Turgor pressure changes trigger characteristic changes in the electrical conductance of the tonoplast and the plasmalemma of the marine alga *Valonia utricularis*

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ABSTRACT

The giant marine alga *Valonia utricularis* is capable of regulating its turgor pressure in response to changes in the osmotic pressure of the sea water. The turgor pressure response comprises two phases, a fast, exponential phase arising exclusively from water shifting between the vacuole and the external medium (time constant about 10 min) and a second very slow, almost exponential phase adjusting (but not always) in the turgor pressure near to the original value by release or uptake of KCl (time constant about 5 h). The changes in the vacuolar membrane potential as well as in the individual conductances of the tonoplast and plasmalemma accompanying turgor pressure regulation were measured by using the vascular perfusion assembly (with integrated microelectrodes, pressure transducers and pressure-regulating valves) as described by Wang *et al.* (*J. Membrane Biology* 157, 311–321, 1997). Measurements on pressure-clamped cells gave strong evidence that the turgor pressure, but not effects related to water flow (i.e. osmosis or streaming potential) or changes in the internal osmotic pressure and in the osmotic gradients, triggers the cascade of osmotic and electrical events recorded after disturbance of the osmotic equilibrium. The findings definitely exclude the existence of osmosensors as postulated for other plant cells and bacteria. There was also evidence that turgor pressure signals were primarily sensed by ion transporters in the vacuolar membrane because conductance changes were first recorded in the many-folded tonoplast and then significantly delayed in the plasmalemma independent of the direction of the osmotic challenge. Consistently, turgor pressure up-regulation (but not down-regulation) could be inhibited reversibly by external addition of the K⁺ transport inhibitor Ba²⁺ and/or by the Cl⁻ transport inhibitor 4,4'-disothiocyanatostilbene-2,2'-disulfonic acid (DIDS). Extensive studies under iso-osmotic and hypo-osmotic conditions revealed that K⁺ influx upon lowering turgor pressure and the less pronounced pressure-dependence of the Cl⁻ influx of *V. utricularis* reported in the literature. The data derived from the blockage experiments under hypo-osmotic conditions were also equally consistent with the experimental findings that the K⁺ efflux is solely passive and progressively increases with increasing turgor pressure due to an increase of the volumetric elastic modulus of the cell wall. However, despite unravelling some of the sequences and other components involved in turgor pressure regulation of *V. utricularis* the co-ordination between the ion transporters in the tonoplast and plasmalemma remains unresolved because of the failure to block the tonoplast transporters by addition of Ba²⁺ and DIDS from the vacuolar side.

Key-words: 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS); barium; charge-pulse relaxation; ion transport; pressure clamp; pressure probe; turgor regulation; turgor sensor; vacuolar membrane potential.

INTRODUCTION

Turgor pressure controls membrane transport and metabolic processes in plant cells as well as the movement of stomata and leaves (Bentrup 1980; Findlay 2001; Hedrich *et al.* 2001). Close control of turgor pressure is therefore a key requirement for plant cells during extension growth (Green, Erickson & Buggy 1971; Cleland 1971, 1977), particularly under fluctuating salinity conditions (Rygol, Zimmermann & Balling 1989). Adjustment of turgor pressure in response to changes in external osmolality is seen impressively in algae inhabiting estuaries and coastal pools (Kesseler 1964, 1965; Steudle & Zimmermann 1971; Bisson & Kirst 1980; Kirst 1980; Kirst 1990; Shepherd, Beilby & Heslop 1999; Stento *et al.* 2000). According to the literature (Gutknecht 1968; Zimmermann & Steudle 1978) giant marine algae such as *Valonia utricularis* (Roth) C. Agardh, *Ventricaria ventricosa* (J. Agardh) Olsen & West and *Halicystis parvula* Schmitz can also regulate turgor pressure over a large range of salinity even though they experience only very slow and weak salinity fluctuations in their natural environment. In
general, algae have developed two mechanisms to adjust their turgor pressure: (1) by uptake or release of inorganic ions such as K\(^+\) or Cl\(^-\); and/or (2) by synthesis and degradation of osmotically active organic compounds such as glyceral, isofloridoside, sucrose, proline, etc. (Greenway & Stetter 1979; Blunden et al. 1992).

Despite extensive experimental work in recent years there are many differing views about the mechanisms that sense turgor pressure changes and use pressure signals to regulate internal osmotic pressure via membrane transport and/or metabolic processes (Gutknecht, Hastings & Bisson 1978; Bisson & Kirst 1995). As pointed out by Findlay (2001) we need simultaneous data on turgor pressure and electrical parameters of plasmalemma as well as tonoplast to describe more precisely the processes following changes in osmotic pressure or turgor pressure. At present, such data are only accessible for V. utricularis and V. ventricosa because the vacuole of these giant algal cells can be perfused under (clamped or variable) turgescent conditions (Wang et al. 1997a; Ryser et al. 1999). Integrated microelectrodes allow measurement of the individual membrane conductances by injection of charge pulses and subsequent measurement of the membrane voltage relaxations. These studies showed (Wang et al. 1997a; Ryser et al. 1999) that the fast voltage relaxation originates from the plasmalemma, whereas the slow one arises from the tonoplast. The analysis showed further that the specific conductance of the tonoplast is low (even lower than the specific conductance of the plasmalemma) and that the vacuolar membrane must be many-folded and/or contains mobile charges or ion transporters, not yet identified. These and other features (Heidecker et al. 2003) and the feasibility of electrorotation and patch clamp measurements on protoplasts derived from the giant ‘mother’ cells (Wang et al. 1997b; Heidecker, Wegner & Zimmermann 1999; Binder et al. 2003) make these species useful for exploring the mechanisms involved in sensing turgor pressure.

Furthermore, there is detailed information on ion and water fluxes in response to changes in osmotic and turgor pressure and about the individual electrical properties of the two membranes of V. utricularis or V. ventricosa. Perturbation of pressure equilibrium results in a biphasic exponential response of turgor pressure. The first turgor pressure relaxation is almost exclusively due to water flow into or out of the cell (Zimmermann & Steudle 1974). Subsequent turgor pressure regulation and return to the original pressure value was not investigated in detail, but is achieved predominantly by vacuolar uptake or release of K\(^+\) and Cl\(^-\). Synthesis or breakdown of organic compounds apparently only plays a role in adjusting the osmotic pressure of the cytosol (Zimmermann 1978). The K\(^+\) fluxes and, to a lesser extent, the Cl\(^-\) fluxes are turgor pressure dependent (Gutknecht 1968; Steudle & Zimmermann 1974). Pressure probe and flux measurements on V. utricularis have shown (Zimmermann & Steudle 1974; Steudle & Zimmermann 1974; Zimmermann, Steudle & Leikes 1976; Zimmermann 1978; Zimmermann & Steudle 1978) that the K\(^+\) influx increases progressively if turgor pressure is lowered from the ‘normal’ turgor pressure value in sea water (about 0.1 to 0.2 MPa) to zero by hyper-osmotic stress. Above 0.2 MPa the K\(^+\) influx was practically independent of turgor pressure. However, there is a progressive increase in K\(^+\) efflux when turgor pressure is increased up to 0.5 MPa by hypo-osmotic stress. Unlike the K\(^+\) influx, the increase in K\(^+\) efflux becomes even more pronounced as the turgor pressure and thus the cell size increases (see following sentence) indicating that the volumetric elastic modulus of the cell wall apparently plays an important role in turgor pressure adjustment under hypo-osmotic conditions (Zimmermann 1978; Zimmermann & Steudle 1978). Size-dependence of fluxes in turgor pressure-regulating algae is not unusual. For example, Bisson and colleagues (Hoffmann & Bisson 1990; Bisson et al. 1995) reported such effects for the Ca\(^{2+}\) influx of Chara buckellii Wood & Imahori (C. longifolia Proctor) upon osmotic stress. Unlike obligate freshwater charophytes (Kamiya & Kuroda 1957; Sanders 1981; Bisson & Bartholomew 1984), but similar to the giant marine algae this salt-tolerant species is capable of regulating its turgor pressure by increasing or decreasing vacuolar KCl in response to a number of different types of osmotic challenges.

Here, we show that the differing responses of the K\(^+\) fluxes to hyper- and hypo-osmotic stress of V. utricularis are probably due to the up-regulation of metabolic energy-dependent K\(^+\) (and Cl\(^-\)) influx transporters occurring only upon a decrease, but not upon an increase in turgor pressure. These data also clearly showed that changes in turgor pressure could be recorded first in the electrical conductance of the tonoplast before pronounced changes in the electrical conductance of the plasmalemma occurred suggesting that this membrane plays a crucial role in the sensing of turgor pressure changes. Turgor pressure regulations under pressure clamp conditions yielded further strong evidence that water-coupled, so-called electrotokinetic effects (see Dainty, Croghan & Fensom 1963; Katchalsky & Curran 1965) as well as osmotic pressure and osmotic pressure gradients can definitely be excluded as possible triggers for the adjustment of turgor pressure by KCl shifting once the rapid water exchange phase had been completed.

**MATERIALS AND METHODS**

**Culture conditions**

Cells of Valonia utricularis (Cladophorales, Chlorophyceae) were collected at Ischia, in the Gulf of Naples, Italy, and kept in 40 L tanks of Mediterranean sea water (MSW, 1127 mosmol kg\(^{-1}\), pH 8.1) under a 12 h light/dark regime (2 × 36 W Fluora lamps; Osram, München, Germany) at 16 °C. The sea water was continuously aerated.

**Experimental conditions**

For the experiments, mature geometrically even cells with volumes between 50 and 160 µL were used. After removal of encrustations the cells were clamped in a small Perspex®
and/or 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS; Fluka, Neu-Ulm, Germany) were used because it is briefly described in detail by Wang (1994) were performed by using the perfusion assembly described in detail by Wang et al. (1997a) and Ryser et al. (1999). Even though perfusion experiments were not performed in the work reported herein, the integrated pressure regulator valve of this apparatus could be used to change turgor pressure and clamp it at non-equilibrium values (for more details, see Wang et al. 1997a). This allows the effects of turgor pressure and trans-membrane water flow on charge-pulse relaxation to be studied separately (see below).

The apparatus can also determine the volume elastic modulus (ε) of the cell wall. This parameter is determined by injecting known volumes into impaled cells and recording the pressure changes (for details, see Zimmermann & Steudle 1974).

Electrical measurements

In charge-pulse experiments the membrane barrier is charged by injection of a current pulse of very short duration. The resulting membrane voltage is forced to decay through the membrane barrier because current flow is prevented by the very high reverse resistance of a diode being part of the external electrical circuit (Benz & Zimmermann 1983). From the voltage decaying (or more in physical terms ‘voltage relaxations’) the electrical parameters of the membranes can be deduced without interference with the resistances of the external circuit.

Briefly, two sharp pressure-tight borosilicate glass micropipettes of about 30 μm diameter were inserted into the centre of the vacuole with micromanipulators. The pipettes were filled with artificial vacuolar sap (AVS) that is usually used for vacuolar perfusion (see Wang et al. 1997a). It contained 210 mM NaCl, 3 mM CaCl₂, 3 mM MgCl₂, 3 mM phosphate buffer and 420 mM KCl (pH 6.3, 1210 mosmol kg⁻¹). Chemicals were purchased from Merck. The microelectrode for charge-pulse injection was a 10-μm-thick platinum wire that was moved slowly through one of the perfusion pipettes deep into the vacuole. The wire was connected to a fast, commercial pulse generator (model 214B; Hewlett Packard, Palo Alto, CA, USA) through a diode with a reverse resistance greater than 10¹⁰ Ω. The reference current-electrode in the bathing ASW consisted of a coined silver wire. A short, rectangular pulse of 1 μs duration was used for charging the membrane barrier. The injected charge could be calculated from the voltage drop across a 10 Ω resistor connected in series with the current microelectrode (monitored by a digital oscilloscope; Model 2440; Tektronix, Beaverton, OR, USA).

The microelectrode for recording voltage relaxation was filled with 3 mM KCl and contained an Ag/AgCl wire. The electrode was integrated into the other pipette as described elsewhere (Wang et al. 1997a; Ryser et al. 1999). The external potential-recording reference electrode consisted of Ag/AgCl and a 3 mM KCl agar bridge, placed in the medium close to the surface of the alga.

Analysis of the voltage relaxation

The semi-logarithmic plot of the voltage relaxation (V(t) versus time (t)) recorded on cells of V. utricularis after the administration of a charge pulse could be fitted by the sum of two exponential decays for a first approximation by using a multiple-exponential-fitting program (Benz & Zimmermann 1983) and/or the Levenberg–Marquardt algorithm of non-linear regression.
\[ V_p(t) = V_1\exp(-t/\tau_1) + V_2\exp(-t/\tau_2) = V_0\left[a_1\exp(-t/\tau_1) + a_2\exp(-t/\tau_2)\right] \]  

(1)

with \( V_0 = V_1 + V_2; a_1 = V_1/V_0 \) and \( a_2 = V_2/V_0 \), where \( V_{1,2} \) are the initial absolute amplitudes of the rapidly and slowly decaying voltages, 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 is a result of the electrical properties of the plasmalemma, whereas the slow one must be attributed to those of the tonoplast. Provided that the surface areas of the plasmalemma and the tonoplast are equal to the geometric surface area of the cell \( (A_{cell}) \), the area-specific resistances and capacitances of the tonoplast and the plasmalemma are given by the following equations:

\[ C_p = Q/(A_{cell}V_1); \quad R_p = 1/G_p = \tau_1/C_p \]  

(2)

\[ C_i = Q/(A_{cell}V_2); \quad R_i = 1/G_i = \tau_2/C_i \]  

(3)

where \( Q \) is the injected charge, \( C_{ip}, G_{ip}, \) and \( R_{ip} \) are the area-specific capacitances, conductances and resistances of the tonoplast and plasmalemma, respectively.

**RESULTS**

**Changes of turgor pressure, vacuolar membrane potential and membrane conductances upon osmotic stress**

About 1 h after insertion of the two capillaries into the vacuole of a *V. utricularis* cell the punctured areas were healed as shown by reading of a constant turgor pressure \( (P) \) and vacuolar membrane potential \( (V_m) \). The turgor pressure of cells bathed in ASW was on average 0.15 ± 0.07 MPa. The corresponding mean vacuolar membrane potential was +4.04 ± 2.01 mV \((n = 200)\). Charge-pulse relaxation experiments yielded a mean value of 103 ± 70 mV for the area-specific conductance of the plasmalemma \( (G_p) \), whereas the area-specific conductance of the tonoplast \( (G_i) \) was on average 61 ± 35 S m⁻² \((n = 182)\). The mean area-specific capacitances of the plasmalemma and the tonoplast were calculated to be \( C_p = 9.5 \pm 2.1 \) mF m⁻² and \( C_i = 81 \pm 34 \) mF m⁻². The values of the electrical parameters were in good agreement with previous data (Wang et al. 1997a).

The charge-pulse relaxation spectrum remained biphasic when the cells were subjected to osmotic challenges or to pressure clamps. The amplitudes of the two voltage relaxations did not change significantly indicating that \( C_p \) and \( C_i \) could roughly be assumed to be constant. Therefore, these data are not given further below.

**Hyper-osmotic conditions**

A typical turgor pressure relaxation and up-regulation experiment is shown in Fig. 1a. The cell was subjected to ASW to which 40 mosmol kg⁻¹ NaCl was added (= +0.1 MPa), turgor pressure decreased exponentially (time constant, \( \tau = 10.6 \) min; volumetric elastic modulus, \( e = 4.9 \) MPa) from a value of \( P_0 = 0.090 \) MPa in ASW and reached a quasi-stationary pressure value of \( P_{min} = 0.035 \) MPa about 50–60 min after addition of NaCl (see inset). This value is expected in the light of the theoretical considerations of Zimmermann & Steudle (1974) if a reflection coefficient of 0.94 is assumed for external NaCl (Heidecker et al. 2003). Apparently, turgor pressure relaxation is solely controlled by water efflux. Twenty minutes after reaching \( P_{min} \) slow up-regulation of turgor pressure was observed presumably due to ion shifting (see below); a value of 562 min was determined for the half time of turgor pressure regulation \( (\tau_{1,2}) \), namely the time after which 50% of the final pressure increase had been established. [It is assumed that the onset of ion regulation coincides with the end of water exchange phase, i.e. at \( P_{min} \)].

Measurements on other cells showed that the time constant of the water exchange phase was independent of the magnitude of the change of the external osmotic pressure (ranging from +0.10 to +0.23 MPa). Therefore, the data were pooled. On average, \( \tau \) was 9.4 ± 3.6 min \((n = 19)\). Subsequent up-regulation occurred usually after 70–100 min, but was only observed in some experiments. Approximately 43% of the cells showed no regulation or only fluctuations in turgor pressure of the order of a few kPa which did not, however, lead ultimately to a continuous (exponential) up-regulation of turgor pressure. When up-regulation occurred, the original turgor pressure was only occasionally reached. In two out of eight experiments, the turgor pressure was completely restored (>95% of \( P_0 \)); in four other experiments, pressure was up-regulated to more than 60% of the original value. The time needed to reach a final constant pressure value as well as \( \tau_{1,2} \) varied also considerably [12–25 h; \( \tau_{1,2} = 339 \pm 178 \) min \((n = 7)\); values ranging from 150 to 562 min]. Therefore, cells were classified as regulating ones when the rate of turgor pressure increase was ≥0.01 MPa after the first 600 min.

Analogous measurements on turgorless cells demonstrated (data not shown) that also only some of these cells (50%) could regenerate turgor pressure. Occasionally, the onset of up-regulation was considerably delayed regardless of the way in which turgor pressure was set to zero (addition of 0.3 MPa NaCl to ASW and/or by release of the turgor pressure via the pressure regulator valve). The onset of the delayed turgor pressure increase was observed 400–500 min after \( P_{min} \).

The finding that only a percentage of cells up-regulated their turgor pressure could be due to the impalement with microelectrodes as morphological changes were often observed after about 20 h (e.g. clustering of chloroplasts). However, there was evidence that the capability for up-regulation was apparently correlated with the initial vacuolar membrane potential. Regulating (turgescent and turgorless) cells exhibited on average a membrane potential of \( V_m = +4.7 \pm 1.6 \) mV \((n = 13)\); see also Table 1) whereas for non-regulating cells \( V_m \) was +1.0 ± 1.7 mV \((n = 10)\).
Turgor pressure changes and electrical membrane conductance in Valonia

Figure 1. Typical hyper-osmotic response of the turgor pressure and the electrical properties of the membranes recorded on a turgor pressure-regulating cell of Valonia utricularis. (a) Response of turgor pressure and vacuolar membrane potential measured with the perfusion apparatus. After replacement of ASW1144 (2.86 MPa) by ASW1184 (2.96 MPa, indicated by the down-headed arrow) the turgor pressure dropped exponentially (time constant $\tau_1 = 10.6$ min) from $P_0 = 0.090$ MPa to $P_{\text{min}} = 0.035$ MPa due to water efflux. $P_{\text{min}}$ was reached after about 50–60 min (see inset). Twenty minutes later an up-regulation of turgor pressure with a half time of 562 min was observed presumably due to ion shifting. The final turgor pressure was reached after about 1300 min. The water exchange phase was accompanied by a decrease in the vacuolar membrane potential from $V_{\text{m0}} = +7.3$ mV to $V_{\text{m1}} = +6.9$ mV within the first 10 min followed by an increase to $V_{\text{m2}} = +8.7$ mV (see inset). During the subsequent 1300 min up-regulation phase the vacuolar membrane potential decreased continuously until a final value of $V_{\text{m3}} = +5.2$ mV was reached. (b) Response of the membrane conductances measured with the perfusion apparatus. The conductance of the plasmalemma, $G_p$, changed slightly from $G_{p0} = 114$ S m$^{-2}$ to $G_{p1} = 104$ S m$^{-2}$ immediately after the hyper-osmotic shock (arrow), then increased continuously to a final value of $G_{p2} = 325$ S m$^{-2}$. Note that this dramatic increase in conductance started during the water exchange phase and that the final value was reached about 600 min before turgor pressure regulation was completed. The osmotically induced initial decrease in the conductance of the tonoplast, $G_t$, was more pronounced than that of the plasmalemma ($G_{t0} = 48$ S m$^{-2}$ and $G_{t1} = 24$ S m$^{-2}$). In contrast to the plasmalemma conductance, the subsequent increase in the tonoplast conductance coincided approximately with the end of the water exchange phase and the plateau value ($G_{t2} = 60$ S m$^{-2}$) was reached about 100 min after the hyper-osmotic shock. Note that this plateau value was not completely constant. A further, but very slight increase was observed after 500 min leading to a final value of $G_{t3} = 70$ S m$^{-2}$ (see Table 1).
In the regulating cells osmotically induced changes of turgor pressure were always associated with strong changes in the vacuolar membrane potential. This can be seen for example from the experiment performed on the regulating cell shown in Fig. 1a. After initiation of the turgor pressure relaxation by addition of 0.1 MPa NaCl, the membrane potential dropped slightly from \( V_{m0} = +6.9 \) mV (see inset) and began increasing after 10 min. A value of \( V_{m2} = +8.7 \) mV was reached about 40 min after the onset of turgor pressure relaxation (arrow), shortly before the end of the water exchange phase (about 60 min). The onset of the up-regulation phase of turgor pressure (i.e. about 70 min after the hyper-osmotic shock) was preceded by a potential decrease that continued during the regulation phase [Note that no conclusions on the causal linkage of processes can be drawn from the sequence of events]. As indicated in Fig. 1a the rate of hyperpolarization varied considerably with time. The initial turgor pressure \( P_b \) in ASW was re-established after about 1300 min and the potential assumed a value of \( V_{m1} = +5.2 \) mV (i.e. not exactly the original value). The corresponding \( V_m \) data measured on this and 12 other cells are listed in Table 1.

Inspection of the \( V_m \) values for non-regulating cells in Table 1 shows that their vacuolar membrane potentials also consisted of three phases, but that the absolute \( V_m \) values were much smaller in comparison to those of regulating cells.

Significant differences between regulating and non-regulating cells were also found for the conductances of the plasmalemma, \( G_p \) and the tonoplast, \( G_t \). In Fig. 1b, the changes in \( G_p \) and \( G_t \) are given for the cell in Fig. 1a. Upon addition of NaCl the plasmalemma conductance dropped slightly from \( G_{p1} = 114 \) S m\(^{-2}\) to \( G_{p2} = 104 \) S m\(^{-2}\) within about 10 min. This decrease in \( G_p \) was apparently correlated with the drop of \( V_{m1} \) to \( V_{m2} \) (see inset of Fig. 1a).

During the increase of the membrane potential from \( V_{m1} \) to \( V_{m3} \) i.e. still during the water exchange phase, the plasmalemma conductance increased, reaching a final value of \( G_{p3} = 325 \) S m\(^{-2}\) after about 700 min. Plasmalemma conductance levelled off before turgor pressure regulation was complete and before the membrane potential reached its final value \( V_{m3} \) (Fig. 1a).

The tonoplast conductance responded somewhat differently. The response after hyper-osmotic shock was delayed compared to \( G_p \). A transient decrease in \( G_t \) occurred during the change of the membrane potential from \( V_{m1} \) to \( V_{m2} \) and was more pronounced (\( G_{t1} = 48 \) S m\(^{-2}\) to \( G_{t2} = 24 \) S m\(^{-2}\)) than that of \( G_p \). Furthermore, a subsequent increase in \( G_t \) levelled off 100 min after addition of NaCl at \( G_t = 60 \) S m\(^{-2}\). Similar changes in \( G_p \) and \( G_t \) were observed for 12 other cells (see Table 1). It should be noted that the plateau value of the tonoplast conductance (reached after about 100 min) remained generally constant for about 400 min before a further, very small increase from \( G_{t3} \) to \( G_t \) in membrane conductance was observed (see also Fig. 1b). Statistical analysis of the data (Table 1) revealed that the characteristic patterns in the time course of the electrical parameters were significant in regulating, but not in non-regulating cells.

Non-regulating cells showed the same trend of changes in plasmalemma and tonoplast conductance. However, the plateau value of the plasmalemma conductance, \( G_{p2} \), was a factor of two smaller and the transient \( G_{t1} \) value of the tonoplast conductance a factor of two larger than in the case of regulating cells (see Table 1).

### Hypo-osmotic conditions

Despite some similarities in the coupling between membrane potential and turgor pressure, the hypo-osmotic regulation phenomena differed significantly from the hyper-osmotic ones. The key difference between hyper- and hypo-osmotic regulation was that all cells down-regulated their turgor pressure. However, 45% of cells did not re-

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**Table 1. Electrical parameters of \( V. utricularis \) after osmotic shocks, in pressure clamp experiments**

<table>
<thead>
<tr>
<th></th>
<th>Hyper-osmotic</th>
<th>Non-regulating</th>
<th>Pressure clamp((-\Delta P))</th>
<th>Hypo-osmotic</th>
<th>Pressure clamp((+\Delta P))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Regulating</td>
<td>Non-regulating</td>
<td>Influx</td>
<td></td>
<td>Efflux</td>
</tr>
<tr>
<td></td>
<td>( n = 13 )</td>
<td>( n = 10 )</td>
<td>( n = 4 )</td>
<td>( n = 10 )</td>
<td>( n = 4 )</td>
</tr>
<tr>
<td>( V_{m0} ) (mV)</td>
<td>4.7 ± 1.6</td>
<td>1.0 ± 1.7</td>
<td>6.2 ± 1.0</td>
<td>4.6 ± 1.5</td>
<td>3.2 ± 0.8</td>
</tr>
<tr>
<td>( V_{m1} ) (mV)</td>
<td>3.9 ± 1.9*</td>
<td>0.6 ± 1.4*</td>
<td>4.8 ± 1.8</td>
<td>5.2 ± 1.5*</td>
<td>3.6 ± 0.6</td>
</tr>
<tr>
<td>( V_{m2} ) (mV)</td>
<td>6.6 ± 1.3*</td>
<td>2.5 ± 1.5*</td>
<td>7.9 ± 1.3</td>
<td>2.2 ± 3.9*</td>
<td>2.9 ± 0.4*</td>
</tr>
<tr>
<td>( V_{m3} ) (mV)</td>
<td>5.0 ± 1.4*</td>
<td>1.5 ± 1.5*</td>
<td>5.7 ± 1.4</td>
<td>5.7 ± 3.9*</td>
<td>4.0 ± 0.6</td>
</tr>
<tr>
<td>( G_p ) (S m(^{-2}))</td>
<td>126 ± 36</td>
<td>112 ± 69</td>
<td>143 ± 32</td>
<td>81 ± 26</td>
<td>78 ± 18</td>
</tr>
<tr>
<td>( G_p ) (S m(^{-2}))</td>
<td>117 ± 36*</td>
<td>106 ± 66</td>
<td>139 ± 46</td>
<td>– –</td>
<td>– –</td>
</tr>
<tr>
<td>( G_t ) (S m(^{-2}))</td>
<td>310 ± 87*</td>
<td>161 ± 74*</td>
<td>247 ± 100</td>
<td>103 ± 27*</td>
<td>91 ± 27</td>
</tr>
<tr>
<td>( G_t ) (S m(^{-2}))</td>
<td>75 ± 31</td>
<td>77 ± 25</td>
<td>84 ± 27</td>
<td>60 ± 20</td>
<td>118 ± 16</td>
</tr>
<tr>
<td>( G_t ) (S m(^{-2}))</td>
<td>32 ± 11*</td>
<td>68 ± 25</td>
<td>44 ± 17*</td>
<td>45 ± 26*</td>
<td>83 ± 22*</td>
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<tr>
<td>( G_t ) (S m(^{-2}))</td>
<td>73 ± 26*</td>
<td>80 ± 32</td>
<td>105 ± 26*</td>
<td>– –</td>
<td>– –</td>
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<tr>
<td>( G_t ) (S m(^{-2}))</td>
<td>85 ± 38</td>
<td>117 ± 69</td>
<td>90 ± 25</td>
<td>50 ± 24</td>
<td>93 ± 23</td>
</tr>
</tbody>
</table>

*The vacuum potentials, \( V_m \), and the conductances of the plasmalemma, \( G_p \) and the tonoplast, \( G_t \) upon hyper- and hypo-osmotic treatment and upon influx and efflux pressure clamp experiments. The data obtained for influx clamp experiments correspond to those measured during hyper-osmotic regulation whereas the outflux pressure clamp data are similar to the hypo-osmotic ones. The data are mean values ± SD. *Difference between this value and the above value is statistically significant (paired Student’s t-test; \( P < 0.05 \)).

establish the original turgor pressure. In these cases pressure decreased continuously until turgor was completely lost after about 20 h. This was not due to tiny leaks induced by the hypo-osmotic shock as indicated by the values of the time constants derived by fitting of the initial part of the regulation curves that were comparable to those found for cells that could re-adjust their original turgor pressure. Down-regulation to \( P = 0 \) was apparently correlated with the morphological changes observed after long-term impalement (see above) and apparently indicated a gradual decrease in vitality. Accordingly, these data were not included in the analysis.

A typical biphasic turgor pressure response induced by lowering the osmotic pressure of the ASW to 2.73 MPa together with the corresponding changes in membrane potential, plasmalemma and tonoplast conductance are shown in Fig. 2. The time constant of \( P \) derived from the semi-logarithmic plot of the water exchange phase (data not shown) was \( \tau_1 = 15.3 \) min. For the subsequent pressure regulation \( \tau_{0.5} \) was determined to be 299 min. As in the hyper-osmotic case the time courses of the two phases were independent of the osmotic pressure in ASW (adjusted to 2.67–2.75 MPa by appropriate dilution with distilled water). On average, \( \tau_1 \) assumed values of \( 8.1 \pm 4.3 \) min (\( n = 8 \)); \( \tau_{0.5} \) was calculated to be \( 318 \pm 110 \) min (\( n = 8 \); values ranging from 92 to 419 min). Note that the time constants derived from the pressure-response to hypo-osmotic treatment agreed well with the corresponding values for hyper-osmotic experiments.

The changes in membrane potential were also tri-phasic, but opposite to those depicted in Fig. 1a (Fig. 2a; see also Table 1). In hypo-osmotic membrane potential curves the durations of the first two phases were significantly longer than under hyper-osmotic conditions (\( V_{m1} \) to \( V_{m2} \): 25 min; Figure 2. Typical hypo-osmotic response of the turgor pressure and the electrical properties of the membranes recorded from a turgor pressure-regulating cell of \( V. utricularis \). (a) Response of turgor pressure and vacuolar membrane potential measured with the perfusion apparatus. After replacement of ASW\(_{1140} \) (2.85 MPa) by ASW\(_{1091} \) (2.73 MPa; down-headed arrow) the turgor pressure increased exponentially from \( P_0 = 0.09 \) MPa to \( P_{\text{max}} = 0.18 \) MPa due to water influx (\( \tau_1 = 15.3 \) min). \( P_{\text{max}} \) was reached after 50 min. Fifteen minutes later a down-regulation occurred with a half time of 299 min until a final pressure value of 0.07 MPa was reached after about 900 min. The changes in the vacuolar membrane potential were tri-phasic, but opposite to those under hyper-osmotic conditions. Furthermore, the duration of the first two phases was significantly longer than in the case of hypo-osmotic treatment and the final membrane potential (reached after 700 min; \( V_{m3} = +6 \) mV) was significantly larger than the original value (\( V_{m0} = +4 \) mV). (b) Response of the membrane conductances measured with the perfusion apparatus. In contrast to hyper-osmotic conditions the plasmalemma conductance, \( G_p \), remained nearly constant. \( G_t \), increased only from 50 to 85 \( \text{S m}^{-2} \) over the entire period of regulation. The tonoplast conductance, \( G_t \), dropped after dilution of ASW, but the decrease occurred relatively slowly compared to hyper-osmotic conditions. Accordingly, the subsequent small increase in conductance also occurred only 150 min after \( P_{\text{max}} \) had been reached. A plateau value of \( G_t = 50 \) \( \text{S m}^{-2} \) was reached after about 700 min.

\[ \begin{array}{|c|c|c|}
\hline
\text{(a)} & \text{(b)} & \text{Time (min)} \\
\hline
\text{200} & \text{350} & \text{1250} \\
\text{10} & \text{80} & \\
\hline
\end{array} \]
under hyper-osmotic conditions. However, the response of the vacuolar membrane potential, \( G_p \), but the dependence of \( G_t \) upon turgor pressure changes could also be demonstrated by relaxation experiments in which cells were subjected to repeated short-term hyper-osmotic regimes (i.e. repetitive addition of NaCl followed by a re-establishment of the original turgor pressure). As shown in Fig. 3, \( G_t \) remained nearly constant, whereas \( G_i \) ‘oscillated’ as expected from the superposition of the data given in Figs 1b and 2b. The same was found for the response of the vacuolar membrane potential (data not shown). The finding that the tonoplast, but not the plasmalemma conductance responded to turgor pressure changes was also demonstrated by short-term experiments in which hyper-osmotic conditions were followed by hypo-osmotic conditions (after intermediate re-establishment of the original turgor pressure; data not shown).

Regulation phenomena of pressure clamped cells

For separate measurements of ‘water flow’ and ‘turgor pressure’ effects the turgor pressure was clamped at the initial value recorded immediately after the healing of the punctured areas around the micropipettes. Then, the pressure within the cell was changed stepwise by appropriate adjustment of the valve of the pressure regulator. Due to the unbalanced osmotic pressure gradient between the vacuole and the bath in clamped cells an inwardly directed water flow is created when the pressure is lowered. On the other hand, an outwardly directed water flow is induced when the turgor pressure is elevated (Fig. 4; for a thorough theoretical treatment, see Wendler & Zimmermann 1982).

For separation of ‘water flow’ and ‘turgor pressure’ effects it is mandatory that the osmolalities of the vacuolar sap and ASW have equilibrated before the clamp is released. In the light of the value of the relaxation time, \( t_r \), derived from osmotically induced water exchange (see above) osmotic equilibration between the tiny vacuole and the large ASW compartment should theoretically be reached after about 50 min. However, in order to ensure that osmotic equilibrium has really been established, the clamp time was prolonged to 70–120 min.

A typical example for the response to a pressure clamp below \( P_n \), the steady-state pressure in ASW, is shown in Fig. 5a. Turgor pressure was clamped at a value of \( P_{cm} = 0.004 \) MPa. When the clamp was released after 70 min (double-headed arrow), no rapid turgor pressure increase could be recorded indicating that the osmotic pressure of the vacuolar sap had actually equilibrated with the osmotic pressure of the bath before the clamp was released. An increase in turgor pressure could first be recorded about 20 min after pressure release indicating that ion shifting was involved in the generation of an osmotic pressure difference. This was also suggested by the following up-regulation of turgor pressure with a half time \( t_{1/2} = 413 \) min. On average, the half time was 302 ± 192 min (n = 4). Values ranged from 45 to 479 min and apparently agreed quite well with the values determined for the turgor pressure regulation phase under hyper-osmotic conditions (Fig. 1). Consistent with this, the tri-phasic vacuolar membrane potential, \( V_m \), was comparable with that recorded under hyper-osmotic conditions (except that the initial decrease of \( V_{m1} \) to \( V_{m2} \) was very much larger in clamped than in non-clamped cells; Fig. 5a). The plasmalemma and tonoplast conductances, \( G_p \) and \( G_t \), were considerably prolonged (up to 120 min). Data for 10 cells are summarized in Table 1; the characteristic changes in the time course of the electrical parameters proved to be statistically significant.

The independence of \( G_m \), but the dependence of \( G_t \) upon turgor pressure changes could also be demonstrated by relaxation experiments in which cells were subjected to repeated short-term hyper-osmotic regimes (i.e. repetitive addition of NaCl followed by a re-establishment of the original turgor pressure). As shown in Fig. 3, \( G_t \) remained nearly constant, whereas \( G_i \) ‘oscillated’ as expected from the superposition of the data given in Figs 1b and 2b. The same was found for the response of the vacuolar membrane potential (data not shown). The finding that the tonoplast, but not the plasmalemma conductance responded to turgor pressure changes was also demonstrated by short-term experiments in which hyper-osmotic conditions were followed by hypo-osmotic conditions (after intermediate re-establishment of the original turgor pressure; data not shown).
and $G_m$ measured under influx clamp conditions also agreed quite well with those derived from hyper-osmotic experiments (Table 1). The agreement of the ‘clamped’ data with those measured under hyper-osmotic conditions suggests that neither water influx nor internal osmotic pressure trigger the changes in the electrical properties of the membrane and the turgor pressure regulation. This conclusion was corroborated by influx clamp experiments at other lowered pressure values (0–0.5 MPa; data not shown). Further support for the dominating effect of turgor pressure changes on membrane potential and conductance was obtained by experiments in which the pressure was clamped to values above $P_o$ (pressure clamps between 0.17 and 0.25 MPa). A typical experiment is shown in Fig. 5b. In this case, the changes in turgor pressure, vacuolar membrane potential (Fig. 5b) and conductances (data not shown) were also comparable with those recorded for hypo-osmotic conditions in Fig. 2 (see also Table 1) except that the initial increase from $V_{m0}$ to $V_{m1}$ was absent in some experiments. In this context, it should be noted that the magnitude of $V_{m0}$ was not related to the clamped pressure levels.

The question remains why the effect of a decrease in turgor pressure on the tonoplast and plasmalemma conductances differs considerably from that observed in response to an increase in turgor pressure. Some evidence was obtained from experiments described in the following section in which $K^+$ and $Cl^-$ transporters as well as ATP synthesis were inhibited.

**Effects of channel and respiratory-chain inhibitors on turgor pressure, vacuolar membrane potential, and charge-pulse relaxations**

Previous studies on *Ventricaria ventricosa* have shown (Ryser *et al*. 1999) that addition of $K^+$ and $Cl^-$ channel blockers ($Ba^{2+}$ and DIDS, respectively) to the vacuolar perfusion solution did not affect the charge-pulse relaxation spectrum. Repetition of these experiments on perfused *V. utricularis* cells confirmed these results (data not shown). Thus, effects of channel blockers (and also of NaCN) were only studied from the external side on regulating cells (Figs 6 & 7). Screening experiments showed that exposure of the cells to the inhibitors resulted in turgor loss and also in irreversible morphological changes to the cells (e.g. clustering of the chloroplasts) when the exposure time exceeded 180 min. Therefore, in most of the experiments an exposure time of 60 min was selected to avoid adverse side-effects. Data were taken 30 min after addition of the inhibitors. At this time, blockage of the channels (or more generally transporters) was complete (see Fig. 6). In general, application of the blockers strongly affected the charge-pulse relaxation spectra from which the plasmalemma and tonoplast conductance were deduced (data not shown). A blockage of plasmalemma transporters resulted in a decrease of the plasmalemma conductance and, in turn, in an increase of the time constant of the fast ‘plasmalemma’ relaxation, $\tau_i$ (Eqn 1). Despite this, in most experiments this component of the spectrum could be clearly resolved and separated from the slow relaxation, $\tau_s$, that must be assigned to the tonoplast (see above). However, in the presence of both channel inhibitors the two relaxations merged into a single exponential curve when the cells were subjected to a hypotonic shock. Since the amplitude of the initial voltage, $V_i$, corresponded to that of the fast, ‘plasmalemma’ relaxation in the absence of the inhibitors and $V_i \gg V_s$, the data were analysed assuming the value of the area-specific capacitance of the plasmalemma.

**Channel inhibitors**

**Iso-osmotic conditions**

When 2 mM $Ba^{2+}$ or 0.2 mM DIDS were added to cells incu-
bated in (iso-osmotic) ASW1125–1145 the turgor pressure remained unaltered within the limits of accuracy (data not shown). While the conductance of the tonoplast, $G_t$, also remained largely unaffected by the inhibitors, the vacuolar membrane potential, $V_m$ (measured over the total membrane barrier) and the plasmalemma conductance, $G_p$, dropped immediately upon addition of DIDS ($V_m$ from $+4.1 \pm 1.1$ to $+3.5 \pm 0.8$ mV, i.e. by $-0.6 \pm 0.6$ mV and $G_p$ from $68 \pm 15$ S m$^{-2}$ to $42 \pm 8$ S m$^{-2}$; $n = 5$) and of Ba$^{2+}$ ($V_m$ from $+4.1 \pm 2.4$ to $+3.1 \pm 2.1$ mV, i.e. by $-1.0 \pm 0.4$ mV and $G_p$ from $81 \pm 24$ to $44 \pm 21$ S m$^{-2}$; $n = 5$). Evaluation of the data shows (Table 2) that the percentage of reduction of the plasmalemma conductance ($G_p$), was similar for DIDS (38%) and for Ba$^{2+}$ (46%). The inhibition of the Cl$^-$ and K$^+$ conductance was nearly additive as indicated by experiments in which Ba$^{2+}$ was added to the ASW 30 min after the addition of DIDS (72% overall inhibition). However, it has to be noted that a background conductance of about $20 \pm 8$ S m$^{-2}$ was recorded ($n = 7$; data not shown) indicating some contribution of an unidentified channel/canrier to the plasmalemma conductance. The effects of the inhibitors on the electrical properties of the membranes were completely reversible as shown by exchange of the inhibitor-containing solution against ASW after 60 min.

**Hyper-osmotic conditions**

The effect of Ba$^{2+}$ on turgor pressure and charge-pulse relaxations under hyper-osmotic conditions differed from that observed in (iso-osmotic) ASW. Typical recordings are shown in Fig.6a & b. Turgor pressure was lowered to about $P = 0.01$ MPa by addition of 0.11 MPa NaCl to ASW1124 (Fig.6; down-headed arrows). After about 80 min up-regulation of turgor pressure occurred. The rate was of

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Turgor pressure changes and electrical membrane conductance in Valonia

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As indicated in the figure, Ba²⁺ immediately stopped turgor pressure increase with the vacuolar membrane potential shifting slightly to less positive values ($\Delta V_m = -1.8 \pm 0.9 \text{ mV}; n = 4$). The effects on $V_m$ and $P$ were completely reversible. Re-establishment of a turgor pressure of 0.07 MPa occurred after 800 min. The pressure increase per time interval was in the same order of magnitude before the addition of Ba²⁺ and after the removal of the blocker. Measurements on three other cells yielded similar results.

As indicated in Fig. 6b, the membrane conductances, $G_p$ and $G_t$, also responded to the hyper-osmotic stress as described above (see Fig. 1b). Upon addition of Ba²⁺ (indicated by the up-headed arrow in Fig. 6b) the tonoplast conductance decreased slightly, but then remained almost constant as under (iso-osmotic) ASW-conditions. In contrast, the reduction in the plasmalemma conductance was much larger than that observed in iso-osmotic ASW. It dropped from 270 to 74 S m⁻². This corresponded to a 73% reduction in conductance with respect to the original value, that is the reduction of the conductance was significantly larger than under iso-osmotic conditions. Four measurements on other cells yielded similar results (188 ± 83 to 60 ± 18 S m⁻²; summarized in Table 2). It should be noted that the Ba²⁺ effect on the conductances was also completely reversible.

Turgor pressure up-regulation was also inhibited by the Cl⁻ channel blocker DIDS. The effects of this inhibitor on turgor pressure and tonoplast conductance (data not shown) were comparable to those observed for Ba²⁺. In contrast to Ba²⁺ the membrane potential increased ($\Delta V_m = +1.9 \pm 0.9 \text{ mV}; n = 5$) immediately after addition of DIDS and the decrease in plasmalemma conductance was
Figure 7. Typical effect of the K⁺ transport blocker Ba²⁺ on turgor pressure and electrical properties of the membranes of a turgor pressure-regulating alga subjected to hypo-osmotic stress. The experiment was performed similar to that shown in Fig. 6, except that the osmolality was changed by dilution with distilled water from ASW₁₁₂₈ to ASW₁₀₇₈ (down-headed arrow). (a) Addition of 2 mM BaCl₂ to the bath (up-headed arrow) did not affect down-regulation of turgor pressure. In contrast, \( V_m \) dropped from about +4.8 to +2.8 mV. The effects of Ba²⁺ on the membrane potential were reversible as shown by removal of the inhibitor (double-headed arrow). (b) During the presence of Ba²⁺ the conductance of the plasmalemma, \( G_p \), decreased from about 110 S m⁻² to about 50 S m⁻² whereas the conductance of the tonoplast, \( G_t \), remained nearly unaffected.

significantly less. On average, \( G_p \) decreased from 215 ± 56 to 120 ± 22 S m⁻² \((n = 5)\) corresponding to a 44% reduction in conductance (see Table 2). This value is apparently comparable with that measured for iso-osmotic conditions indicating that the relative contribution of Cl⁻ conductance to the total plasmalemma conductance was not changed in response to hyper-osmotic stress.

Hypo-osmotic conditions

Analogous experiments under hypo-osmotic conditions revealed similarities, but also characteristic features distinct from those described above for hyper-osmotic conditions. Down-regulation of the turgor pressure was not inhibited when Ba²⁺ (Fig. 7a) or DIDS (data not shown) were added temporarily about 150 min after establishment of an elevated, quasi-stationary turgor pressure value by dilution of ASW. Addition of both Ba²⁺ (\( \Delta V_m = -1.1 \pm 1.0 \text{ mV}; n = 5 \)) and DIDS (\( \Delta V_m = -1.6 \pm 0.6 \text{ mV}; n = 5 \)) induced a decrease in \( V_m \) during hypo-osmotic regulation. The tonoplast conductance was not significantly changed in the presence of Ba²⁺ (Fig. 7b) and DIDS (again similar to iso- and hyper-osmotic conditions, data not shown). In contrast, \( G_p \) was decreased by addition of Ba²⁺ from 113 to 50 S m⁻² in the experiment shown in Fig. 7b [over all from 112 ± 32 to 54 ± 29 S m⁻² (48%); \( n = 5 \)]. In addition the Cl⁻ conductance assumed values as recorded in iso-osmotic ASW [Table 2; DIDS: from 83 ± 25 to 46 ± 18 S m⁻² (55%); \( n = 6 \)].

Inhibition by NaCN

Measurements were performed in the presence of 0.3 mM NaCN in the dark over a time period of 60 min. As in the case of the channel inhibitors longer exposure times resulted in cell damage associated with turgor pressure loss. Effects of NaCN on the electrical properties of cells (vacuolar membrane potential as well as plasmalemma and tonoplast conductance; see Table 2) were evaluated by comparing the data before and 30 min after addition of the inhibitor. However, as already mentioned above it has to
be noted that blockage was largely complete after 10 min exposure.

CN⁻ had similar effects on the turgor pressure as those described above for the two channel inhibitors (data not shown). Turgor pressure regulation upon hyper-osmotic stress was stopped, whereas the turgor pressure in iso-osmotic ASW was unaffected. Under hypo-osmotic stress turgor pressure down-regulation occurred with the same time constant as in untreated cells. Independent of the external osmotic conditions, the membrane potential always dropped from +3 to +6 mV to a final value of 0 mV (data not shown). In contrast to the effects of Ba²⁺ and DIDS the tonoplast conductance decreased dramatically (i.e. by about 70%) from 90 ± 13 to 27 ± 12 S m⁻² (iso-osmotic), from 75 ± 10 to 22 ± 10 S m⁻² (hyper-osmotic) and from 69 ± 27 to 23 ± 5 S m⁻² (hypo-osmotic) in five experiments each.

In contrast, the effect of NaCN on the plasmalemma conductance was much less under hypo- than under iso- and hyper-osmotic conditions. For iso- and hyper-osmotic conditions the reduction in \( G_p \) was 64% (change from 95 ± 16 to 34 ± 12 S m⁻²; \( n = 5 \)) and 61% (change from 191 ± 71 to 75 ± 34 S m⁻²; \( n = 5 \)), respectively (Table 2). For hypo-osmotic conditions the reduction in \( G_p \) was much less (34%; change from 94 ± 34 to 62 ± 16 S m⁻²; \( n = 5 \); Table 2). Comparison of the \( G_p \) data in Figs 6 and 7 as well as in Table 2 suggests that the transporters blocked by Ba²⁺ and DIDS must, at least partly, be dependent on ATP and that the contribution of the ATP-dependent transporters to the total plasmalemma conductance differed under hyper- and hypo-osmotic conditions. This conclusion was also supported by experiments in which CN⁻ together with the channel inhibitors was added to ASW. Under iso- and hyper-osmotic conditions the reduction of the plasmalemma conductance was about 80% as found for Ba²⁺ and DIDS in the absence of CN⁻ (see above). Similar results were obtained for hypo-osmotic conditions (82%).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>( G_p ) (S m⁻²) before addition mean ± SD, ( n = 5 )</th>
<th>( G_p ) (S m⁻²) after addition mean ± SD, ( n = 5 )</th>
<th>( \Delta G_p ) (S m⁻²) induced by the blocker mean ± SD, ( n = 5 )</th>
<th>Percentage of residual conductance</th>
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</thead>
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<tr>
<td>Iso-osmotic</td>
<td>DIDS</td>
<td>68 ± 15</td>
<td>42 ± 8</td>
<td>-26 ± 8</td>
</tr>
<tr>
<td></td>
<td>Ba²⁺</td>
<td>81 ± 24</td>
<td>44 ± 21</td>
<td>-37 ± 11</td>
</tr>
<tr>
<td></td>
<td>CN⁻</td>
<td>95 ± 16</td>
<td>34 ± 12</td>
<td>-61 ± 16</td>
</tr>
<tr>
<td>Hypo-osmotic</td>
<td>DIDS</td>
<td>83 ± 25</td>
<td>46 ± 18</td>
<td>-37 ± 11</td>
</tr>
<tr>
<td></td>
<td>Ba²⁺</td>
<td>112 ± 32</td>
<td>54 ± 29</td>
<td>-58 ± 36</td>
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<td>CN⁻</td>
<td>94 ± 34</td>
<td>62 ± 16</td>
<td>-32 ± 20</td>
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<td>Hyper-osmotic</td>
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<td>120 ± 22</td>
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<tr>
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</tr>
<tr>
<td></td>
<td>CN⁻</td>
<td>191 ± 71</td>
<td>75 ± 34</td>
<td>-116 ± 52</td>
</tr>
</tbody>
</table>

*Data were derived from five independent experiments as shown in Figs 6 and 7 (mean ± SD). \( G_p \) values were taken before and 30 min after addition of the inhibitors. In the case of hyper- and hypo-osmotic turgor pressure regulation the blockers were added about 150 min after the onset of up- or down-regulation. For comparison of the data the percentages of the residual conductances are given. *Note that the conductance of the plasmalemma under hyper-osmotic conditions is higher than under iso- and hypo-osmotic conditions in accordance with the data in Fig. 1.

### DISCUSSION

Separate measurements of the individual conductances of the tonoplast and the plasmalemma upon turgor pressure changes have given some insight into the structural components and ion transporters involved in turgor pressure adjustment in the marine alga *Valonia utricularis*. The pressure clamp experiments provided clear-cut evidence (Fig. 5a & b) that several parameters can be excluded as possible candidates for triggering the cascade of osmotic and electrical events recorded during turgor pressure regulation. Release of the clamp after establishment of an osmotic equilibrium between the bath and the vacuole resulted in turgor pressure regulation kinetics as observed under hyper- or hypo-osmotic conditions. The sequence of the accompanying changes in the individual conductances of tonoplast and plasmalemma as well as in the vacuolar membrane potential (measured over the two barriers) correlated very well with those recorded by osmotic challenges (Table 1). This finding is not consistent with the hypothesis that electro-osmosis or streaming effects arising from the initial, osmotically induced net water flow (see, e.g. Dainty et al. 1963 and Katchalsky & Curran 1965) were involved in turgor pressure regulation.

The pressure clamp experiments also ruled out changes in the internal and external osmotic pressure as well as in osmotic pressure gradients as trigger signals for turgor pressure regulation. This becomes evident when the directions of the turgor and internal osmotic pressure changes under clamp conditions are compared with those under osmotic conditions. As shown in the schematic diagram of Fig. 4, pressure clamps below the steady state turgor pressure lead to a dilution of the internal concentration of osmotically active ions because of an inwardly directed water flow. With regard to the direction of water flow, pressure clamp below \( P_o \) (Fig. 5a) and hypo-osmotic treatment (Fig. 2) can be considered as equivalent. However, after water equilibra-
tion and release of the clamp the cells responded with a turgor pressure up-regulation and with associated changes in the electrical properties of the individual membranes as found upon a hyper-osmotic challenge. Similarly, when the pressure was clamped above the original turgor pressure value (Fig. 5b), up-concentration of the internal osmotically active ions occurs as observed under hyper-osmotic conditions (see Fig. 1). The subsequent pressure and electrical regulation phenomena upon clamp release, however, were very similar to those observed upon hypo-osmotic challenge. These findings are not consistent with the hypothesis of the existence of osmosensors in *V. utricularis* must be coupled with the electromechanical features of the membranes (Coster & Zimmermann 1976; Zimmermann, Beckers & Coster 1977; Coster, Steudle & Zimmermann 1978). Pressure-induced potential changes across the individual membranes could not be recorded because of technical difficulties. The changes of the vacuolar membrane potentials were rather small, but it has to be noted that even small changes can induce large changes in the intrinsic membrane electric fields and, in turn, in ion transport (Coster & Zimmermann 1976; see also further below). Furthermore, there is a body of evidence (Falke et al. 1988; Morris 1990; Okazaki & Tazawa 1990; Cosgrove & Hedrich 1991; Opsahl & Webb 1994; Garrill, Tyerman & Findlay 1994; Bisson & Kirst 1995; Awaysa et al. 1995; Awaysa & Subramanyam 1998; Maingret et al. 1999) that pressure signal transduction can be mediated by integral membrane proteins, particularly by stretch-activated, displacement-sensitive and shear-stress-sensitive ion channels. A stretch-activated channel has also been reported for protoplasts of *V. utricularis* (Heidecker et al. 1999). Mechano-sensitive ion channels may modulate cytoskeletal dynamics thus activating the regulation pathway that ultimately adjusts the original turgor pressure. The tonoplast of *V. utricularis* has a sponge-like appearance that greatly enlarges its surface area (Ryser et al. 1999; Heidecker et al. 2003). This suggests an important role of the cytoskeleton in turgor pressure regulation because this peculiar structure must be stabilized against the transient, relatively large unbalanced turgor pressures upon osmotic challenge. A biological membrane can only expand by about 3% before rupture occurs (Nichol & Hutter 1996). Thus mechano-protection must involve localized strengthening and remodelling of the cytoskeleton. The measurements of the membrane conductances are consistent with this view. Upon hypo-osmotic challenge, changes in the conductance of the tonoplast, *G*<sub>T</sub>, always occurred before significant changes in the plasmalemma conductance, *G*<sub>P</sub>, were observed (Fig. 2). Under hyperosmotic conditions, the transient initial decrease in *G*<sub>T</sub> was much more pronounced (both in absolute and relative terms) than the small (but significant) change of *G*<sub>P</sub> (Fig. 1). Similar observations were made in pressure clamp experiments (Table 1). This can be taken as evidence that the sponge-like tonoplast is involved in early events that are related to the transduction of the turgor signal (Coster et al. 1978). This conclusion contrasts the assumption of Hastings & Gutknecht (1974) based on hyperbaric experiments on *Valonia macrophysa* that turgor pressure is sensed at the plasmalemma.

Even though the transformation of a turgor pressure signal into ion transport in the tonoplast and plasmalemma has yet to be explored in more detail, it seems clear that these processes must be highly co-ordinated to regulate turgor pressure once water movement has disturbed it. It is therefore not surprising that the capability for regulation is subject to physiological variations and depends on the direction of the osmotic challenge (e.g. Rabinowitch, Grover & Ginzburg 1975). As shown here, cells of *V. utricularis* in hypo-osmotic stress always down-regulated their turgor pressure. In contrast, only half of the cells subjected to hyper-osmotic challenges showed up-regulation of turgor pressure. The capability for up-regulation did not depend on the magnitude of the turgor pressure. It occurred even in turgorless cells (although sometimes considerably delayed).

Down- and up-regulation did not always return turgor pressure to its original value. Under hypo-osmotic conditions, 45% of the cells responded with a continuous decrease of turgor pressure that ended ultimately in turgor pressure loss. The mechanism for turgor loss is unclear, but the decrease was so slow (14–18 h) that tiny leaks around the microcapillaries can probably be excluded. The up-regulation kinetics of regulating cells varied considerably and only rarely was the original turgor pressure restored. In non-regulating cells the turgor pressure remained more or less at the value (*P* > 0 MPa) established after pressure equilibration between the cell and the external artificial sea water (ASW). Potential implement artefacts of the microelectrode technique could be excluded (see above). The capability of regulation apparently depended on the magnitude of the initial value of the vacuolar membrane potential, *V*<sub>mem</sub> (Table 1) and thus on metabolic energy [which may be subjected to considerable variations in differently aged cells; see Stento et al. (2000)]. Evidence that *K*<sup>+</sup> and *Cl*<sup>−</sup> transport processes involved in turgor regulation are dependent on metabolic energy comes from the observation that the reduction of plasmalemma conductance by *CN*<sup>−</sup> apparently overlaps with the blockage of *K*<sup>+</sup> and *Cl*<sup>−</sup> transport across the plasmalemma by *Ba*<sup>2+</sup> and DIDS, respectively. Alternatively, it is also conceivable that channels and carriers contributing to the conductance require ATP for activation as shown previously for both animal and plant membranes (e.g. Nichols & Lederer 1990; Spalding & Goldsmith 1993).

The iso-osmotic blocker experiments clearly demonstrated (see Results and Table 2) that a large part of the plasmalemma conductance (70–100 S m<sup>−2</sup>) can be assigned to *K*<sup>+</sup> (= 46% inhibition) and *Cl*<sup>−</sup> (= 38% inhibition) trans-

port. In the presence of either $\text{Ba}^{2+}$ or DIDS the conductance was about 40 S m$^{-2}$. When the two channel inhibitors were added together the conductance decreased to 20 S m$^{-2}$ ($\approx 72\%$ inhibition). CN$^-$ induced a similar reduction of the conductance to about 30 S m$^{-2}$ ($\approx 64\%$ inhibition in the absence and $77\%$ in the presence of the channel inhibitors). Because of the striking similarity of the values it seems likely that both ion transport processes depend on metabolic energy or require ATP for activation. Under hyper-osmotic conditions, the absolute value of the plasmalemma conductance of up-regulating cells increased by a factor of 2–3 to about 220 S m$^{-2}$ (Figs 1b & 6b; Table 1). This suggests that activation of existing transporters or formation of new ones was required for turgor pressure up-regulation. The finding that the increase in turgor pressure and plasmalemma conductance could be blocked by $\text{Ba}^{2+}$ and/or DIDS as well as by CN$^-$ is consistent with both assumptions. The contribution of $\text{K}^+$ and $\text{Cl}^-$ channels to the conductance corresponded to about 150 S m$^{-2}$ ($\approx 68\%$ reduction upon $\text{Ba}^{2+}$ addition) and 100 S m$^{-2}$ ($\approx 44\%$ reduction upon DIDS addition). Surprisingly, the conductances of $\text{K}^+$ and $\text{Cl}^-$ were not additive because a remaining conductance of about 30 S m$^{-2}$ ($\approx 20\%$) was recorded in the presence of both inhibitors. This finding could be due to a voltage-dependence of the conductances blocked by DIDS and/or $\text{Ba}^{2+}$ since both inhibitors had opposite effects on the vacuolar membrane potential (+2 mV and –2 mV in the presence of DIDS and $\text{Ba}^{2+}$, respectively) under hyper-osmotic challenge. Alternatively, it is also conceivable that mutual interactions between $\text{K}^+$ and $\text{Cl}^-$ transport are induced under hyper-osmotic conditions.

Under hypo-osmotic conditions, supply of metabolic energy to turgor pressure regulation should play a minor role because $\text{K}^+$ (and $\text{Cl}^-$) ions are passively released from the vacuole (see Zimmermann 1978). In agreement with this, the plasmalemma conductances that could be blocked by $\text{Ba}^{2+}$ (about 60 S m$^{-2}$; 52%) or DIDS (about 40 S m$^{-2}$; 45%) were comparable to those recorded under iso-osmotic conditions (about 40 S m$^{-2}$; 46% and about 30 S m$^{-2}$; 38%, respectively), but the percentage of transporters that could be blocked by CN$^-$ was significantly less (about 30 S m$^{-2}$; 34%) than under iso-osmotic conditions (about 60 S m$^{-2}$; 64%; see Table 2).

In the light of the above considerations, it is clear that some of the processes and components involved in turgor pressure regulation of $V.\ utricularis$ could be unravelled. However, the co-ordination between the ion transporters in the tonoplast and plasmalemma remains open because of the failure to block ion transporters in the tonoplast from the vacuolar side by $\text{Ba}^{2+}$ and DIDS. A great challenge for the future is also to identify the ions that contribute to the residual plasmalemma conductance in blockage experiments seen both under iso-, hyper- and hypo-osmotic conditions. In the light of the work of Shepherd et al. (1999) and Stento et al. (2000) the role of cytosolic $\text{Ca}^{2+}$ as a possible messenger of turgor pressure signals should be examined.

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