM external NPPB on turgor pressure and the electrical properties of a *V. utricularis* cell under hypo-osmotic conditions. (**A**) Response of turgor pressure (*P, black trace*) and vacuolar membrane potential (V_m, *gray trace*) upon lowering the osmolality of the bath from 1,144 to 1,118 mosmol · kg^{-1} (*upward arrow*). Due to water influx, the turgor pressure increased from P_0 = 0.100 MPa to P_1 = 0.146 MPa. After 50 min, downregulation started. When 300 μM NPPB was added at t = 201 min (*double-headed arrow*), turgor regulation ceased about 60 min later and continued to decrease somewhat delayed after removal of the inhibitor (downward arrow). A final stationary turgor pressure of P = 0.075 MPa was reached after 310 min. After acute osmotic challenge, vacuolar membrane potential decreased transiently from V_{m0} = + 7.0 mV to + 3.5 mV in order to assume a value of V_{m1} = + 5.5 mV after 160 min. Upon addition of NPPB, the vacuolar membrane potential dropped to V_{m2} = −0.5 mV. After removal of the inhibitor, a biphasic response of V_m was observed (with a peak value of + 4.3 mV) before the vacuolar membrane potential continuously increased, reaching the original value of V_{m3} = + 7.5 mV after 350 min. (**B**) Time course of the plasmalemma conductance, G_p (■), and the tonoplast conductance, G_t (□). G_p remained unaffected by hypo-osmotic shock (*upward arrow*). By contrast, hypo-osmotic challenge induced a decrease of G_t from G_{t0} = 76 S · m^{-2} to a minimum value of 33 S · m^{-2}, followed by a slight increase to G_{t1} = 49 S · m^{-2}. Addition of NPPB (*double-headed arrow*) resulted in a decrease both of G_p from G_{p1} = 160 S · m^{-2} to G_{p2} = 19 S · m^{-2} and of G_t to G_{t2} = 14 S · m^{-2}. After removal of the inhibitor, both conductances increased again and reached G_{p3} = 110 S · m^{-2} and G_{t3} = 95 S · m^{-2}, almost the original values once a stationary pressure had been established.

Removal of the inhibitor resulted in a rapid transient increase of V_m followed by a slow continuous increase until a final value of V_{m3} = +7.5 mV was reached.

Figure 5B shows the corresponding time courses of G_p and G_t. It is obvious that G_p remained largely unaffected by the hypo-osmotic shock, whereas G_t decreased from G_{t0} = 76 S · m^{-2} to G_{t1} = 49 S · m^{-2} (i.e., by about 35%) in agreement with previous data (Heidecker et al., 2003b). Upon exposure to 300 μM NPPB, G_p and G_t dropped dramatically from G_{p0} = G_{p1} = 160 S · m^{-2} to G_{p2} = 19 S · m^{-2} (by 141 S · m^{-2} or 88%) and from G_{t1} = 49 S · m^{-2} to G_{t2} = 14 S · m^{-2} (by 35 S · m^{-2} or 71%). The reduction of the conductances was at least partly reversible upon removal of the inhibitor. When the pressure was readjusted, the plasmalemma and tonoplast conductances assumed stable values of G_{p3} = 110 S · m^{-2} and G_{t3} = 95 S · m^{-2}. Similar results were obtained in three other independent experiments (*see* Table 3).

Concentrations of 50, 100 and 200 μM NPPB failed to inhibit turgor pressure regulation in ASW. A typical experiment in the presence of 100 μM is depicted in Figure 6A. It is obvious that P and V_m remained almost unaffected. Reduction of G_p at 100 μM NPPB was less than at 300 μM NPPB, whereas G_t was reduced to the same extent. G_p and G_t decreased by 79 S · m^{-2} (or 52%) and 27 S · m^{-2} (or 60%, *see* Fig. 6B). The values of five independent experiments are summarized in Table 3. When 100 μM NPPB was applied simultaneously with 200 μM DIDS, an inhibitor of plasmalemma Cl^- transport, turgor pressure regulation was delayed or ceased completely in the four cells investigated. Note that G_p was strongly reduced by the combination of these blockers (on average from 98 ± 34 S · m^{-2} to 15 ± 6 S · m^{-2}, i.e., by about 85%), whereas G_t dropped from 24 ± 9 S · m^{-2} to 8 ± 2 S · m^{-2}, i.e., by about 66%. This value corresponded to G_t reduction obtained with 100 μM NPPB alone.

Interestingly, hypo-osmotic turgor regulation remained unaffected when (nonperfused) cells were simultaneously exposed to 200 μM DIDS and 2 mM $BaCl_2$ during pressure readjustment (n = 3). Under these conditions, G_p was strongly reduced (from 117 ± 5 S · m^{-2} to 25 ± 4 S · m^{-2}, i.e., by about 79%), whereas no response of G_t was observed. V_m was also hardly affected. Surprisingly, no or only minor effects on turgor pressure regulation were also found when $BaCl_2$ (up to 5 mM) and DIDS (up to 400 μM) were added to cells (n = 6) that had been perfused with AVS containing 40 mM K_2SO_4 (which inhibits Cl^- transport in the tonoplast, as shown above). Under these conditions, G_p also decreased strongly while G_t remained constant. Exposure of the cells to 2 mM $BaCl_2$ and 100 μM NPPB also did not affect turgor pressure readjustment of nonperfused cells and of cells perfused before with sulfate-containing AVS. Changes in G_p and G_t were similar to those observed in iso-osmotic ASW in the presence of the two inhibitors.

Table 3. Effects of external NPPB on V_m, G_p and G_t recorded under hypo-osmotic conditions or during pressure readjustment after imposing a pressure jump*

C_{NPPB} (μm)	V_{m0} (m*V*)	V_{m1} (m*V*)	V_{m2} (m*V*)	V_{m3} (m*V*)	G_{p1} ($S \cdot m^{-2}$)	G_{p2} ($S \cdot m^{-2}$)	G_{p3} ($S \cdot m^{-2}$)	G_{t0} ($S \cdot m^{-2}$)	G_{t1} ($S \cdot m^{-2}$)	G_{t2} ($S \cdot m^{-2}$)	G_{t3} ($S \cdot m^{-2}$)
100	+7.9	+6.1	+5.1	+6.5	166	78	157	47	34	13	45
(n = 5)	±2.3	±1.7	±2.3	±1.3	±35	±29	±25	±16	±9	±5	±12
200	+5.9	+6.2	+2.5	+3.9	139	27	128	23	17	7	30
(n = 3)	±1.9	±2.8	±3.1	±1.9	±43	±7	±30	±3	±4	±3	±5
300	+5.9	+4.4	−0.4	+5.6	165	20	124	57	38	12	83
(n = 4)	±0.8	±0.9	±1.8	±2.6	±12	±10	±13	±19	±12	±4	±11

*Data were derived from three to five independent experiments, as shown in Figures 5 and 6 (mean values ± SD). Values were taken before hypo-osmotic shock was applied or pressure was clamped at a higher level (V_{m0}, G_{t0}), before addition of the blocker (V_{m1}, G_{p1}, G_{t1}), about 50 min after application of the blocker (V_{m2}, G_{p2}, G_{t2}) and when the initial pressure was readjusted after washing away the blocker (V_{m3}, G_{p3}, G_{t3}). The blocker was added about 150 min after challenge with a hypo-osmotic shock or release of pressure clamp, respectively.

Effect of NPPB on Turgor Pressure Relaxation in Low-Cl^- and K^+-Free ASW after Imposing a Pressure Jump

Studies of hypo-osmotic turgor pressure down-relaxations in low-Cl^- and K^+-free ASW were performed on pressure-clamped cells using the approach of Heidecker et al. (2003b). In contrast to acute hypo-osmotic challenges, pressure clamp conditions allowed us to separate clearly interfering effects of low-Cl^- ASW or K^+ removal on the electrical properties of the membranes from those of NPPB. The effects of low-Cl^- ASW and of a subsequent positive pressure step on V_m, G_t and G_p are shown in Figure 7. Micropipettes were inserted into the cell in normal high-Cl^- ASW. When a steady-state pressure of P_0 = 0.104 MPa had been attained, the turgor was clamped to this value. Then, ASW was replaced by low-Cl^- ASW. After 60 min, the pressure was increased by 0.065 MPa and the new pressure value was clamped for 80 min. This time was sufficient to establish osmotic equilibrium between the cell and the bath (Heidecker et al., 2003b). Upon release of the pressure clamp, a hypo-osmotic down-relaxation of the turgor pressure occurred (Fig. 7A). Compared with Figures 5 and 6 this decrease was much faster than that in high-Cl^- ASW. Interestingly, the pressure decrease did not level off at a value close to the original value as expected from measurements in high-Cl^- ASW (Figs. 5 and 6; *see also* Heidecker et al., 2003b). Rather, the pressure decreased continuously until complete turgor pressure loss occurred.

Exposure of the cell to low-Cl^- ASW was accompanied by changes in the membrane potential and conductances which were similar to those observed in Figure 3 (V_{m0} = +8.5 m*V* *vs.* V_{m1} = +15.6 m*V*, Fig. 7A; G_{p0} = 100 $S \cdot m^{-2}$ *vs.* G_{p1} = 33 $S \cdot m^{-2}$ and G_{t0} = 64 $S \cdot m^{-2}$ *vs.* G_{t1} = 37 $S \cdot m^{-2}$, Fig. 7B). When the positive pressure step was imposed, G_p remained unaltered whereas V_m and G_t responded to the pressure increase as under hypo-osmotic conditions in high-Cl^- ASW; i.e., V_m decreased to V_{m2} = +10.9 m*V* (Fig. 7A) and G_t to G_{t2} = 24 $S \cdot m^{-2}$ (Fig. 7B). After release of the pressure clamp, V_m and G_t slowly recovered in order to reach final values of V_{m3} = +21.3 m*V* and G_{t3} = 63 $S \cdot m^{-2}$. Similar results were obtained with two other cells (G_{t1} = 47 ± 16 $S \cdot m^{-2}$ *vs.* G_{t2} = 21 ± 10 $S \cdot m^{-2}$ corresponding to a decrease in G_t of about 30% with respect to G_{t0}; V_{m1} = +20.8 ± 4.8 m*V* *vs.* V_{m2} = +5.5 ± 7.5 m*V*; n = 3).

In the following set of experiments, *Valonia* cells bathed in K^+-free ASW were subjected to the same pressure regime. When the pressure was stepwise increased by values ranging 0.050–0.070 MPa, the electrical parameters of the alga remained completely unaffected (n = 4). In two other experiments, only a marginal decrease in G_t (by about 5 $S \cdot m^{-2}$) and V_m (by about 0.5 m*V*) was observed (*data not shown*). [4]. When the clamp was released subsequently, the turgor pressure dropped continuously to zero with a time course comparable to that in low-Cl^- ASW.

Addition of 300 μM NPPB after pressure clamp release always strongly retarded turgor pressure down-relaxation in low-Cl^- ASW as well as in K^+-free ASW. Data for a typical experiment performed in low-Cl^- ASW are given in Figure 8. In the presence of 200 μM NPPB, turgor pressure relaxation was slowed down only in part of the cells (one out of four cells when kept in low-Cl^- ASW and three out of four cells when exposed to K^+-free ASW). Addition of 100 μM NPPB had no effect on the time course of pressure down-relaxation in either low-Cl^- or K^+-free ASW (n = 3, *data not shown*).

[4]Note that when the pressure was increased while cells were still bathed in ASW, subsequent K^+ removal did not affect G_t (n = 6) or caused only a slight decrease of G_t (by 7 $S \cdot m-2$ or less, n = 2)

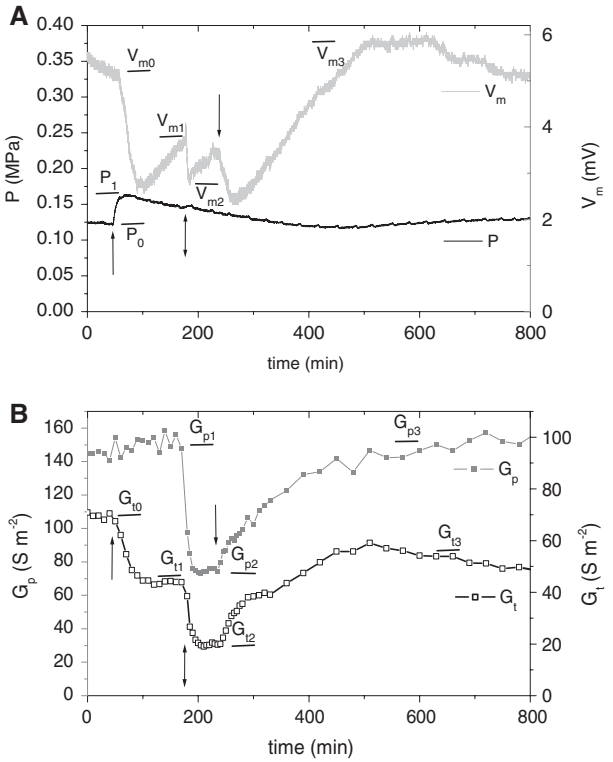

Fig. 6. Effect of 100 μM external NPPB on turgor pressure and the electrical properties of a *V. utricularis* cell under hypo-osmotic conditions. (**A**) Response of turgor pressure (*P, black trace*) and vacuolar membrane potential (V_m, *gray trace*) upon lowering the osmotic pressure of the bath from 1,136 to 1,110 mosmol · kg^{-1} (*upward arrow*). Pressure downregulation remained unaffected by addition of NPPB at t = 175 min (*double-headed arrow*). Removal of the inhibitor about 60 min later had no effect. A final stationary turgor pressure of P = 0.117 MPa was reached after about 400 min. After acute osmotic challenge, vacuolar membrane potential decreased transiently from V_{m0} = +5.1 mV to +2.7 mV in order to assume a slightly higher value of V_{m1} = +3.8 mV after 130 min. Upon addition of NPPB, vacuolar membrane potential dropped to V_{m2} = +2.8 mV. After removal of the inhibitor, a biphasic response of V_m was observed (with a minimum value of +2.4 mV) before increasing again to reach the original value of V_{m3} = +5.7 mV after 350 min. (**B**) Time course of plasmalemma conductance, G_p (■), and tonoplast conductance, G_t (□). G_p remained unaffected by hypo-osmotic shock (*upward arrow*). By contrast, hypo-osmotic challenge induced a decrease of G_t from G_{t0} = 70 S · m^{-2} to 33 S · m^{-2}, followed by a slight increase to G_{t1} = 45 S · m^{-2}. Addition of NPPB (*double-headed arrow*) resulted in a decrease both of G_p from G_{p1} = 152 S · m^{-2} to G_{p2} = 73 S · m^{-2} and of G_t to G_{t2} = 18 S · m^{-2}. After removal of the inhibitor, both conductances increased again and reached G_{p3} = 150 S · m^{-2} and G_{t3} = 55 S · m^{-2}, almost the original values once a stationary pressure had been established.

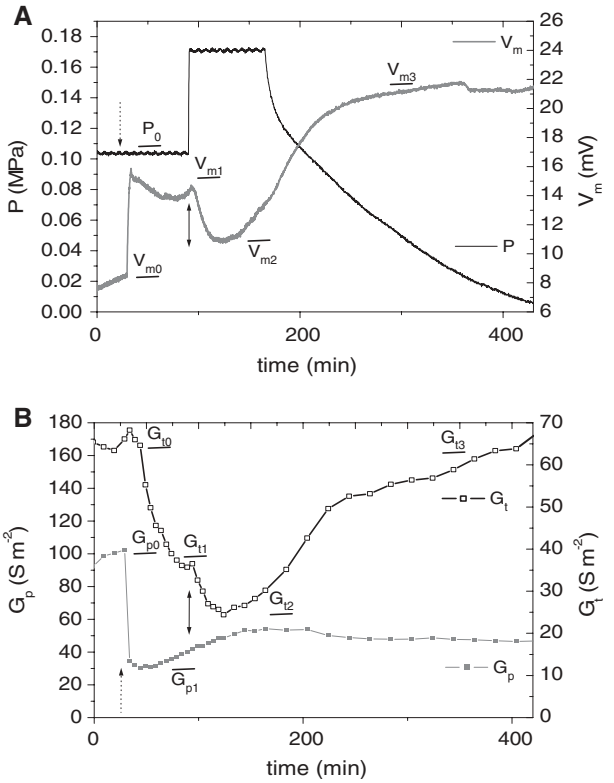

Fig. 7. Changes in the electrical properties of a *V. utricularis* cell induced by low-Cl$^-$ ASW and pressure increments. (**A**) Time courses of turgor pressure, P (*black trace*), and vacuolar membrane potential, V_m (*gray trace*). After impalement in ASW, turgor pressure was clamped at the initial steady-state value P_0 = 0.104 MPa (*not shown*). At t = 25 min, ASW was replaced by low-Cl$^-$ ASW (*dotted arrow*). Sixty minutes later, the pressure was clamped at 0.169 MPa (*double-headed arrow*) and arrrested at that value for 80 min. Subsequently, the clamp was released. This led to a fast decrease of the pressure and finally to total turgor pressure loss of the cell. Upon exposure of the cell to low-Cl$^-$ ASW, V_m increased from V_{m0} = +8.5 mV to V_{m1} = +15.6 mV; it responded to the pressure jump by a transient decrease to V_{m2} = +10.9 mV, followed by a continuous increase which was not interrupted by release of the pressure clamp. The final value was V_{m3} = +21.3 mV, considerably more positive than the original one. (**B**) Time courses of the conductances of plasmalemma, G_p (■), and tonoplast, G_t (□). G_p and G_t decreased from G_{p0} = 100 S · m^{-2} to G_{p1} = 33 S · m^{-2} and from G_{t0} = 64 S · m^{-2} to G_{t1} = 37 S · m^{-2} after exposure to low-Cl$^-$ ASW. When the positive pressure step was imposed, G_p remained unchanged whereas G_t dropped further to G_{t2} = 24 S · m^{-2}. G_t increased again slowly after release of the clamp and reached a final value of G_{t3} = 63 S · m^{-2} about 220 min later.

Discussion

Recent measurements of the individual conductances of the tonoplast and the plasmalemma of *V. utricularis* upon turgor pressure changes together with investigations of structural changes associated with turgor pressure regulation have given clear-cut evidence that the sponge-like tonoplast and apparently not the plasmalemma is involved in the very early events of sensing and transduction of the turgor pressure signal (Heidecker et al., 2003b). Upon hyper-osmotic shock, a transient decrease in G_t occurred that was much more pronounced (in both absolute and relative terms) than the rapid but very small change of G_p. During the subsequent upregulation phase, G_t and, in particular, G_p increased continuously. The increase in G_p was

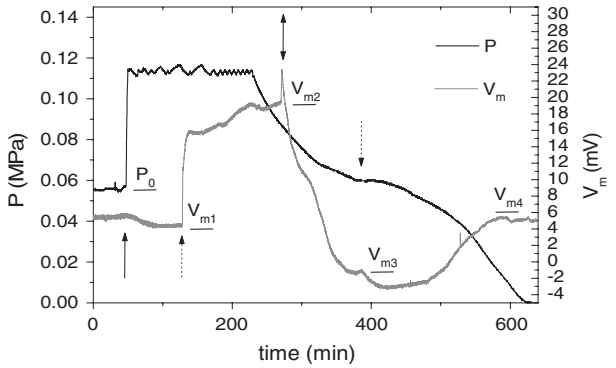

Fig. 8. Inhibition of turgor pressure down-relaxation in low-Cl^- ASW by 300 μM external NPPB. After impalement in ASW (*not shown*), pressure was clamped at the initial steady-state value $P_0 = 0.056$ MPa. After 76 min, pressure was increased and clamped at 0.115 MPa (*arrow*). Eighty-one minutes later, ASW was replaced by low-Cl^- ASW (*upward dotted arrow*). This was accompanied by an increase of V_m from $V_{m1} = +4.2$ mV to $V_{m2} = +19.2$ mV. Release of the pressure clamp at $t = 258$ min resulted in down-relaxation of pressure. Retardation of pressure relaxation occurred upon addition of 300 μM NPPB at $t = 279$ min (*double-headed arrow*). After 106-min exposure to the inhibitor, a stationary turgor pressure of 0.060 MPa was reached. The corresponding value of the vacuolar membrane potential was $V_{m3} = -1.2$ mV. Removal of NPPB led to an increase of V_m to $V_{m4} = +5.2$ mV and – somewhat delayed – to the continuation of the pressure relaxation until complete turgor pressure loss occurred.

quite dramatic. Under hypo-osmotic conditions, G_t dropped after dilution of ASW and increased only relatively slowly after the peak value in turgor pressure had been reached. The drop in G_t was on average about 30%. In contrast, G_p remained nearly constant and increased only slightly during the period of downregulation of turgor pressure. Similar observations were made in pressure clamp experiments. In this respect, the response of *V. utricularis* is different from that of the marine alga *Lamprothamnium papulosum*, where hypo-osmotic stress induces an increase in the plasmalemma conductance that apparently facilitates salt efflux from the cell (Shepherd et al., 1999; Beilby, Cherry & Shepherd, 1999).

The differences in the response of the conductances of *V. utricularis* to hypo- and hyper-osmotic challenge are apparently linked with the upregulation of K^+ and Cl^- transporters in the plasmalemma during turgor pressure adjustment following a hyper-osmotic shock. These transporters could be inhibited by Ba^{2+} and DIDS as well as by external Cyanide, indicating a direct or indirect dependence on metabolic energy. In contrast, under hypo-osmotic challenge, ions are apparently passively released from the vacuole through the cytoplasm to the medium because pressure readjustment was insensitive to Cyanide, indicating that the process of regulation did not depend on metabolic energy. Interestingly, Ba^{2+} and DIDS could not inhibit hypo-osmotic turgor pressure regulation, even though the ion conductance of the plasmalemma was reduced by about 80%.

The effects of the inhibitors NPPB and sulfate on the conductances of the tonoplast and plasmalemma of *V. utricularis* cells in the absence and presence of Ba^{2+} and DIDS led to a refinement of our view about salt transport under hypo-osmotic turgor pressure regulation conditions.

Evidence for Turgor Pressure Effects on K^+, but not on Cl^-, Transporters in the Tonoplast

Exposure of cells to sulfate and low-Cl^- and K^+-free ASW demonstrated that the increase in turgor pressure upon hypo-osmotic challenge inhibits K^+, but not Cl^-, transporters in the tonoplast.

External sulfate had no effect on the tonoplast and plasmalemma conductances and, in turn, on the ion transporters of *V. utricularis*. However, addition of sulfate to the perfusion medium reduced G_t dramatically. There is no doubt that this reduction in G_t must be ascribed to the increase in the sulfate concentration and not to the decrease in the Cl^- concentration. In Figure 1, it is clearly shown that upon reduction of the vacuolar Cl^- concentration from 522 to 12 mM in the presence of 60 mM sulfate, no response of G_t was observed, whereas replacement of native vacuolar sap (about 3 mM sulfate) by AVS containing 60 mM sulfate (associated with a reduction of vacuolar Cl^- from about 640 to only 522 mM) induced a drop in G_t of about 60%. Furthermore, about 45% reduction of G_t occurred when sulfate-free AVS was replaced by media containing 10 mM sulfate (Fig. 1C). Under these conditions, Cl^- was only slightly reduced from 642 to 622 mM. The sulfate-mediated dramatic reduction in G_t shows that this anion had penetrated the innumerable interconnected vacuolar entities encircled by cytoplasmic strands because the electrical signal of the tonoplast seen in charge-pulse experiments is governed by the huge area of the vacuolar invaginations (Wang et al., 1997a; Ryser et al., 1999) and not by the area of the central vacuole. Unstirred layers and/or mucopolysaccharides within the invaginations obviously did not interfere with sulfate penetration as was apparently the case for the larger molecule TEA (ionic radius about 5 Å; Yanochko & Yool, 2004) because replacement of vacuolar K^+ by TEA did not lead to a clear reduction in G_t. The effect of sulfate on G_t was reversible when vacuolar concentrations of up to 40 mM were applied; however, with 60 mM sulfate G_t remained constant when the vacuole was subsequently perfused with sulfate-free AVS (Fig. 1B). The reason is probably that sulfate could not be removed effectively from the vacuolar invaginations when it had been applied at such a high concentration. Whereas sulfate had a great ef-

fect on tonoplast conductance, G_p remained unaffected. Lowering the Cl^- concentration in ASW to 20 mM had a similar effect on G_t. Therefore, it seems most likely that sulfate predominantly inhibits a vacuolar Cl^- transporter. Inhibition became more pronounced with increasing sulfate concentration in high-Cl^- ASW and reached a plateau value at concentrations around 40 mM. The dependence of inhibition on the sulfate concentration could be fitted by the Hill equation (Weiss, 1997). Analysis of the Hill plot yielded a Hill coefficient of 4. This value suggests that the interaction of sulfate with the vacuolar Cl^- transporter is rather cooperative than competitive. This would be consistent with the assumption that either binding sites within a Cl^- channel are blocked by sulfate or sulfate binds to a protein regulating the activity of the transporter. Such interactions of sulfate with anion channels were reported by Frachisse et al. (1999) for *Arabidopsis* hypocotyl cells. However, in contrast to inhibition of transport in *Valonia*, Cl^- transport in *Arabidopsis* is enhanced by sulfate. This is explained by an increase of the channel density in the plasma membrane and/or an increase in the open probability in the presence of sulfate, possibly by interaction with a membrane protein that stabilized anion channel activity. Inhibition of Cl^- channels by sulfate was reported by Taylor and Brownlee (2003) for the marine alga *Coccolithus pelagicus*.

In *V. utricularis*, analysis of the Hill plot yielded an 50% inhibitory concentration value for the reduction of G_t of 7.7 mM. At this concentration, G_t is reduced by about 30%. A value of 7.7 mM is higher by about a factor of 2 than the concentration of free sulfate found usually in the vacuoles of cells of *V. utricularis* (Spiess, 1996). Obviously, the vacuolar sulfate concentration must be kept as low as 3–4 mM in order to avoid permanent inhibition of the Cl^- transporters in the tonoplast. A low concentration of sulfate is apparently achieved by binding to the mucopolysaccharide network of filaments found in the central vacuole of *V. utricularis* (Zimmermann et al., 2002; Heidecker et al., 2003a). A significant change in the concentration of sulfate during hypo-osmotic (but also during hyper-osmotic) turgor pressure regulation is not expected because of the high value of the volumetric elastic modulus of the cell wall (20–50 MPa; Zimmermann & Steudle, 1974, 1978). Using the Philip equation (Philip, 1958) it can be readily shown that the relative volume changes are $< 0.5\%$. Even if we take into account volume changes of the central vacuole due to local relocations of the cytoplasm because of the disproportionate swelling of the vacuolar entities located close to the plasmalemma (Mimietz et al., 2003), changes in sulfate concentrations should be negligible. Significant changes in sulfate can only be expected by synthesis or breakdown of mucopolysaccharides, which depend strongly on environmental stimuli (Evans & Holligan, 1972; Evans, Simpson & Callow, 1973; Evans & Callow, 1974; Evans et al., 1974; Millard & Evans, 1982). How far synthesis or breakdown of this network occurs during pressure adjustment is unknown. Known is only from the work of Heidecker et al. (2003a), that the vacuolar mucopolysaccharide network is involved in turgor pressure regulation.

Even though more investigations in this direction are certainly required, at the present stage it is quite unlikely that regulation of Cl^- transporters in the tonoplast plays a crucial role in hypo-osmotic turgor pressure adjustment. First, increase of the vacuolar sulfate concentrations to 60 mM did not change the kinetics of turgor pressure regulation. This is, however, expected if Cl^- transporters in the tonoplast are involved in regulation. Second, the changes in G_t upon clamping of the turgor pressure at elevated values are also inconsistent with the assumption of a contribution of vacuolar Cl^- transporters to turgor pressure regulation. As shown in detail by Heidecker et al. (2003b), clamping at elevated pressures is equivalent to acute hypo-osmotic challenge because release of the clamped pressure results in normal hypo-osmotic turgor pressure regulation. Interestingly, even though the G_t value in low-Cl^- ASW (i.e., in the presence of low-Cl^- cytosol) was by about 50% lower than in standard ASW, application of a positive pressure clamp led to a comparable drop of G_t in both media (by about 30–40% of the original value in ASW). This finding obviously rules out a significant effect of pressure on the Cl^- transporters but, rather, suggests an effect on the K^+ transporters in the tonoplast. This conclusion was supported by G_t measurements in K^+-free ASW. Cells immersed in K^+-free ASW exhibited a reduction in G_t value of 32%. The rapid deterioration of the cells observed frequently upon transfer to K^+-free medium suggests that this resulted from an exchange of cytosolic K^+ against external Na^+. When a positive pressure clamp was applied to cells exposed to K^+-free ASW, G_t did not respond, in contrast to low-Cl^- ASW conditions. This suggests that the low cytosolic K^+ concentration inactivates the pressure-dependent K^+ transport into the vacuole (Gutknecht, 1968; Zimmermann et al., 1982) but not the release of vacuolar KCl because hypo-osmotic turgor pressure regulation was not affected by the decrease in G_t. This conclusion is consistent with previous assumptions (Zimmermann & Steudle, 1974; Hastings & Gutknecht, 1976; Beilby & Bisson, 1999; Bisson & Beilby, 2002) that turgor pressure directly affects tonoplast K^+ uptake. The explanation also leads to a straightforward interpretation of the concentration-dependent effects of NPPB on the tonoplast and plasmalemma conductances as well as on hypo-osmotic turgor pressure regulation.

Inhibition of Hypo-osmotic Turgor Pressure Regulation by NPPB

The NPPB data are of great relevance for unraveling the mechanism underlying hypo-osmotic turgor pressure regulation because they demonstrate the involvement not only of transporters in the tonoplast but also of transporters of the plasmalemma in turgor pressure adjustment. The results clearly show that above a threshold concentration of about 300 μM hypo-osmotic turgor pressure regulation could be completely prevented, even in low-Cl^- and K^+-free ASW, i.e., in the presence of steep ion concentration gradients that favor salt efflux (Fig. 8). Inhibition of hypo-osmotic turgor pressure regulation was associated with a reduction of G_t and G_p by about 85%. The extreme low conductances of the tonoplast and the plasmalemma can be taken as an indication that the K^+ as well as Cl^- transporters in both membranes are inhibited. At 200 μM NPPB hypo-osmotic turgor pressure regulation could be retarded in some experiments, whereas at 100 μM NPPB no effect on turgor pressure adjustment could be observed. At this concentration, G_p and G_t were reduced by about 53% and 65%, respectively, under hypo- as well as iso-osmotic conditions. Interestingly, pronounced inhibition of the membrane conductances and of turgor pressure regulation could be achieved at 100 μM NPPB provided that 200 μM DIDS were added simultaneously to the ASW. DIDS inhibits the Cl^- transporters of the plasmalemma but not those of the tonoplast. Thus, we are driven to the conclusion that NPPB at this concentration inhibits specifically the K^+ transporters in the plasmalemma. Consistent with this, addition of 100 μM NPPB had no further effect on G_p of cells pretreated with 2 mM Ba^{2+}; Ba^{2+} is a potent inhibitor of K^+ transporters in the plasmalemma. Furthermore, in K^+-free ASW the tonoplast, but not the plasmalemma, conductance could be reduced by addition of 100 μM NPPB. However, it must be noted that this decrease in G_t was not observed when cells were pretreated with 100 μM NPPB before transfer to K^+-free ASW. A possible reason for this may be that inhibition of the K^+ transporters in the plasmalemma by NPPB prevents or retards K^+ depletion of the cytosol, thus leaving the K^+ gradients across the tonoplast unchanged. Measurements in low-Cl^- ASW gave evidence that NPPB – at least at a concentration of 100 μM – interacts specifically with Cl^- transporters in the tonoplast. In low-Cl^- ASW, 100 μM NPPB reduced G_p to the same extent as in standard (high-Cl^-) ASW, whereas G_t remained unchanged. No effect on G_t could also be recorded on cells perfused with AVS containing sulfate that inhibits the Cl^- transporters in the tonoplast, as shown above.

Postulation of (concentration-dependent) interactions of NPPB with both cation and anion transporters does not necessarily contradict the general assumption that NPPB is a potent anion channel inhibitor in animal and plant cells (Greger, 1990; Schroeder et al., 1993). There are also some reports, especially on plant cells, that K^+ channel activity can be affected by this inhibitor (Garrill et al., 1996; Giles et al., 2003).

Mechanism of Hypo-osmotic Turgor Pressure Regulation

Inhibition of KCl efflux through both membranes seems to be an important prerequisite for inhibition of hypo-osmotic pressure regulation. This explains why simultaneous application of 2 mM Ba^{2+} and DIDS to ASW did not affect hypo-osmotic turgor pressure regulation (Heidecker et al., 2003b). These two membrane-impermeable inhibitors also reduced G_p by about 85%, but not G_t, in contrast to 300 μM NPPB or 100 μM NPPB plus 200 μM DIDS. The conclusion that turgor pressure adjustment under hypo-osmotic challenge provides inhibition of the ion transporters in both membranes is somewhat puzzling because inhibition of the pathways in one of the membranes, particularly of the plasmalemma, should be sufficient. Also puzzling is the finding that simultaneous addition of vacuolar sulfate und external Ba^{2+} and DIDS did not inhibit (or retard) turgor pressure regulation even though the conductances of the two membranes were reduced by about 80%.

A possible and very likely explanation is that KCl efflux does not exclusively occur via electrogenic K^+ and Cl^- transporters but also via electrically silent transporter systems. Such redundant efflux systems would protect the cells efficiently against long-lasting high turgor pressures. Candidates for a bypass mechanism for KCl efflux could be vesicles that can directly communicate with the plasmalemma as postulated for ion transport in *Actabularia acetabulum* (Mummert & Gradmann, 1991). Work on protoplasts of *V. utricularis* prepared from the giant mother cells has shown that tiny lipid vesicles seem to be the precursors of the huge and strongly folded central vacuole (Wang et al., 1997b). As mentioned above, charge-pulse experiments, but also electron microscopic studies (Mimietz et al., 2003), have given clear-cut evidence that the vacuolar invaginations are interconnected between each other and with the central vacuole. However, this does not exclude the possibility that part of the original vesicles is still disconnected or newly formed upon hypo-osmotic challenge because of the disproportionate swelling of the vacuolar entities located close to the plasmalemma (Mimietz et al., 2003). A dynamic exchange between disconnected and interconnected vacuolar entities can also not be excluded, particularly under hypo-osmotic conditions. Disconnected vesicles will not contribute to the charge-pulse relaxation spec-

trum and will also not be inhibited by membrane-impermeable vacuolar and external inhibitors. By contrast, NPPB should bind to these vacuolar entities because it is membrane-permeable, as indicated by the rapid and almost isochronous response in G_t and G_p upon external addition. The only assumption that has to be made is that NPPB binding changes trafficking and/or the fusion properties of the vacuolar entities with the plasmalemma and, in turn, secretion of KCl into ASW. The finding that not only 300 μM NPPB but also 100 μM NPPB in the presence of DIDS prevents hypo-osmotic turgor pressure regulation suggests further that the sites of plasmalemma fusion are the areas where the Cl^- transporters are located.

Although the assumption of vesicular transport of salt during hypo-osmotic turgor pressure regulation can explain quite straightforwardly the data presented here, a challenge for the future is to identify the ion transporter(s) that contributed to the residual conductances of the tonoplast and plasmalemma upon inhibition with 300 μM NPPB or 100 μM NPPB and 200 μM DIDS. It cannot be excluded that these residual conductances on the order of about 20% reflect an ion transporter (e.g., for Ca^{2+}) that also plays a role in hypo-osmotic turgor pressure regulation. Further insight into the mechanism of hypo-osmotic turgor pressure regulation is expected if progress is made in the separate measurements of the potential changes of the plasmalemma and the tonoplast. Changes in the vacuolar membrane potential are difficult to interpret because an unambiguous assignment to the individual membranes is not possible.

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Zi 99/13-3) to U. Z. We thank Mrs. Katja Schwuchow for excellent technical assistance.

References

Beilby, M.J., Bisson, M.A. 1999. Transport systems of *Ventricaria ventricosa*: I/V analysis of both membranes in series as a function of $[K^+]_o$. *J. Membr. Biol.* **171:**63–73

Beilby, M.J., Cherry, C.A., Shepherd, V.A. 1999. Dual turgor regulation response to hypotonic stress in *Lamprothamnium papulosum*. *Plant Cell Environ.* **22:**347–359

Bentrup, F.W. 1980. Electrogenic membrane transport in plants. A review. *Biophys. Struct. Mech.* **6:**175–189

Benz, R., Zimmermann, U. 1983. Evidence for the presence of mobile charges in the cell membrane of *Valonia utricularis*. *Biophys. J.* **43:**13–26

Binder, K.-A., Wegner, L.H., Heidecker, M., Zimmermann, U. 2003. Gating of Cl^- currents in protoplasts from the marine alga *Valonia utricularis* depends on the transmembrane Cl^- gradient and is affected by enzymatic cell wall degradation. *J. Membr. Biol.* **191:**165–178

Bisson, M.A., Beilby, M.J. 2002. The transport systems of *Ventricaria ventricosa*: Hypotonic and hypertonic turgor regulation. *J. Membr. Biol.* **190:**43–56

Bisson, M.A., Kirst, G.O. 1980. *Lamprothamnium*, a euryhaline charophyte. II Time course of turgor regulation. *J. Exp. Bot.* **31:**1237–1244

Brooks, S.P.J., Storey, K.B. 1992. Bound and determined: A computer program for making buffers of defined ion concentrations. *Anal. Biochem.* **201:**119–126

Cleland, R. 1971. Cell wall extension. *Annu. Rev. Plant Physiol.* **22:**197–222

Cleland R. 1977. The control of cell enlargement. *In*: Integration of Activity in the Higher Plant. D. Jennings, editor. Cambridge University Press, Cambridge, pp. 101–115

Evans, L.V., Callow, M.E. 1974. Polysaccharide sulphation in *Laminaria*. *Planta* **117:**93–95

Evans, L.V., Callow, M.E., Percival, E., Fareed, V. 1974. Studies on the synthesis and composition of extracellular mucilage in the unicellular red alga *Rhodella*. *J. Cell. Sci.* **16:**1–21

Evans, L.V., Holligan, M.S. 1972. Correlated light and electron microscopic studies on brown algae I. Localisation of alginic acid and sulphated polysaccharides in *Dictyota*. *New Phytol.* **71:**1161–1172

Evans, L.V., Simpson, M., Callow, M.E. 1973. Sulphated polysaccharide synthesis in brown algae. *Planta* **110:**237–252

Findlay, G.P. 2001. Membranes and the electrophysiology of turgor regulation. *Aust. J. Plant Physiol.* **28:**617–634

Frachisse, J.M., Thomine, S., Colcombet, J., Guern, J., Barbier-Brygoo, H. 1999. Sulphate is both a substrate and an activator of the voltage-dependent anion channel of *Arabidopsis* hypocotyl cells. *Plant Physiol.* **121:**253–261

Garrill, A., Tyerman, S.D., Findlay, G.P., Ryan, P.R. 1996. Effects of NPPB and niflumic acid on outward K^+ and Cl^- currents across the plasma membrane of wheat root protoplasts. *Aust. J. Plant Physiol.* **23:**527–534

Giles, K.R., Humphries, M., Abell, A., Garrill, A. 2003. The synthesis of NPPB and NPBB by reductive amination and the effects of these compounds on K^+ channels of the alga *Nitella hookeri*. *Bioorg. Med. Chem. Lett.* **13:**293–295

Green, P.B., Erickson, R.O., Buggy, J. 1971. Metabolic and physical control of cell elongation rate. *Plant Physiol.* **47:**423–430

Greger, R. 1990. Chloride channel blockers. *Methods Enzymol.* **191:**793–810

Gutknecht, J. 1968. Salt transport in *Valonia*: Inhibition of potassium uptake by small hydrostatic pressures. *Science* **160:**68–70

Hastings, D.F., Gutknecht, J. 1976. Ionic relations and the regulation of turgor pressure in the marine alga, *Valonia macrophysa*. *J. Membr. Biol.* **28:**263–275

Hedrich, R., Spidola, N., Savchenko, G., Felle, H.H., Kaiser, W.M., Heber, U. 2001. Changes in apoplastic pH and membrane potential in leaves in relation to stomatal responses to CO_2, malate, abscisic acid or interruption of water supply. *Planta* **213:**594–601

Heidecker, M., Mimietz, S., Wegner, L.H., Zimmermann, U. 2003a. Structural peculiarities dominate the turgor pressure response of the marine alga *Valonia utricularis* upon osmotic challenges. *J. Membr. Biol.* **192:**123–139

Heidecker, M., Wegner, L.H., Binder, K-A., Zimmermann, U. 2003b. Turgor pressure changes trigger characteristic changes in the electrical conductance of the tonoplast and the plasmalemma of the marine alga *Valonia utricularis*. *Plant Cell Environ.* **26:**1035–1051

Kesseler, H 1964. Die Bedeutung einiger anorganischer Komponenten des Seewassers für die Turgorregulation von *Chaetomorpha linum*. *Helgol. Wiss. Meeresunters.* **10:**73–90

Kesseler, H. 1965. Turgor, osmotisches Potential und ionale Zusammensetzung des Zellsaftes einiger Meeresalgen verschiedener Verbreitungsgebiete. *Bot. Gothob.* **3:**103–111

Kirst, G.O. 1990. Salinity tolerance of eukaryotic marine algae. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **41:**21–53

Millard, P., Evans, L.V. 1982. Sulphate uptake in the unicellular marine red alga *Rhodella maculata*. *Arch. Microbiol.* **131:**165–169

Mimietz, S., Heidecker, M., Krohne, G., Wegner, L.H., Zimmermann, U. 2003. Impact of hypoosmotic challenges on spongy architecture of the cytoplasm of the giant marine alga *Valonia utricularis*. *Protoplasma* **222:**117–128

Mummert, H., Gradmann, D. 1991. Ion fluxes in *Acetabularia*: Vesicular shuttle. *J. Membr. Biol.* **124:**255–263

Philip, J.R. 1958. Propagation of turgor and other properties through cell aggregations. *Plant Physiol.* **33:**271–274

Ryser, C., Wang, J., Mimietz, S., Zimmermann, U. 1999. Determination of the individual electrical and transport properties of the plasmalemma and the tonoplast of the giant marine alga *Ventricaria ventricosa* by means of the integrated perfusion/charge-pulse technique: Evidence for a multifolded tonoplast. *J. Membr. Biol.* **168:**183–197

Schroeder, J.I., Schmidt, C., Sheaffer, J. 1993. Identification of high-affinity slow anion channel blockers and evidence for stomatal regulation by slow anion channels in guard cells. *Plant Cell* **5:**1831–1841

Shabala, S.N., Lew, R.R. 2002. Turgor regulation in osmotically stressed *Arabidopsis* epidermal root cells. Direct support for the role of inorganic ion uptake as revealed by concurrent flux and cell turgor measurements. *Plant Physiol.* **129:**290–299

Shepherd, V.A., Beilby, M.J., Bisson, M.A. 2004. When is a cell not a cell? A theory relating coenocytic structure to the unusual electrophysiology of *Ventricaria ventricosa* (*Valonia ventricosa*). *Protoplasma* **223:**79–91

Shepherd, V.A., Beilby, M.J., Heslop, D.J. 1999. Ecophysiology of the hypotonic response in the salt-tolerant charophyte alga *Lamprothamnium papulosum*. *Plant Cell Environ.* **22:**333–346

Shihira-Ishikawa, I., Nawata, T. 1992. The structure and physiological properties of the cytoplasm in intact *Valonia* cell. *Jpn. J. Phycol. (Sorui)* **40:**151–159

Spiess, I. 1996. Untersuchungen an perfundierten turgeszenten Algenzellen von Valonia utricularis: Elektrophysiologische Messungen zur Charakterisierung der elektrischen Eigenschaften von Plasmalemma und Tonoplasten. PhD Dissertation, University of Würzburg

Spiess, I., Wang, J., Benz, R., Zimmermann, U. 1993. Characterization of the chloride carrier in the plasmalemma of the alga *Valonia utricularis*: The inhibition by 4,4'-diisothiocyanatostilbene- 2,2'-disulfonic acid. *Biochim. Biophys. Acta* **1149:**93–101

Stento, N.A., Gerber Ryba, N., Kiegle, E.A., Bisson, M.A. 2000. Turgor regulation in the salt-tolerant alga *Chara longifolia*. *Plant Cell Environ.* **23:**629–637

Steudle, E., Zimmermann, U. 1971. Cell turgor and selective ion transport of *Chaetomorpha linum*. *Z. Naturforsch.* **26:**1276–1282

Taylor, A.R., Brownlee, C. 2003. A novel Cl^- inward-rectifying current in the plasma membrane of the calcifying marine phytoplankton *Coccolithus pelagicus*. *Plant Physiol.* **131:**1391–1400

Thomine, S., Guern, J., Barbier-Brygoo, H. 1997. Voltage-dependent anion channel of *Arabidopsis* hypocotyls: Nucleotide regulation and pharmacological properties. *J. Membr. Biol.* **159:**71–82

Wang, J., Spiess, I., Ryser, C., Zimmermann, U. 1997a. Separate determination of the electrical properties of the tonoplast and the plasmalemma of the giant-celled alga *Valonia utricularis*: Vacuolar perfusion of turgescent cells with nystatin and other agents. *J. Membr. Biol.* **157:**311–321

Wang, J., Sukhorukov, V.L., Djuzenova, C.S., Zimmermann, U., Müller, T., Fuhr, G. 1997b. Electrorotational spectra of protoplasts generated from the giant marine alga *Valonia utricularis*. *Protoplasma* **196:**123–134

Wang, J., Wehner, G., Benz, R., Zimmermann, U. 1991. Influence of external chloride concentration on the kinetics of mobile charges in the cell membrane of *Valonia utricularis*. Evidence for the existence of a chloride carrier. *Biophys. J.* **59:**235–248

Wangemann, P., Wittner, M., Di Stefano, A., Englert, H.C., Lang, H.J., Schlatter, E., Greger, R. 1986. Chloride channel blockers in the thick ascending limb of the loop of Henle structure activity relationship. *Pfluegers Arch.* **407:**S128–S141

Weiss, J.N. 1997. The Hill equation revisited: Uses and misuses. *FASEB J.* **11:**835–841

Yanochko, G.M., Yool, A.J. 2004. Block by extracellular divalent cations of *Drosophila* big brain channels expressed in *Xenopus* oocytes. *Biophys. J.* **86:**1470–1478

Zimmermann, U. 1978. Physics of turgor- and osmoregulation. *Annu. Rev. Plant Physiol.* **29:**121–148

Zimmermann, U., Büchner, K.H., Benz, R. 1982. Transport properties of mobile charges in algal membranes: Influence of pH and turgor pressure. *J. Membr. Biol.* **67:**183–197

Zimmermann, U., Schneider, H., Wegner, L.H., Haase, A. 2004. Water ascent in tall trees: Does evolution of land plants rely on a highly metastable state? *New Phytol.* **162:**575–615

Zimmermann, U., Steudle, E. 1974. The pressure-dependence of the hydraulic conductivity, the membrane resistance and membrane potential during turgor pressure regulation in *Valonia utricularis*. *J. Membr. Biol.* **16:**331–352

Zimmermann, U., Steudle, E. 1978. Physical aspects of water relations of plant cells. *Adv. Bot. Res.* **6:**45–117

Zimmermann, U., Wagner, H., Heidecker, M., Mimietz, S., Schneider, H., Szimtenings, M., Haase, A., Mitlöhner, R., Kruck, W., Hoffmann, R., König, W. 2002. Implications of mucilage on pressure bomb measurements and water lifting in trees rooting in high-salinity water. *Trees* **16:**100–111