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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

C. Ryser, J. Wang, S. Mimietz, U. Zimmermann
Lehrstuhl für Biotechnologie, Biozentrum, Universität Würzburg, Am Hubland, D-97074 Würzburg, Germany

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Abstract. The charge-pulse relaxation spectrum of nonperfused and perfused (turgescent) cells of the giant marine alga *Ventricaria ventricosa* showed two main exponential decays with time constants of approximately 0.1 msec and 10 msec, respectively, when the cells were bathed in artificial sea water (pH 8). Variation of the external pH did not change the relaxation pattern (in contrast to other giant marine algae). Addition of nystatin (a membrane-impermeable and pore-forming antibiotic) to the vacuolar perfusion solution resulted in the disappearance of the slow exponential, whereas external nystatin decreased dramatically the time constant of the fast one. This indicated (by analogy to corresponding experiments with *Valonia utricularis*, J. Wang, I. Spieß, C. Ryser, U. Zimmermann, *J. Membrane Biol.* **157:** 311–321, 1997) that the fast relaxation must be assigned to the RC-properties of the plasmalemma and the slow one to those of the tonoplast. Consistent with this, external variation of $[K^+]_o$ or of $[Cl^-]_o$ as well as external addition of K^+- or Cl^--channel/carrier inhibitors (TEA, Ba^{2+}, DIDS) affected only the fast relaxation, but not the slow one. In contrast, addition of these inhibitors to the vacuolar perfusion solution had no measurable effect on the charge-pulse relaxation spectrum. The analysis of the data in terms of the "two membrane model" showed that K^+- and (to a smaller extent) Cl^--conducting elements dominated the plasmalemma conductance. The analysis of the charge-pulse relaxation spectra also yielded the following area-specific data for the capacitance and the conductance for the plasmalemma and tonoplast (by assuming that both membranes have a planar surface): (plasmalemma) $C_p = 0.82 * 10^{-2}$ F m^{-2}, $R_p = 1.69 * 10^{-2}$ Ω m^2, $G_p = 5.9 * 10^4$ mS m^{-2}, (tonoplast) $C_t = 7.1 * 10^{-2}$ F m^{-2}, $R_t = 14.9 * 10^{-2}$ Ω m^2 and $G_t = 0.67 * 10^4$ mS m^{-2}. The electrical data for the tonoplast show that (in contrast to the literature) the area-specific membrane resistance of the tonoplast of these marine giant algal cells is apparently very high as reported already for *V. utricularis*. The exceptionally high value of the area-specific capacitance could be explained — among other interpretations — by assuming a 9-fold enlargement of the tonoplast surface. The hypothesis of a multifolded tonoplast was supported by transmission electronmicroscopy of cells fixed under maintenance of turgor pressure and of the electrical parameters of the membranes. This finding indicates that the tonoplast of this species exhibited a sponge-like appearance. Taking this result into account, it can be easily shown that the tonoplast exhibits a high-resistance (1.1 Ω m^2).

Vacuolar membrane potential measurements (performed in parallel with charge-pulse relaxation studies) showed that the potential difference across the plasmalemma was mainly controlled by the external K^+-concentration which suggested that the resting membrane potential of the plasmalemma is largely a K^+-diffusion potential. After permeabilization of the tonoplast with nystatin the potential of the intact membrane barrier dropped from about slightly negative or positive (−5.1 to +18 mV, $n = 13$) to negative values (−15 up to −68 mV; $n = 8$). This indicated that the cytoplasm of *V. ventricosa* was apparently negatively charged relative to the external medium. Permeabilization of the plasmalemma by addition of external nystatin resulted generally in an increase in the potential to

Correspondence to: U. Zimmermann

Abbreviations: ACS, artificial cytoplasma sap; ASW, artificial sea water; AVS, artificial vacuolar sap; NSW, natural sea water; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; TEA, tetraethylammonium; MES^-, 2-(N-morpholino)ethanesulfonate

slightly more positive values (−0.8 to +4.3 mV; $n = 5$), indicating that the vacuole is positively charged relative to the cytoplasm.

These findings apparently end the long-term debate about the electrical properties of *V. ventricosa.* The results presented here support the findings of Davis (*Plant Physiol.* **67:** 825–831, 1981), but are contrary to the results of Lainson and Field (*J. Membrane Biol.* **29:** 81–94, 1976).

Key words: Membrane capacitance — Membrane resistance — Membrane folding — Nystatin — Tonoplast — Plasmalemma — Vacuolar perfusion

Introduction

Marine and pond water giant-celled algae have been used extensively to study the electrical (and transport) properties of the membrane barrier (Hope & Walker, 1975; Walker, 1976; Lainson & Field, 1976; Findlay & Hope, 1976; Zimmermann, 1978; Zimmermann & Steudle, 1974; Davis, 1981; Tazawa & Shimmen, 1982; Wendler, Zimmermann & Bentrup, 1980; Beilby, 1989). However, a major problem which is encountered with microelectrode measurements in giant algal cells is the interpretation of the data because of the presence of two membranes in series, i.e., the tonoplast and the plasmalemma. The vacuole of giant algal cells occupies usually more than 95% of the total cell volume. In contrast to the impalement of the microelectrodes into the thin cytoplasmic layer, vacuolar placement of microelectrodes is easy. Furthermore, vacuolar electrodes block — if any — only occasionally whereas cytoplasmic microelectrodes can be covered rapidly with a thick seal (Findlay & Hope, 1976). The disadvantage of vacuolar microelectrodes is that they do not allow to measure separately the electrical properties of the tonoplast and the plasmalemma (for exceptions, *see* Davis, 1981). To circumvent this problem, it is, therefore, assumed in the analysis of the electrophysiological data (Beilby, 1989) that the tonoplast of giant algal cells is substantially more conductive than the plasmalemma (Beilby, 1990; Blatt, 1987).

Very recently, we have demonstrated for the giant cells of the marine alga *Valonia utricularis* (Wang et al., 1997) that vacuolar microelectrodes can be used to determine the electrical properties of the tonoplast and the plasmalemma separately. This progress in the electrophysiology of giant-celled algae was achieved by measurements of the charge-pulse relaxation spectrum of nonperfused and perfused turgescent cells in the presence or absence of vacuolar and external nystatin (or amphotericin B), respectively.

Nystatin (or amphotericin B) is a pore-forming antibiotic which permeabilizes membranes, but does not penetrate them (*see also* Marty & Finkelstein, 1975; Kleinberg & Finkelstein, 1984; Bolard, 1986; Horn & Marty, 1988; Shvinka & Caffier, 1994; Marty & Neher, 1995). Nystatin can, therefore, be used to increase separately the conductance of the tonoplast and the plasmalemma depending on the site of addition. Vacuolar nystatin resulted in the disappearance of the slow relaxation of the charge-pulse relaxation spectrum of *V. utricularis*, whereas external nystatin decreased dramatically the time constant of the fast relaxation. This finding demonstrated that the fast relaxation must be assigned exclusively to the RC-properties of the plasmalemma and the slow one exclusively to the RC-properties of the tonoplast.

The analysis of the charge-pulse relaxation spectrum of *V. utricularis* in terms of the "two membrane model" led to the following conclusions: (i) that the tonoplast resistance is very high (comparable to that of the plasmalemma), (ii) that the area of the tonoplast is considerably larger than the geometric area and/or contains "mobile charges" associated with electrogenic transport systems and (iii) that the plasmalemma conductance is mainly determined by Cl^--channels/carriers.

In this communication we applied this novel method to the giant-celled alga *Ventricaria ventricosa.* This species (like *V. utricularis*) belongs to related genera of the class of *Bryopsidophyceae* (together with *Chaetomorpha* and *Valoniopsis*) which seems to differ from the mainstream plant model (Steudle & Zimmermann, 1971). These species can be characterized by a large tonoplast resistance and a positive potential difference across the tonoplast (Findlay et al., 1971; Findlay et al., 1978) respectively. This large potential difference is expected to arise from a unusual K^+ ATPase (Gutknecht, 1967).

V. ventricosa also shows the phenomenon of turgor regulation (like *V. utricularis* and *Chaetomorpha;* Steudle & Zimmermann, 1971; Zimmermann & Steudle, 1974; Zimmermann, Steudle & Lelkes, 1976; Zimmermann, 1978), i.e., the cells have the ability to maintain constant turgor pressure over a large range of salinity (Gutknecht, 1968; Hastings & Gutknecht, 1974).

Experiments with vacuolar and external pore-forming antibiotics gave strong support to the view that the two relaxations of the charge-pulse relaxation spectrum of this species also arise from the RC-properties of the two membranes. Evidence for this interpretation came additionally from the demonstration that the variation of the concentration of the major external ions, in particular of K^+, only affected the fast relaxation (arising from the plasmalemma) and the membrane potential. External addition of transport inhibitors revealed that DIDS-sensitive Cl^--channels/carriers and particularly TEA/Ba^{2+}-sensitive K^+-channels contributed to about 93% of the conductance of the plasmalemma. In con-

trast, the tonoplast conductance remained unaltered upon vacuolar addition of these inhibitors.

The analysis of the data in terms of the "two membrane model" also showed that the area of the tonoplast of *V. ventricosa* must be much larger than the geometric one. This could be supported by transmission electronmicroscopy which suggested that the tonoplast of *V. ventricosa* (as well as of *V. utricularis*) exhibits a spongelike appearance.

Materials and Methods

Harvest and Culture Conditions

Giant, spherical cells of *Ventricaria ventricosa* (Siphonocladales, Chlorophyceae; formerly named *Valonia ventricosa, see* Olsen & West, 1988) were collected from the coast of the island of Oahu, HI. The size of the cells varied considerably. For the experiments, freshly collected cells of extremely large size (1.2 to $7.3 * 10^3$ mm^3) and of smaller size (60 to 500 mm^3) were used. Before use, the cells were kept for 3 to 11 weeks in 5-liter glass containers filled with natural sea water (NSW, 1024 mosmol kg^{-1}, pH 8.1) under a 12 hr light/dark regime (2 × 36 W Fluora lamps, Osram, München, Germany) at a temperature of 20°C (293 K).

Cells of *V. ventricosa* grow very slowly. Part of the freshly collected cells formed aplanospores. Cells originated from these aplanospores were cultured. The cultured cells were also used for charge-pulse relaxation experiments when they had grown to about 2 to 6 mm in diameter corresponding to volumes of about 4.2 to 113 mm^3.

Experimental Conditions

For the experiments the cells were bathed in artificial sea water (ASW; 1024 mosmol kg^{-1}) containing 510 mM NaCl, 11 mM KCl, 10 mM $CaCl_2$ and 9 mM $MgCl_2$. The pH of ASW was adjusted to 8.1 by addition of 10 mM HEPES/NaOH (N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)/NaOH).

For the study of the effect of external K^+, Cl^- and Na^+ on the charge-pulse relaxation spectrum, media were used in which the concentration of the ions was varied accordingly by replacing Na^+ by cholin, K^+ by Na^+ and Cl^- by MES^- (2-(N-morpholino)ethanesulfonate) in the ASW. The osmolality of the various ASW-solutions was adjusted to 1024 mosmol kg^{-1} (measured by freezing point depression using an Osmomat (model 030-M, Gonotec GmbH, Berlin, Germany)).

For evaluation of the contribution of K^+-channels and of Cl^--transport systems to the charge-pulse relaxation spectrum, ASW was used which contained additionally TEA (tetraethylammonium), $BaCl_2$ and/or DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; for experimental details, *see* Spieß et al., 1993).

For charge-pulse experiments at pH 7 and 6, the ASW was buffered with 10 mM PIPES/NaOH (piperazine-N,N′-bis(ethanesulfonic acid)) and MES-/NaOH (2-(N-morpholino)ethanesulfonic acid), respectively. Lower pH-values were adjusted by addition of 10 mM malic acid and appropriate amounts of NaOH.

For vacuolar perfusion, artificial vacuolar sap (AVS; pH 6.1; 1340 mosmol kg^{-1}) was used containing 22 mM Na_2SO_4, 625 mM KCl, 3 mM $CaCl_2$, 3 mM $MgCl_2$ and 3 mM phosphate buffer. The concentrations of the major ions of AVS corresponded to those of the normal vacuolar sap of *V. ventricosa* (Gutknecht, 1966).

Separate permeabilization of the tonoplast and the plasmalemma was achieved by addition of nystatin (or amphotericin B) to the external or vacuolar solution. The antibiotics were pre-dissolved in dimethyl sulfoxide (DMSO). A portion of the 5 M-stock solution was added to artificial cytoplasm sap (ACS; pH 7.3; osmolality 1024 mosmol kg^{-1}; containing 40 mM NaCl, 80 mM KCl, 408 mM K_2SO_4, 5 mM $CaCl_2$, 10 mM $MgCl_2$, 10 mM EGTA and 10 mM HEPES buffer). The final concentration of DMSO in the artificial cytoplasm sap was 1%. Control experiments showed that this concentration of DMSO did not significantly affect the charge-pulse relaxation spectra of the membranes of *V. ventricosa.*

All salts were obtained from Merck (Darmstadt, Germany). The buffers, nystatin, amphotericin B, TEA and DMSO were purchased from Sigma (Deisenhofen, Germany). DIDS was obtained from Fluka (Neu-Ulm, Germany).

A fiber-optic light source (model KL 150B, Schott, Mainz, Germany) equipped with a halogen lamp (150 W, Osram, München, Germany), a heat protecting filter KG 1 and a fiber-optic bundle (Schott, Mainz, Germany) was used to illuminate the cells during the experiments with white light (40 W m^{-2}). The experiments were performed at 20°C (293 K) if not otherwise stated.

Electrical Measurements

The experimental conditions for charge-pulse relaxation studies on nonperfused giant algal cells have been described in detail elsewhere (Benz & Zimmermann, 1983; Wang, Zimmermann & Benz, 1994). Briefly, two pressure-tight pipettes (outer tip diameter < 30 μm) were inserted deeply into the vacuole of the cell. The shank of the pipette containing the current microelectrode was sealed by a rubber 'O'-ring to an oil-filled perspex chamber in which a pressure transducer was mounted for recording of the turgor pressure (Zimmermann & Steudle, 1974). The current microelectrode consisting of a 10 μm thick platinum wire was moved through the pipette 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 larger than 10^{10} Ω. The reference current electrode in the bathing solution consisted of a rectangular steel plate of 28 × 8 mm. The membrane barrier was charged with a short, rectangular pulse of 1-μsec duration. The injected charge, *Q*, was calculated from the voltage drop (monitored by a digital oscilloscope, model 2440, Tektronix, Beaverton, OR) across a 10 Ω resistor connected in series with the current microelectrode.

For recording of the resting and generated potential across the membrane barrier a second pipette-microelectrode was used which was filled with 3 M KCl and contained an Ag/AgCl wire. The external potential-recording reference electrode consisted of Ag/AgCl and a 3 M KCl agar bridge. This electrode was placed in the bulk medium close to the surface of the alga. The voltage relaxation spectrum after a charge-pulse was recorded by a fast, differential amplifier of high input impedance (connected to the potential-measuring electrodes) and stored on a Nicolet 2090 or 490 digital oscilloscope (Frankfurt, Germany) in 4096 data points with 12-bit amplitude resolution. These were then transferred to a PC/AT computer for further analysis. The bandwidth of the whole electrical system was 0–50 kHz (Wang et al., 1993). The complete setup was shielded against electromagnetic waves by a Faraday cage.

For charge-pulse experiments on perfused cells the electrophysiological setup was combined with a vacuolar perfusion assembly as described in detail recently (Wang et al., 1997). Briefly, the potential-recording microelectrode was integrated into an inflow glass pipette which was completely filled with perfusion solution prior to insertion into the vacuole. The inflow pipette was connected to a syringe containing the perfusion solution. The perfusion solution in the syringe

was forced into the vacuole by a perfusion pump (PERFUSOR™ SECURA FT, B. Braun Melsungen AG, Melsungen, Germany). The outflow pipette contained the current microelectrode and was connected to a pressure chamber in which a pressure regulator was mounted to adjust the pressure in the cell and in the other part of the hydraulic system as required.

The perfusion setup allowed to change the perfusion flow rate and the turgor pressure independently of each other (while maintaining the internal and external ion concentration) and to exchange perfusion solutions without exposure of the cell to a pressure shock.

Analysis of the Voltage Relaxation

The semilogarithmic plot of the voltage relaxation, V_m, *vs.* time, t, recorded on cells of *V. ventricosa* after the administration of a charge-pulse could be fitted by the sum of two exponential decays to a first approximation (*see* Fig. 1), using a multiple-exponential-fitting program (Benz & Zimmermann, 1983) and/or the Levenberg-Marquardt algorithm of nonlinear regression:

$$V_m(t) = V_1\exp(-t/\tau_1) + V_2\exp(-t/\tau_2)$$

$$= V_0(a_1\exp(-t/\tau_1)) + (a_2\exp(-t/\tau_2)) \quad (1)$$

with

$$V_0 = V_1 + V_2$$

and

$$a_1 = V_1/V_0;\ a_2 = V_2/V_0$$

where $\tau_{1,2}$ are the relaxation time constants, $V_{1,2}$ the initial absolute amplitudes of the rapidly and slowly decaying voltages and $a_{1,2}$ the initial relative amplitudes of the rapidly and slowly decaying voltages, respectively. The fit to the experimental data was very significant under all circumstances ($P < 0.001$, checked by using Student's t-test). Furthermore, in 10 successive experimental runs (taken on the same algal cell at time intervals of 30 sec) the relaxation parameters did not vary by more than 3%. This demonstrates the high reproducibility of the measurements.

It has to be noted that a significantly better fit of the charge-pulse relaxation spectrum could frequently (but not always; *see* below) be achieved by assuming that a third exponential relaxation of very small initial amplitude occurred (*see* e.g., Fig. 1). The time constant of this relaxation was between τ_1 and τ_2. The occasional occurrence of a third relaxation was also reported for *V. utricularis* (Zimmermann, Büchner & Benz, 1982). Because of this variability in the occurrence of the third, intermediate relaxation, the voltage relaxation spectrum was analysed in terms of two exponentials if not stated otherwise.

The experiments presented below suggest very strongly (*see also* Wang et al., 1997) that the fast relaxation arises from the electrical properties of the plasmalemma, whereas the slow one must be attributed to those of the tonoplast. In terms of this "two membrane model" the area-specific resistances and capacitances of the tonoplast and the plasmalemma, respectively, are given by the following equations (provided that the surface area of the plasmalemma and the tonoplast are equal to the geometric surface area, A_{cell}, of the algae):

$$C_p = Q/(A_{cell}\,V_1);\ R_p = 1/G_p = \tau_1/C_p \quad (2)$$

and

$$C_t = Q/(A_{cell}\,V_2);\ R_t = 1/G_t = \tau_2/C_t \quad (3)$$

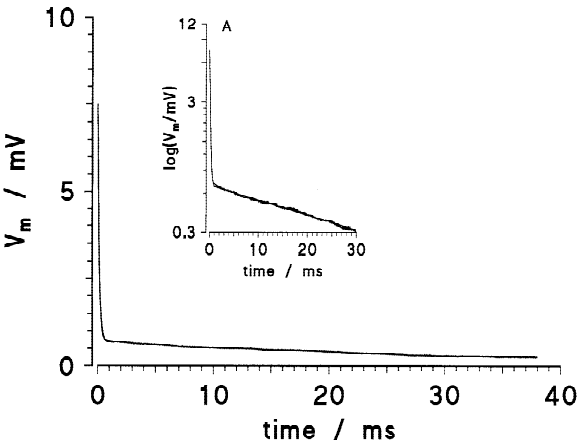

Fig. 1. Typical charge-pulse relaxation spectrum of a cultivated, nonperfused cell of the giant marine alga *Ventricaria ventricosa* bathed in ASW (1024 mosmol kg^{-1}, pH 8.1). The spectrum was recorded by injection of a charge-pulse of 1-μsec duration via a microelectrode into the vacuole. The injected charge was $Q = 7.32$ nAsec. The vacuolar resting potential was $V_{vo} = +8.2$ mV. The spherical cell had a surface area of $A_{cell} = 80$ mm^2, a volume of $V_{cell} = 66$ mm^3 and a turgor pressure of $P = 0.16$ MPa. The semilogarithmic plot of the biphasic voltage relaxation spectrum versus time is shown in the inset. The relaxations in the inset were fitted to the sum of three exponentials by using the expanded Eq. 1.: $V_m(t) = V_o(a_1\exp(-t/\tau_1)) + (a_2\exp(-t/\tau_2)) + (a_3\exp(-t/\tau_3))$. For further details, *see* text.

where Q is the injected charge, $C_{t,p}$, $G_{t,p}$ and $R_{t,p}$ are the area-specific capacitances, conductances and resistances of the tonoplast and plasmalemma, respectively. It should be pointed out that the actual surface area of the two membranes may not necessarily be equal to the geometric one (A_{cell}; *see* below).

Results

Effects of External and Vacuolar Nystatin on the Charge-Pulse Relaxation Spectrum: Assignment of the Relaxations to the Tonoplast and Plasmalemma Properties

The charge-pulse experiments on the algal cells of *V. ventricosa* were started 1–2 hr after the insertion of the two microelectrodes (or of the in-/outflow pipettes) into the vacuole. This time was sufficient to heal the punctured areas as shown by the constancy of the cell turgor pressure (in average 0.2 MPa) over time and the reproducibility of the charge-pulse relaxation spectra in repeated experimental runs.

A typical voltage relaxation spectrum of a charge-pulse experiment performed on a cultivated nonperfused cell of *V. ventricosa* is shown in Fig. 1. The vacuolar resting potential of the cell was $V_{vo} = +8.2$ mV. The membrane barrier was charged by a short current pulse of 1-μsec duration to an initial voltage of about $V_o =$

Table 1. Results of charge-pulse experiments on cells of *Ventricaria ventricosa*

Cell parameter					Relaxation parameter					Plasmalemma		Tonoplast			
Cell	V_{cell} mm^3	A_{cell} mm^2	P MPa	V_{vo} mV	Q nAs	τ_1 μsec	V_1 mV	τ_2 msec	V_2 mV	C_p $10^{-2}Fm^{-2}$	G_p $mScm^{-2}$	C_t $10^{-2}Fm^{-2}$	G_t $mScm^{-2}$	A_t^* mm^2	G_t^* $mScm^{-2}$
1	14	28	0.19	−6	4.22	172	16.7	4.57	2.39	0.902	5.25	6.31	1.38	196	0.197
2	27	44	0.21	3	4.78	190	13.4	5.56	1.77	0.811	4.27	6.14	1.10	333	0.146
3	51	69	0.22	5	8.07	151	14.4	4.38	2.51	0.812	5.38	4.66	1.06	396	0.185
4	62	61	0.21	2	6.56	146	14.2	3.39	1.99	0.757	5.19	5.40	1.59	435	0.223
5	160	141	0.32	−6	21.9	434	20.9	15.4	2.34	0.743	1.71	6.64	0.431	1260	0.048
6	290	220	0.27	−3	15.8	144	8.94	48.9	0.84	0.803	5.58	8.55	0.175	2340	0.016
7	840	430	0.14	22	33.4	71	10.3	14.0	0.98	0.754	10.6	7.93	0.566	4520	0.054
8	1700	680	0.19	2	24.2	118	4.84	63.9	0.52	0.735	6.23	6.84	0.107	6330	0.012
9	2540	900	0.21	17	57.4	144	8.20	71.8	0.74	0.778	5.40	8.62	0.120	9970	0.011
10	3870	1160	0.10	4	53.4	112	4.23	54.3	0.47	1.09	9.70	9.78	0.180	10500	0.020
Mean		373	0.21	4						0.818	5.93	7.09	0.672	3628	0.0913
±SD		±407	±0.06	±9						±0.106	±2.55	±1.60	±0.565	±4029	±0.0865

Charge-pulse experiments were performed on over 90 cells bathed in ASW. Only a part of data was shown. For a first approximation, the voltage relaxation spectra after the injection of the charge-pulse was fitted to a function of the sum of two exponentials (Eq. 1). The time constants and the initial amplitudes of the fast (τ_1 and V_1) and the slow exponential relaxation (τ_2 and V_2) were obtained from least-square-fits. Q is the injected charge, A_{cell} is the surface area of the alga, V_{vo} is the resting vacuolar potential and P is the turgor pressure. The area-specific membrane capacitances and conductances of the plasmalemma (C_p and G_p) and that of the tonoplast (C_t and G_t) respectively were calculated by assuming that the surface area of the plasmalemma (A_p) and the tonoplast (A_t) is equal to that of the cell ($A_p = A_t = A_{cell}$). The real surface area and the real area-specific conductance of the tonoplast (A_t^* and G_t^*) were calculated by using $A_t^* = A_{cell}(C_t/C_t^*)$ and $G_t^* = G_t(A_{cell}/A_t^*)$ under the assumption that the real area-specific capacitance of the tonoplast is equal to that of the plasmalemma ($C_t^* = C_p$). For further details, *see* text.

+14 mV (in reference to the resting potential). By analogy to *V. utricularis* and *H. parvula* (Zimmermann et al., 1982; Benz, Büchner & Zimmermann, 1988) the semilogarithmic plot of the voltage relaxation spectrum suggested (*data not shown*) that the voltage relaxation could be fitted by the sum of two exponential curves according to Eq. 1 (correlation coefficient $r = 0.99865$). The following parameters (±SD) were obtained from the least square fitting of the relaxation spectrum: for the fast relaxation, $V_1 = 11.2 \pm 0.3$ mV and $\tau_1 = 256 \pm 4$ μsec; for the slow relaxation, $V_2 = 1.52 \pm 0.01$ mV and $\tau_2 = 3.75 \pm 0.02$ msec. Similar results were obtained for approx. 90 other (nonperfused) cells of *V. ventricosa.* Some of these data are listed in Table 1.

The fit of the voltage relaxation spectrum in Fig. 1 could be slightly, but significantly improved by assuming three exponential decays (Fig. 1, inset *A;* correlation coefficient $r = 0.99986$). In this case, the following parameters (±SD) could be derived for the fast relaxation: $V_1 = 12.3 \pm 0.3$ mV and $\tau_1 = 219 \pm 6$ μsec; for the slow relaxation: $V_2 = 1.21 \pm 0.06$ mV and $\tau_2 = 4.11 \pm 0.07$ msec; for the third intermediate exponential: $V_3 = 0.82 \pm 0.06$ mV and $\tau_3 = 1.37 \pm 0.17$ msec. The third, intermediate relaxation could not always be resolved (only in about 50% of the experimental runs performed on different nonperfused cells).

Charge-pulse experiments on cells perfused with artificial vacuolar sap (AVS) yielded similar results for the relaxation parameters ($n = 36$, data not shown; *see also* Wang et al., 1997).

In the following set of experiments the effects of external and vacuolar nystatin on the charge-pulse relaxation spectrum of perfused and nonperfused cells of *V. ventricosa* were investigated in order to identify the membrane from which the fast, the slow and the third decay curves originated. The turgor pressure of the vacuolar perfused cells was adjusted to 0.2 MPa as measured in the nonperfused cells. For the nystatin-induced permeabilization of the plasmalemma and the tonoplast, respectively, the ASW or the AVS, respectively, were replaced by the artificial cytoplasmic sap (ACS, *see* Materials and Methods). In contrast to AVS and ASW which contained about 600 mM Cl^-, the Cl^--concentration in ACS was only 140 mM in order to match the cytoplasmic Cl^--concentration. This procedure avoids lethal effects due to diffusion of high concentrations of Cl^- through the permeabilized membranes into the cytoplasm (Wyn Jones, Brady & Speirs, 1978; Wang et al., 1997).

Lowering of the Cl^--concentration in the bulk medium or in the perfusion solution resulted in an increase of the time constants of the relaxations of freshly collected cells (curve 1 in Fig. 2*A; see also* below). Upon external addition of nystatin the time constant of the fast exponential, τ_1, decreased dramatically (curve 2 in Fig. 2*A* and Fig. 3*A*). After 40 min the time constant of the fast relaxation process was too short to be resolved (Fig. 3*A*). Interestingly, the time constant of the slow exponential, τ_2, did not change significantly over a period of more than 1 hr (Fig. 3*A*). Fitting of the charge-pulse

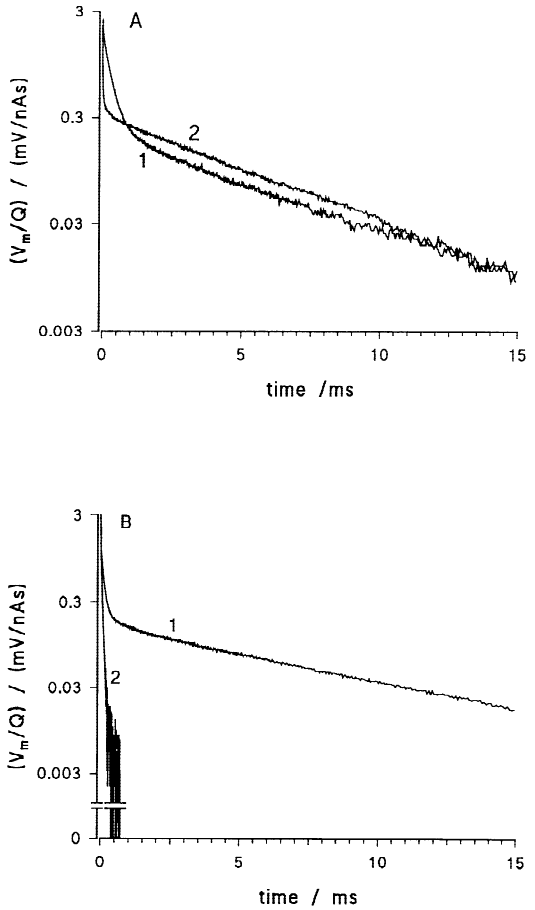

Fig. 2. Typical effect of nystatin on the charge-pulse relaxation spectra of freshly collected cells of *V. ventricosa* after external (*A*) and vacuolar (*B*) addition. (*A*) Charge-pulse relaxation spectra measured on a nonperfused cell (A_{cell} = 80 mm^2, V_{cell} = 66 mm^3, P = 0.23 MPa, and V_{vo} = +6.8 mV); curve 1: control measurements in ASW, injected charge Q = 7.1 nAsec; curve 2: relaxation spectrum 48 min after replacing the bath solution by ACS containing 50 μM nystatin (injected charge Q = 8.14 nAsec). (*B*) Charge-pulse experiments performed on a vacuolar perfused cell bathed in ASW (A_{cell} = 132.7 mm^2, V_{cell} = 143.8 mm^3 and V_{vo} = −0.4 mV). The flow rate of the perfusion solution (AVS) was adjusted to 20 mm^3 min^{-1} and the turgor pressure to 0.12 MPa. Recordings were performed before (curve 1, Q = 19.8 nAs) and 100 min after changing the perfusion solution from AVS to ACS plus 50 μM nystastin (curve 2, Q = 9.48 nAsec). Note that for the evaluation of the nystatin effect on the voltage spectrum the voltage relaxations were normalized to the injected charge, *Q*. Furthermore, it should be noted that similar results were obtained from eight independent measurements by using different freshly collected or cultivated cells.

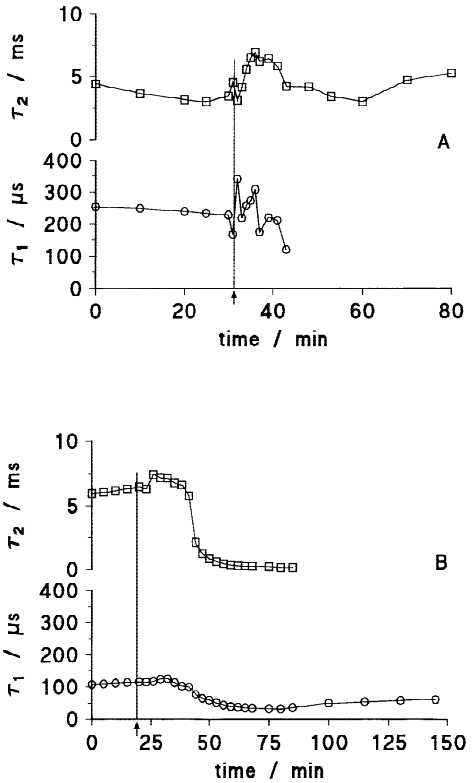

Fig. 3. Time course of the effect of nystatin on the time constants of the fast (τ_1) and the slow relaxation (τ_2) after external (*A*) and vacuolar (*B*) addition. For calculation of the time constants data were taken from the corresponding charge-pulse relaxation studies presented in Fig. 2*A* and *B*. The arrows marked the changes of ASW (Fig. 3*A*) and of AVS (Fig. 3*B*) to ACS plus 50 μM nystatin, respectively. Note that only a part of the data are shown because of clarity (even though the charge-pulse relaxation spectra were recorded approximately every one minute, *see* Fig. 2). For further details, in particular for the effect of lowered Cl^--concentration in the ACS medium on the voltage relaxations, see text.

spectrum by three exponentials showed (curve 2 in Fig. 2*A*) that in the presence of external nystatin the third intermediate relaxation (amplitude of about 0.5 mV and a time constant of about 1 msec) occurred regularly.

The opposite results were found when nystatin was added to the vacuolar perfusion solution (Fig. 2*B*). Under these conditions, the time constant of the slow exponential relaxation, τ_2, decreased rapidly and the slow relaxation disappeared after about 30 min, whereas τ_1 remained nearly constant for more than 1 hr (curve 2 in Figs. 2*B* and 3*B*). In the presence of vacuolar nystatin, an intermediate exponential could not be resolved.

Equivalent concentrations of amphotericin B yielded similar, membrane-depending effects, but this compound was less efficient and acted more slowly than nystatin (*data not shown*). Furthermore, when the above experiments with nystatin or amphotericin B were repeated with cultured cells, similar results were obtained as with freshly collected cells.

Therefore, the specific, side-dependent effect of pore-forming antibiotics on the charge-pulse relaxation spectrum of *V. ventricosa* suggested strongly that the fast voltage relaxation originates from the plasmalemma, whereas the slow and intermediate ones arise from the electrical properties of the tonoplast.

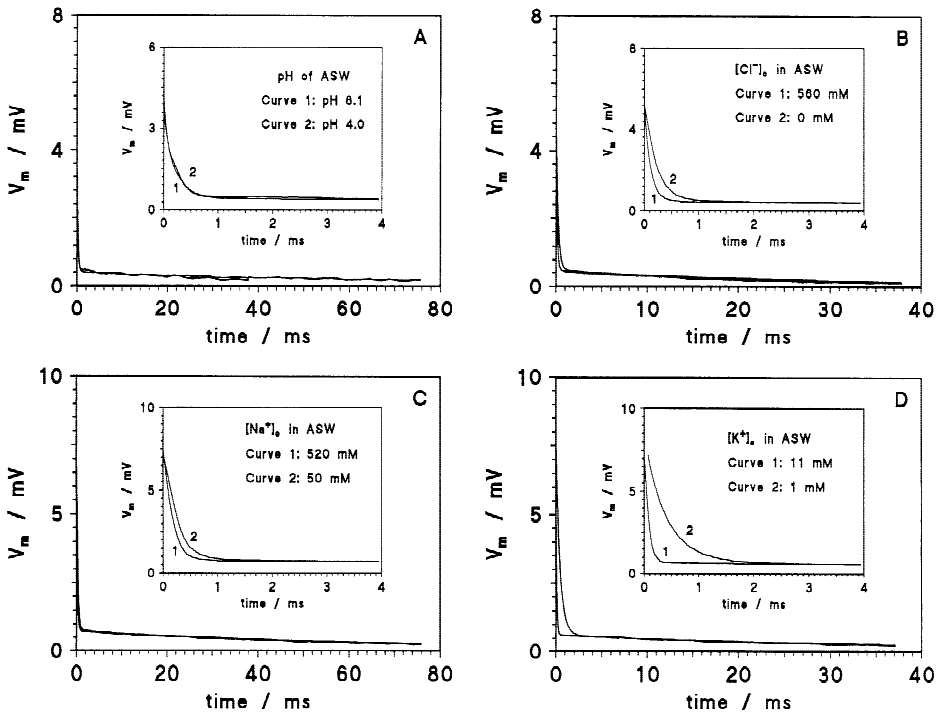

Fig. 4. Influence of an external pH of 4 (*A*) as well as of the external K^+ (*B*), Cl^- (*C*) and Na^+ (*D*) concentration on the charge-pulse relaxation spectra of *V. ventricosa*. Charge-pulse experiments were performed on cultured cells bathed in ASW (curves 1) and 15 min later after replacing ASW by the pH- or ion-manipulated solutions (curves 2). Cell parameters: (*A*) A_{cell} = 1680 mm^2, V_{cell} = 6440 mm^3, P = 0.12 MPa, and V_{vo} = +12 mV; (*B*) A_{cell} = 680 mm^2, V_{cell} = 1800 mm^3, P = 0.15 MPa, and V_{vo} = +8.5 mV; (*C*) A_{cell} = 900 mm^2, V_{cell} = 2540 mm^3, P = 0.22 MPa, and V_{vo} = +17 mV; and (*D*): A_{cell} = 400 mm^2, V_{cell} = 780 mm^3, P = 0.20 MPa, and V_{vo} = +11 mV. Insets: magnified plots of the corresponding charge-pulse relaxations in the time range of 0–4 msec.

Effects of the pH and the Major Ions of the External Medium on the Conductance and Potential of the Plasmalemma

Figure 4*A* represents a typical charge-pulse experiment on a cultivated cell of *V. ventricosa* in which the pH of the ASW was lowered to a value of 4. As indicated, the charge-pulse relaxation spectrum was not influenced significantly by this low pH value. A similar finding was found for freshly collected (small and large) cells.

In contrast, lowering of $[K^+]_o$, $[Cl^-]_o$ or $[Na^+]_o$ in the ASW affected the charge-pulse relaxation spectrum of *V. ventricosa*. As can be seen from typical recordings on cultured cells in Fig. 4*B–D* the (fast) exponential which arises from the passive electrical properties of the plasmalemma (*see above*) was influenced significantly. The (slow) relaxation of the tonoplast remained nearly unaltered. It is evident that the effect of $[K^+]_o$ on the plasmalemma relaxation was much more pronounced than that of $[Cl^-]_o$ or $[Na^+]_o$. The replacement of external chloride by MES^- only resulted in an increase of the time constant of the fast exponential by a factor of about 2.

Analysis of curves as shown in Fig. 4 in terms of the "two membrane model" by using Eqs. 1–3 showed that the capacitance of the plasmalemma (and that of the tonoplast) was independent of the external ion concentrations (*data not shown*). Plots of the dependence of the area-specific plasmalemma conductance (normalized to the respective values in ASW) on $[K^+]_o$, $[Cl^-]_o$ and $[Na^+]_o$, respectively, are depicted in Fig. 5. Data are given for cultured as well as for freshly collected, small and large sized cells.

It is evident from the regression analysis in these figures that $[K^+]_o$ affected significantly the area-specific conductance of the plasmalemma of *V. ventricosa* (Fig. 5*A;* 7% increase in conductance per 1 mM $[K^+]_o$*;* $P < 0.0001$). The conductance changes induced by $[Cl^-]_o$ were much smaller (Fig. 5*B;* 0.1% per 1 mM; $P < 0.01$), but significant. As shown in Figs. 5*A* and *B*, the effects of external K^+ and Cl^- were size-independent and similar for cultured and freshly collected algae. In contrast, $[Na^+]_o$ did not influence the conductance of the plasmalemma of small and large sized, freshly collected cells (Fig. 5*C;* $P > 0.3$ and $P > 0.5$, respectively). However, external Na^+ had (like $[Cl^-]_o$) a small, but significant effect on the area-specific plasmalemma conductance of cultivated cells (Fig. 5*C;* 0.08% per 1 mM; $P < 0.03$).

$[K^+]_o$, $[Cl^-]_o$ and $[Na^+]_o$ had no effect on the con-

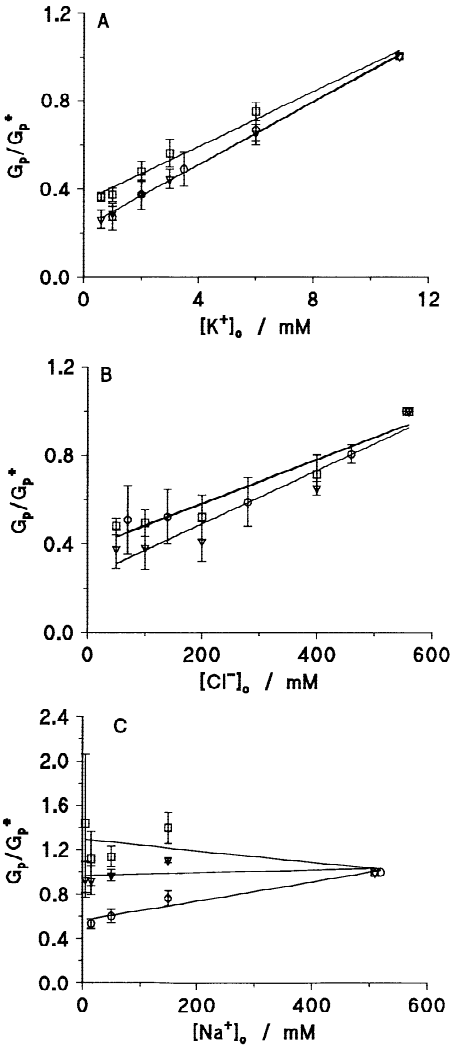

Fig. 5. Dependence of the electrical area-specific conductance of the plasmalemma, G_p, of *V. ventricosa* on $[K^+]_o$ (*A*), $[Cl^-]_o$ (*B*) and $[Na^+]_o$ (*C*) calculated from charge-pulse relaxation spectra (as shown in Fig. 4) by using Eqs. 1 and 2. The plasmalemma conductance, G_p, was normalized to the control value, G_p^*, measured in ASW. Symbols: open circles: cultivated cells; open squares: freshly collected large cells and open triangles: freshly collected small cells. Each data point represents the mean ± SD of 3 to 9 independent experiments. The straight lines were obtained by linear regression analysis of the data. For further explanations, *see* text.

ductance of the tonoplast of cultured and freshly collected cells — at least within the limits of accuracy (*data not shown*).

Effects of Nystatin and External Ions on the Vacuolar Resting Membrane Potential

The addition of nystatin was accompanied with changes in the vacuolar resting membrane potential, V_{vo}. This potential is the algebraic sum of the potential across the tonoplast, V_{vc}, and of the potential across the plasmalemma, V_{co} (Davis, 1981). V_{vo} varied between −5.1 and +18 mV ($n = 13$). The response of the membrane potential upon vacuolar or external nystatin addition was also subject to a large variability. Despite this, it is clear from Fig. 6*A* that the membrane potential dropped to more negative values when nystatin was added to the perfused cells from the vacuolar side (−15 to −68 mV, $n = 8$). Addition of nystatin to nonperfused cells from the outside (Fig. 6*B*) resulted generally in an increase of the potential to slightly more positive values of −0.8 to +4.3 mV ($n = 5$). The potentials recorded under these conditions, apparently reflected the potential difference across the plasmalemma, i.e., V_{co} in Fig. 6*A*, and across the tonoplast, i.e., V_{vo} in Fig. 6*B*. Under both conditions the potentials relaxed back to zero or to values around zero, presumably because of ionic changes in the cytoplasm due to the high permeability of the plasmalemma and the tonoplast, respectively. Independent of this, it seems to be clear that the cytoplasm is negatively charged relatively to the external medium and the vacuole.

Recordings of the resting vacuolar membrane potential, V_{vo}, of nonperfused cells in dependence on $[K^+]_o$, $[Cl^-]_o$ and $[Na^+]_o$, respectively are represented in Fig. 7. The measurements were conducted during the course of the charge-pulse experiments shown in Fig. 5. The regression analysis of the data obtained on cultivated and freshly collected cells showed that V_{vo} did not depend significantly on $[Na^+]_o$ (Fig. 7*C*, $P > 0.05$). A weak response in the potential was observed when $[Cl^-]_o$ was changed (Fig. 7*B*, $P < 0.03$). In contrast, a pronounced hyperpolarisation response of V_{vo} of both cultivated and freshly collected cells was recorded upon a decrease in $[K^+]_o$ (Fig. 7*A; P* < 0.0005).

Since the passive electrical properties of the tonoplast were not affected by $[K^+]_o$ (*see above*), the $[K^+]_o$-effects on V_{vo} apparently reflected the changes in the plasmalemma potential difference.

In contrast to $[Cl^-]_o$ and $[Na^+]_o$, the changes in the plasmalemma potential difference with $[K^+]_o$ (about 40 mV per decade, *see* Fig. 7*A*) agreed quite well with the Nernst equilibrium potential difference for $[K^+]_o$ (dashed lines in Fig. 7). This suggested that the plasmalemma potential difference was mainly due to a K^+-diffusion potential.

The conclusions in this section agree quite well with data reported for this species in the literature (Gutknecht, 1966; 1967; Davis, 1981).

Temperature-Dependence of the Electrical Properties of the Plasmalemma and Tonoplast

Measurements of the plasmalemma and tonoplast conductance as a function of temperature suggested that ion

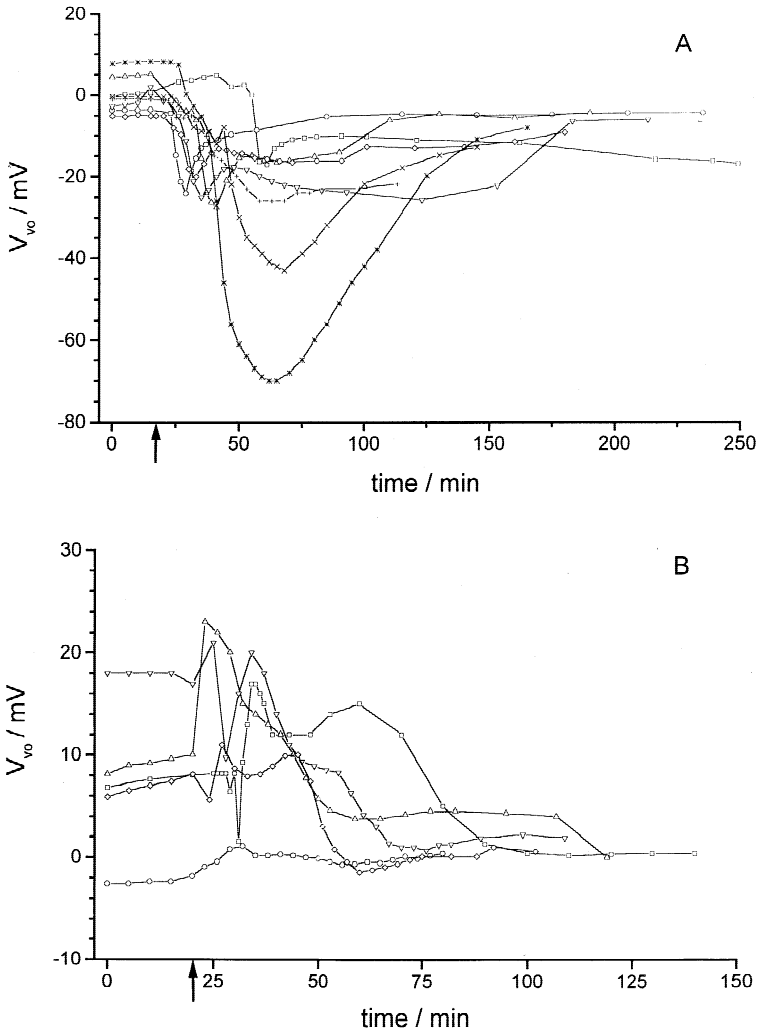

Fig. 6. Changes of the vacuolar resting membrane potential V_{vo} of *V. ventricosa* after vacuolar (*A*) and external (*B*) addition of 50 μM nystatin dissolved in ACS (*see* arrows). Measurements are represented for five to eight different cells (as indicated by the different symbols). For further details, *see* text.

channels play a major role in ion transport across the two membranes. Charge-pulse experiments were performed on cultured cells of *V. ventricosa* at different temperatures in the following way. The temperature was increased first from 20°C to 33°C (293 K to 306 K) and then lowered to 6°C (279 K). Hysteresis phenomena were only observed (*data not shown*) when the temperature was increased again from 6°C to 20°C (279 K to 293 K; similarly to *V. utricularis;* Büchner, Rosenheck & Zimmermann, 1985). An Arrhenius plot of the area-specific conductances of the plasmalemma and the tonoplast is shown in Fig. 8. From the slopes of the semilogarithmic plots the activation energy for the plasmalemma and tonoplast conductances were calculated to be 6.9 kcal mol^{-1} and 11.5 kcal mol^{-1}, respectively. These values are expected if ion diffusion occurs through open channels in cell membranes (Hille, 1984).

It should be noted that the vacuolar membrane potential, V_{vo}, varied only slightly with temperature (about +20 mV at 35°C (308 K) and about +5 mV at 5°C (278 K)).

Evidence for K^+- and Cl^--Channels/Carriers in the Plasmalemma

Addition of K^+-channel-blockers (TEA and Ba^{2+}) and of Cl^--channel/carrier-blockers (DIDS) to the vacuolar perfusion solution did not reveal any effect on the charge-pulse relaxation spectrum (*data not shown*). In contrast, these inhibitors changed the spectra significantly when added to the external medium.

Upon addition of increasing concentrations of TEA to the ASW the time constant of the fast relaxation (arising from the plasmalemma) decreased accordingly, whereas the slow one (arising from the tonoplast) remained nearly unaltered. Figure 9 shows plots of the average area-specific conductance G_p (normalized to the corresponding G_p^*-value in ASW) in dependence on $[TEA]_o$ for cultivated and freshly collected cells. It is evident that the plasmalemma conductance decreased markedly with increasing concentrations of $[TEA]_o$. This is expected if K^+-channels were present in the plasmalemma. The inhibitory effect of TEA on the K^+-

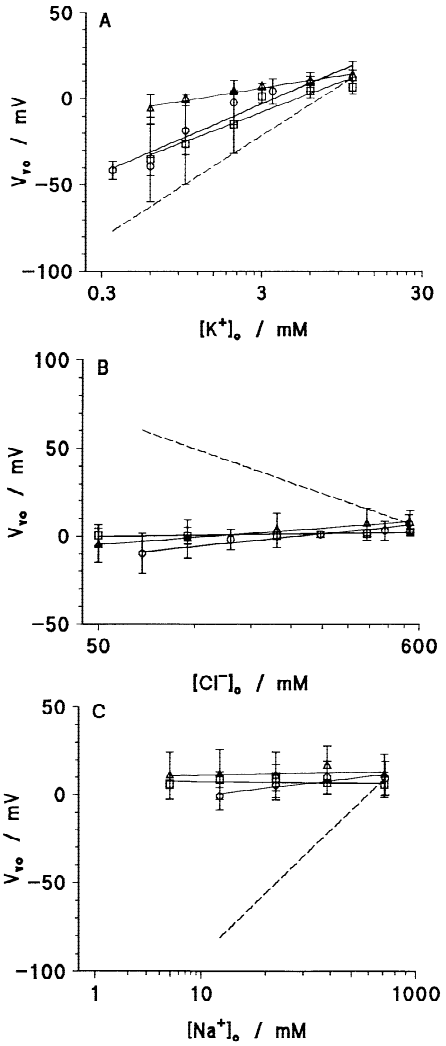

Fig. 7. Logarithmic plot of the concentration-dependence of the resting vacuolar potential, V_{vo}, of *V. ventricosa* on $[K^+]_o$ (*A*), $[Cl^-]_o$ (*B*) and $[Na^+]_o$ (*C*) measured simultaneously during the course of the charge-pulse experiments evaluated in Fig. 6. The straight lines represent the Nernst equilibrium potentials for the corresponding ions.

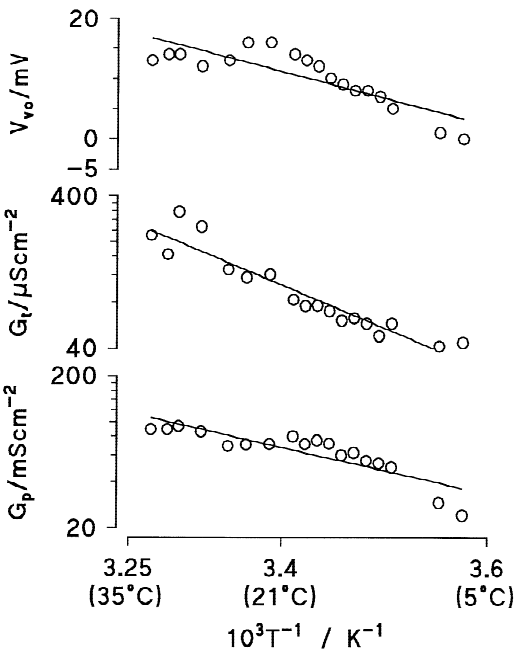

Fig. 8. Arrhenius plot of the area-specific plasmalemma (*A*) and tonoplast (*B*) conductance and the resting vacuolar potential of a cultivated cell of *V. ventricosa* (A_{cell} = 940 mm^2, V_{cell} = 2600 mm^3, P = 0.12 MPa, and V_{vo} = +4.8 mV) bathed in ASW. Data were deduced from charge-pulse experiments as shown in Figs. 1 and 5 and analyzed by using Eqs. 1–3. From the slope of the regression lines the activation energy is calculated to be 6.9 kcal mol^{-1} for the plasmalemma conductance and 11.5 kcal mol^{-1} for the tonoplast conductance.

channels was completely reversible within 30 min after removal of TEA. However, above a concentration of 100 mM $[TEA]_o$ was lethal to the cells.

Nonlinear regression analysis (by using Fig P, Biosoft, Cambridge, UK) of the data given in Fig. 9 showed that the curves could be fitted satisfactorily by assuming a Langmuir or Michaelis-Menten kinetics, respectively:

$$G_p/G_p^* = I_{max}c/(IC_{50} + c) \quad (4)$$

where I_{max} is the maximal inhibition at saturation, c the concentration of the inhibitor and IC_{50} the concentration of the blocker at which a 50%-inhibition is observed.

Since the concentration-dependent, inhibitory effect of TEA on freshly collected and cultivated cells was very similar (*see* Fig. 9), they were pooled and analyzed by using Eq. 4. This yielded an average IC_{50}-value of 11.5 ± 2.5 mM and an average I_{max}-value of 45 ± 8.2% (n = 9). As indicated by the standard deviations, both values were subject to some variations (IC_{50}: 6.9 to 13.9 mM; and I_{max}: 35 to about 55%).

External $[Ba^{2+}]_o$ yielded similar results as $[TEA]_o$ although much lower concentrations of Ba^{2+} were required to achieve a corresponding decrease in the area-specific plasmalemma conductance (*see also* Walter, Büchner & Zimmermann, 1988). The inhibitory effect of $[Ba^{2+}]_o$ on the plasmalemma conductance was also completely reversible — at least 1 hr after removal of Ba^{2+} from the ASW. Concentrations > 5 mM were lethal to the cells.

Typical plots of the average normalized area-specific plasmalemma conductance G_p/G_p^* for cultivated and freshly collected cells are shown in Fig. 10. In contrast to TEA, cultivated and freshly collected cells exhibited some differences in the response of the plasmalemma conductance upon addition of increasing $[Ba^{2+}]_o$. However, as indicated in Fig. 10 the effects were size-independent for freshly collected cells. Analysis of the

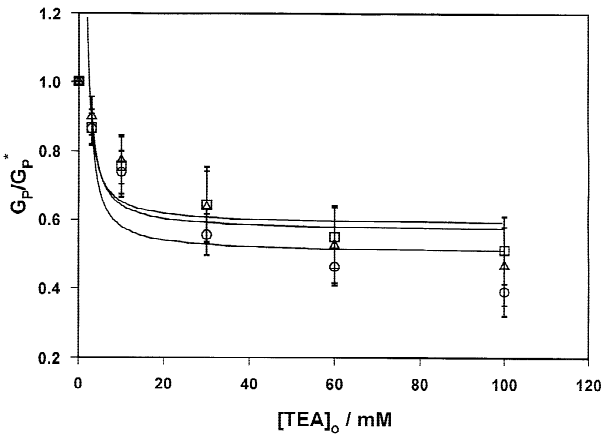

Fig. 9. Plots of the area-specific plasmalemma conductance, G_p, (normalized to the value G_p^* measured in ASW) as a function of the TEA-concentration in the external medium. Measurements were performed with cultivated (open circles) as well as with freshly collected large (open squares) and small (open triangles) cells of *V. ventricosa.* TEA was added 1 hr after insertion of the microelectrodes into the vacuole. After performance of 5–7 charge-pulse relaxation experiments within a time interval of 2–5 min the ASW was replaced successively by ASW containing increasing concentrations of TEA. The corresponding biphasic relaxations were fitted to Eq. 1, and the plasmalemma conductance, G_p, was calculated by using Eq. 2. The data points represent the average (±SD) of three independent measurements at each TEA-concentration and were fitted to the Langmuir kinetics (Eq. 4; correlation coefficient $r = 0.3$).

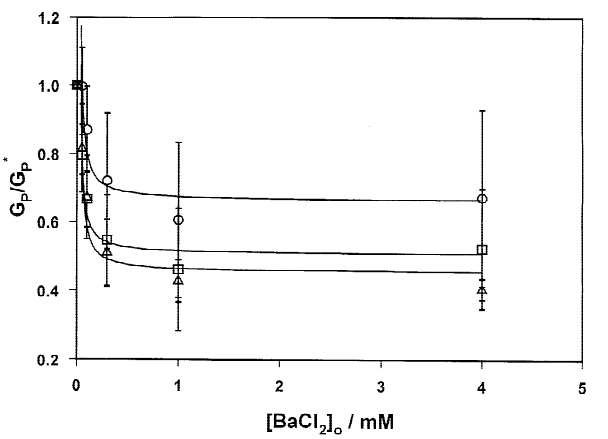

Fig. 10. Plots of the area-specific plasmalemma conductance, G_p (normalized to the value G_p^* measured in ASW) as a function of the Ba^{2+}-concentration in the external medium. Measurements were performed with cultivated (circles) as well as with freshly collected large (squares) and small (triangles) cells of *V. ventricosa.* Experimental conditions and analysis were performed as described in the legend to Fig. 9. (correlation coefficient $r = 0.3$).

data in Fig. 10 in terms of Eq. 4 yielded the following values for the IC_{50} and I_{max}-parameters: 0.17 ± 0.11 mM; 33 ± 21.6% ($n = 3$) for cultivated cells and 0.2 ± 0.06 mM; 51 + 11.4% ($n = 7$) for freshly collected algae.

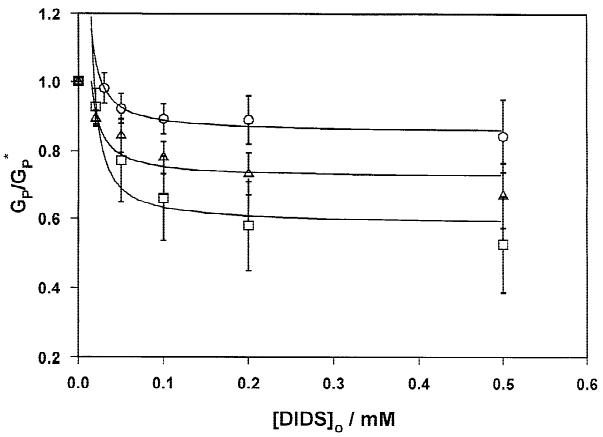

Fig. 11. Plots of the area-specific plasmalemma conductance, G_p, (normalized to the value G_p^* measured in ASW) as a function of the DIDS-concentration in the external medium. Measurements were performed with cultivated (circles) as well as with freshly collected large (squares) and small (triangles) cells of *V. ventricosa.* Experimental conditions and analysis were performed as described in the legend to Fig. 9. (correlation coefficient $r = 0.5$).

It is evident that the parameters for Ba^{2+}-inhibition were also subjected to large variations.

The variable response of the plasmalemma conductance to $[TEA]_o$ and $[Ba^{2+}]_o$ was obviously due to variations in the physiological state of the cells. This could be demonstrated in experiments in which the effects of TEA and Ba^{2+} on the fast relaxation of the charge-pulse relaxation spectrum were studied successively on the same (cultivated) cell. Under these conditions it was found that the I_{max}-values for $[TEA]_o$ and $[Ba^{2+}]_o$ agreed quite well (*data not shown; n* = 3).

Similarly to $[TEA]_o$ and $[Ba^{2+}]_o$, $[DIDS]_o$ did only affect the fast relaxation, but not the slow one. The effect of DIDS on the fast relaxation was, however, only partially reversible as shown by experiments performed 1 hr after removal of DIDS from the ASW (*data not shown*). Concentrations > 0.5 mM were lethal to the cells.

The concentration-dependent effect of the Cl^--channel/carrier inhibitor DIDS on the normalized area-specific plasmalemma conductance G_p/G_p^* of cultivated and freshly collected cells of *V. ventricosa* is given in Fig. 11. As indicated in the figure, the normalized G_p/G_p^*-value decreased with increasing $[DIDS]_o$. The nonlinear regression analysis showed that the data given in the figure could also be fitted satisfactorily by Eq. 4. Comparable with $[Ba^{2+}]_o$, the average I_{max}-values of the cultivated and the freshly collected algae showed significant differences (13 ± 6.7% for the cultivated cells (n = 3) and 35 ± 11.6% for the freshly collected ones (n = 6)). In contrast, the average IC_{50}-values for both types of cells were quite similar (0.02 ± 0.01 mM for the cultivated cells ($n = 3$) and 0.06 ± 0.03 mM for the freshly

Table 2. Calculation of the experimentally yielded inhibition, *I*, of the blocking agents TEA and DIDS upon the area-specific plasmalemma conductance determined on 15 different cells of *V. ventricosa*

	I [%]		
Cell	TEA	DIDS	Σ
1	26	60	86
2	52	24	76
3	47	19	66
4	30	73	103
5	51	37	88
6	44	52	96
7	62	27	89
8	62	26	88
9	68	33	101
10	80	27	107
11	71	42	113
12	77	29	106
13	54	22	76
14	72	35	107
15	55	44	99
Mean ± SD	57 ± 11	37 ± 15	93 ± 15

Calculation of the *I*-values, i.e., the percentage of the inhibition of the plasmalemma conductance of *V. ventricosa* (cultivated and freshly collected cells) in the presence of 100 mM $[TEA]_o$ and 0.5 mM $[DIDS]_o$, respectively. Experimental conditions: After control experiments (yielding $G_{p,max}$) the ASW was replaced by ASW containing 100 mM TEA. Charge-pulse experiments in the presence of this medium yielded $G_{p,min}$(TEA). After 30 min washing with ASW, charge-pulse experiments were performed in ASW containing 0.5 mM DIDS in order to determine $G_{p,min}$ (DIDS). $I_{(TEA)}$ and $I_{(DIDS)}$ were calculated from these values by $I = (G_{p,max} - G_{p,min})/G_{p,max}$. The total inhibition, Σ_I, of the electrical plasmalemma conductance is given by the algebraic sum of $I_{(TEA)} + I_{(DIDS)}$. For further details, *see* text.

collected cells ($n = 6$)). The large standard deviations show that the effect of $[DIDS]_o$ on the plasmalemma conductance was also subjected to large variations.

A possible explanation of the large cell-to-cell variations of the I_{max}-values for the TEA/Ba^{2+}- and the DIDS-inhibition is that the contribution of the K^+- and Cl^--channels to the total plasmalemma conductance varied from cell to cell, but that the algebraic sum of the contribution of the K^+- and Cl^--channels to the total conductance is the same for all cells. The verification of this assumption provides that the values yielded for the inhibition, *I*, in the presence of 100 mM $[TEA]_o$ and 0.5 mM $[DIDS]_o$ are determined for the same cell in successive experimental runs. The data for $I_{(TEA)}$ and $I_{(DIDS)}$ for 15 cultivated and freshly collected large and small cells (calculated from TEA/DIDS experiments successively performed on a given cell) are represented in Table 2. As expected, $I_{(TEA)}$ and $I_{(DIDS)}$ were quite variable for the 15 cells (57 ± 11% and 37 ± 15%, respectively), but the algebraic sum of them ($\Sigma_I = I_{(TEA)} + I_{(DIDS)}$) was nearly the same (93 ± 15%). This finding supports the above conclusion that the plasmalemma conductance is determined nearly exclusively by K^+- and Cl^--channels (or carriers), whereby the K^+-channel seemed to be in general the major conducting element.

Discussion

The charge-pulse relaxation spectrum measured on cells of *V. ventricosa* resembled those reported previously for *V. utricularis* (Zimmermann et al., 1982; Benz & Zimmermann, 1983; Wang et al. 1991) and *H. parvula* (Benz et al., 1988). It consisted of a fast ($\tau_1 = 168 \pm 99$ μsec; $a_1 = 0.89 \pm 0.02$) and a slow exponential ($\tau_2 = 29 \pm 28$ msec and $a_2 = 0.11 \pm 0.01$). Frequently, a third intermediate relaxation ($\tau_3 = 1$ msec and $a_3 = 0.05$) was observed.

The experiments in which the effect of nystatin (and amphotericin B) on the charge-pulse relaxation spectrum were investigated confirmed the "two membrane model" which predicts that the antibiotics should affect exclusively either the slow or the fast voltage decays depending on the side of addition (vacuolarly or externally, for a detailed discussion, *see* Wang et al., 1997). Thus, it is justified to assign the fast exponential relaxation of *V. ventricosa* to the RC-properties of the plasmalemma, and the slow voltage decay to the RC-properties of the tonoplast. The finding that the third intermediate relaxation was never observed upon vacuolar, but upon external addition of antibiotics suggest that this relaxation also arose from the tonoplast. It is conceivable that this relaxation arose from (hitherto unidentified) conducting elements (such as mobile charges of an electrogenic transport system) or from cable properties of the tonoplast which seems to be folded (*see below*).

Consistent with the interpretation of the charge-pulse relaxation spectrum in terms of the "two membrane model" was the finding that external changes of $[K^+]_o$ and $[Cl^-]_o$ or external addition of K^+- and Cl^--channel/carrier inhibitors, respectively, did only affect the fast relaxation, but not the slow (and intermediate) one. In contrast to *V. utricularis* and *H. parvula* (Graves & Gutknecht, 1997*a,b;* Wang et al., 1991; Spieß et al., 1993) K^+ seemed to be the dominating element of the plasmalemma conductance of *V. ventricosa.* This was evident from the pronounced decrease of the plasmalemma conductance (up to 80%) when $[K^+]_o$ was decreased or when TEA or Ba^{2+} were added externally.

$[Cl^-]_o$ contributed to the plasmalemma conductance of *V. ventricosa,* but significantly less than found for cells of *V. utricularis* and *H. parvula.* As seen elsewhere (Wang et al., 1991; *unpublished data*) replacement of external Cl^- by MES^- increased the time constant of the plasmalemma exponential of these species by a factor of > 10 (in contrast to a factor of 2 as found for *V. ventricosa*). Furthermore, external addition of DIDS resulted

only in a decrease of the plasmalemma conductance of up to 16% for cultivated and 47% for freshly collected cells. The percentage of both ions to the plasmalemma conductance was apparently subject to some (partly large) physiological variations; however, a clear-cut relationship with volume, turgor pressure and/or age of the cells (as found for other membrane and wall parameters of giant algae; Zimmermann & Steudle, 1974; 1978; Steudle, Zimmermann & Lelkes, 1977; Zimmermann, 1978) could not be revealed.

In this context, it is also interesting to note that external pH had no significant effect on the charge-pulse relaxation spectrum of *V. ventricosa* in contrast to *V. utricularis* and *H. parvula.* In the case of the latter two species, lowering of the external pH from 8.2 to 4 resulted in a dramatic increase of τ_1 and τ_2 (by a factor of 5–10; Zimmermann et al., 1982; Benz et al., 1988; Wang, Benz & Zimmermann, 1992).

The apparent differences in the electrical conducting elements of the plasmalemma of *V. ventricosa* and *V. utricularis* are interesting for two reasons. First, they justified retrospectively the recent re-naming of this species (formerly *Valonia ventricosa;* Olsen & West, 1988). Second, they also strengthen considerably the view that the two voltage decays of the charge-pulse relaxation spectra arise rather from the RC-properties of the tonoplast and the plasmalemma than from specific ion transport systems as discussed previously (Zimmermann et al., 1982; Wang et al., 1991).

By using Eqs. 2 and 3 the values of the area-specific capacitances, resistances and conductances of the individual membranes of a given cell could be calculated from the charge-pulse relaxation spectra (*see* Table 1 and Figs. 5, 8–11). By assuming that the surface area is equal to the geometric one, the calculations yield the following average values for the corresponding average area-specific parameters: (plasmalemma) $C_p = 0.82 * 10^{-2}$ F m^{-2}, $R_p = 1.69 * 10^{-2}$ Ωm^2, $G_p = 5.9 * 10^4$ mS m^{-2} (tonoplast) $C_t = 7.1 * 10^{-2}$ F m^{-2}, $R_t = 14.9 * 10^{-2}$ Ωm^2 and $G_t = 0.67 * 10^4$ mS m^{-2}. It is obvious that the C_p-, but not the C_t-value agrees with the area-specific capacitance of about 10^{-2} F m^{-2} reported for cell membranes with smooth surface (Hille, 1984; Fuhr, Zimmermann & Shirley, 1996). In contrast, the value of C_t is apparently by a factor of about 9 too high.

Following the line of previous arguments for the interpretation of the high area-specific value of the tonoplast capacitance of *V. utricularis* (Wang et al., 1997) we must assume either the presence of mobile charges of an (*unknown*) electrogenic transport system and/or an area enlargement. If we assume that the apparent increase in the area-specific capacitance of the tonoplast is exclusively due to an area enlargement (i.e., that the real area-specific capacitance value, C_t^*, corresponds to that of the plasmalemma, $C_t^* = C_p$), the real surface area of the tonoplast must be about 9 times larger than the geometric surface (*see* calculations in Table 1). Taking this value into account, the real average area-specific resistance, R_t^*, and conductance, G_t^*, of the tonoplast are estimated to be $R_t^* = 110 * 10^{-2}$ Ωm^2 and $G_t^* = 0.091 * 10^4$ mS m^{-2}, respectively.

Preliminary electronmicroscopy studies of cells of *V. ventricosa* and *V. utricularis* suggested that the tonoplast of these two species exhibits a spongelike appearance. The cell shown in Fig. 12 was fixed under maintenance of turgor pressure; thus artificial dissolution of the plasmalemma from the cell wall and/or folding of the tonoplast is avoided. It is obvious from the figure that the tonoplast envelopes the chloroplasts and the nuclei resulting in a dramatic area enlargement. At the present stage of information we cannot decide whether this area enlargement seen in the electronmicroscopy is large enough to account for the area-specific capacitance value of the tonoplast (C_t) or if additionally the presence of mobile charges of electrogenic transport systems must be taken into consideration.

However, independent of this open question, the data show clearly that the area-specific resistance of the tonoplast of *V. ventricosa* (like that of *V. utricularis,* Wang et al., 1997) is high. The values calculated here for the real area-specific resistance (R_t^*) are close to those reported for isolated vacuoles of higher plants (approximately 1 Ωm^2 (Weiser & Bentrup, 1994)). Taking the results obtained on *V. utricularis* and *V. ventricosa* together it seems to be clear that the tonoplast of marine giant algae is apparently not highly conductant as is generally assumed in data analysis of former vacuolar microelectrode measurements (Beilby, 1989).

Another interesting result obtained here is the finding that the cytoplasm of *V. ventricosa* is negatively charged relative to the external medium and the vacuole. The potential changes recorded after addition of nystatin from the vacuolar or external side were quite variable, presumably due to ion exchange between the different compartments through the permeabilized tonoplast and plasmalemma, respectively. However, the trend in the potential changes observed immediately after the addition of nystatin support very strongly the data of Davis (1981) and Gutknecht (1966). Davis measured directly V_{vc} and V_{co} in mature cells by placing in a very elegant way a microelectrode into the cytoplasm of *V. ventricosa.* Gutknecht measured V_{co} in aplanospores that consist almost entirely of cytoplasm and subtracted this value from V_{vo} measured in mature cells. Both authors found that the cytoplasmic region represents a highly negative potential region. This finding contrasted results obtained by Lainson and Field (1976) who claimed that the cytoplasm is not charged.

Evidence was also presented in this communication that the potential across the plasmalemma must be a K^+

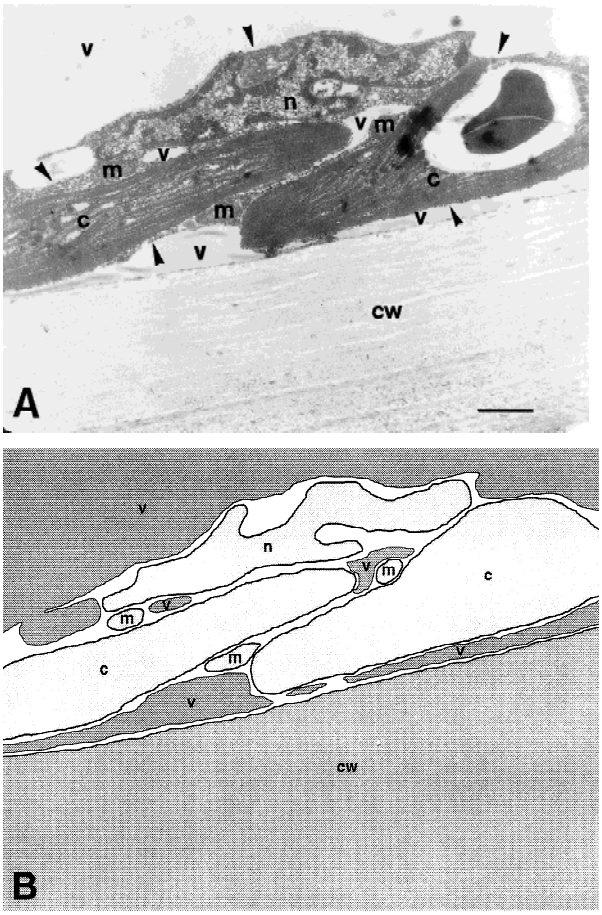

Fig. 12. (*A*) Transmission electron microscopy of small size cells of *V. utricalaris.* The cells were fixed in ASW containing 2% glutardialdehyde, 35 mM cacodylic acid and 1% tannic acid for 2 hr at room temperature. The osmolality of the fixation solution was adjusted to the osmolality of the culture medium (1024 mosmol kg^{-1}) by appropriate dilution with distilled water. During fixation the cells remained turgescent as revealed by charge-pulse experiments, turgor pressure measurements and light microscopy. Then the cells were washed with ASW containing 35 mM cacodylic acid solution (osmolality as above). Afterwards the cells were post-fixed at 4°C (277 K) for 1 hr by using ASW/cacodylic acid solution to which 1% OsO_4 solution had been added. The fixed cells were gradually dehydrated with ethanol by using a gradient pump (16 ml/hr for 24 hr, Model 380, Instrumentation Specialities, CO). The samples were then embedded in Spurr's resin. Sections of 60–90 nm thickness were made with a microtom (MT-7000, Ultra, RMC, Tucson, AZ) and then viewed in a transmission electron microscope (EM 900, Zeiss, Oberkochen, Germany). For further details, *see* Mimietz, Ryser & Zimmermann (*manuscript in preparation*). The nucleus (*n*), the mitochondria (*m*) and the chloroplasts (*c*) are surrounded by a layer of cytoplasma (white area). The vacuole (*v*) is bounded by the tonoplast (marked by arrows). The cell wall (*cw*) is located at the bottom. Scale bar: 1.1 μm. (*B*) Schematic diagram of Fig. 12*A.*

diffusion potential, because $[K^+]_o$ changed the potential as expected in the light of the Nernst equation.

A question which could not be answered here is the finding that vacuolar TEA, Ba^{2+} and DIDS had no effect on the conductance of the tonoplast. This can have two reasons: (i) these inhibitors react only from the cytoplasmic side and/or (ii) the K^+- and Cl^--channels are insensitive to these inhibitors.

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