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Separate Determination of the Electrical Properties of the Tonoplast and the Plasmalemma of the Giant-Celled Alga *Valonia utricularis:* Vacuolar Perfusion of Turgescent Cells with Nystatin and Other Agents

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Abstract. In the giant-celled marine algae *Valonia utricularis* the turgor-sensing mechanism of the plasmalemma and the role of the tonoplast in turgor regulation is unknown because of the lack of solid data about the individual electrical properties of the plasmalemma and the vacuolar membrane. For this reason, a vacuolar perfusion technique was developed that allowed controlled manipulation of the vacuolar sap under turgescent conditions (up to about 0.3 MPa). Charge-pulse relaxation studies on vacuolarly perfused cells at different turgor pressure values showed that the area-specific resistance of the total membrane barrier (tonoplast and plasmalemma) exhibited a similar dependence on turgor pressure as reported in the literature for nonperfused cells: the resistance assumed a minimum value at the physiological turgor pressure of about 0.1 MPa. The agreement of the data suggested that the perfusion process did not alter the transport properties of the membrane barrier.

Addition of 16 μM of the H^+-carrier FCCP (carbonylcyanide *p*-trifluoromethoxyphenyhydrazone) to the perfusion solution resulted in a drop of the total membrane potential from +4 mV to −22 mV and in an increase of the area-specific membrane resistance from 6.8 $\times 10^{-2}$ to 40.6 $\times 10^{-2}$ Ωm^2. The time constants of the two exponentials of the charge pulse relaxation spectrum increased significantly. These results are inconsistent with the assumption of a high-conductance state of the tonoplast (R. Lainson and C.P. Field, *J. Membrane Biol.* **29:**81–94, 1976).

Depending on the site of addition, the pore-forming antibiotics nystatin and amphotericin B affected either the time constant of the fast or of the slow relaxation (provided that the composition of the perfusion solution and the artificial sea water were replaced by a cytoplasma-analogous medium). When 50 μM of the antibiotics were added externally, the fast relaxation process disappeared. Contrastingly, the slow relaxation process disappeared upon vacuolar addition. The antibiotics cannot penetrate biomembranes rapidly, and therefore, the findings suggested that the fast and slow relaxations originated exclusively from the electrical properties of the plasmalemma and the tonoplast respectively. This interpretation implies that the area-specific resistance of the tonoplast is significantly larger than that of the plasmalemma (consistent with the FCCP data) and that the area-specific capacitance of the tonoplast is unusually high (6.21×10^{-2} Fm^{-2} compared to 0.77×10^{-2} Fm^{-2} of the plasmalemma). Thus, we have to assume that the vacuolar membrane of *V. utricularis* is highly folded (by a factor of about 9 in relation to the geometric area) and/or contains a fairly high concentration of mobile charges of an unknown electrogenic ion carrier system.

Key words: Electrical measurements — Marine alga — Membrane barrier — Turgor pressure — Vacuolar perfusion — Nystatin

Introduction

One of the most remarkable characteristics of certain marine algae is their ability to maintain turgor pressure over a large range of salinity (Gutknecht, 1968; Zimmermann & Steudle, 1974; Hastings & Gutknecht, 1974, 1976; Zimmermann, 1978, 1989; Kirst, 1990). This phenomenon is termed turgorregulation. In the giant celled marine alga *Valonia utricularis* constant turgor pressure

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Abbreviations: ASW, artificial sea water; AVS, artificial vacuolar sap; ACS, artificial cytoplasma sap; FCCP, carbonylcyanide *p*-trifluoromethoxyphenyhydrazone

is achieved by regulation of the K^+- and Cl^--transport systems in response to external osmotic stress. Recent electrophysiological studies on cells of *V. utricularis* (with a microelectrode inserted into the large central vacuole) under various external medium conditions have greatly improved our knowledge about several steps presumably involved in sensing turgor pressure and in transducing of hydrostatic pressure signals into membrane ion transport (Zimmermann, Büchner & Benz, 1982; Wang et al., 1991). However, interpretation of electrophysiological data is not always straightforward because of the presence of two membranes (the tonoplast and the plasmalemma) arranged in series (*see* e.g., Lainson & Field, 1976; Davis, 1981; Benz & Zimmermann, 1983). Despite many efforts (e.g., Davis, 1981) the electrical properties of the plasmalemma (or the tonoplast) of mature cells of *V. utricularis* were not amenable to separate measurements using microelectrodes in the cytoplasm, because the wall was too tough and the cytoplasmic layer too thin.

Thus, it is still an open question whether the two exponentials of the charge pulse relaxation spectrum measured on *V. utricularis* after the injection of very short charge pulses into the vacuole arise from the RC-properties of the two membranes or from mobile charges in the plasmalemma which are linked with a Cl^- carrier system (Zimmermann et al., 1982; Benz & Zimmermann, 1983; Wang et al., 1991). Interpretation of the charge pulse relaxation spectrum in terms of two membranes requires the hypothesis that the tonoplast surface is 5–10-fold folded, whereas the analysis of the spectra in terms of the ''mobile charge concept'' is based on the hypothesis that the tonoplast is highly electrically conductive, but salt impermeable. Both hypothesis contrast with findings on isolated vacuoles of higher plants (Hedrich & Schroeder, 1989; Martinoia, 1992; Weiser & Bentrup, 1994). However, a high conductance state of the tonoplast (in relation to the plasmalemma) is assumed commonly in the analysis of electrophysiological data performed on giant algal cells (Spanswick & Costerton, 1967; Spanswick, 1972; Lainson & Field, 1976; Findlay & Hope, 1976; Coster & Smith, 1977; Smith, 1983; Beilby, 1989; Wang et al., 1991).

Progress can be expected in electrophysiological studies on cells of *V. utricularis* if the permeability of the tonoplast could be manipulated from the vacuolar side. Such manipulations (in combination with independent changes of the composition of the external artificial sea water) should facilitate the separation of the effects of the tonoplast and the plasmalemma on electrophysiological spectra. In principle, the vacuolar environment can be modified by intracellular perfusion with media containing permeabilizing agents, inhibitors, channel blockers etc. Intracellular perfusion was used in electrophysiological measurements on the giant squid axon (Baker, Hodgkin & Shaw, 1961), on the very large cells of *Halicystis parvula* and *Ventricaria ventricosa* (Blount & Levedahl, 1960; Gutknecht, 1967, 1968; Hastings & Gutknecht, 1974) as well as on the internodal cells of *Characeae* (Tazawa, 1964; Kishimoto, 1965; Kikuyama & Tazawa, 1976; Shimmen, Kikuyama & Tazawa, 1976).

The perfusion experiments on algae cells reported in the literature are usually performed under turgorless conditions or in the presence of very small hydrostatic pressure gradients. However, elucidation of the turgor pressure-dependent electrical properties of the membrane barrier of *V. utricularis* requires a more sophisticated perfusion approach because the manipulations of the vacuolar sap must be performed under highly turgescent conditions. The work of our group and others (Zimmermann & Steudle, 1974; Zimmermann, Steudle & Lelkes, 1976; Zimmermann, 1978; Zimmerman et al., 1982; Nawata, Kikuyama & Shihira-Ishikawa, 1993; Reid, Tester & Smith, 1993; McCulloch & Beilby, 1995) has clearly shown that both the turgor pressure and the elastic properties of the cell wall influence ion in- and effluxes as well as the electrophysiological properties of *V. utricularis* and other giant algal cells.

In this communication we report the successful development of a vacuolar perfusion device which allows charge pulse relaxation studies on mature cells of *V. utricularis* under turgescent conditions. The technique is capable of monitoring the charge pulse relaxation spectra and, thus, the electrical parameters of the membrane barrier with very high time resolution (<10 μsec). Turgor pressure can be adjusted without changes of the internal ionic concentration up to about 0.3 MPa. Thus, problems arising from changes in the ion gradients required for adjustment of turgor pressure in nonperfused cells (Zimmermann & Steudle, 1974) are avoided.

We used the perfusion device to study the effects of the H^+-carrier FCCP as well as of the polyene antibiotics nystatin and amphotericin B on the inner side of the tonoplast of *V. utricularis.* These agents were selected to answer the question about the conductance state of the tonoplast and its contribution to the charge pulse relaxation spectrum.

Vacuolarly added FCCP could have a dramatic effect on the membrane potential and resistance of the tonoplast as well as on the charge pulse relaxation spectrum of the cell. This would happen if the vacuolar membrane is not highly electrically conductive (Benz & McLaughlin, 1983), but more comparable to vacuoles of higher plants (Hedrich & Schroeder, 1989; Martinoia, 1992; Weiser & Bentrup, 1994).

Nystatin and amphotericin B permeabilize sterol-containing lipid bilayer membranes and cell membranes by forming ion selective channels. Incorporation of these antibiotics into the membrane leads, therefore, to

an increase in the electrical conductance of several orders of magnitude (Marty & Finkelstein, 1975; Kleinberg & Finkelstein, 1984; Bolard, 1986; Horn & Marty, 1988; Shvinka & Caffier, 1994; Marty & Neher, 1995). The antibiotics do not penetrate (at least rapidly) the membrane and thus allow manipulation of the conductance states of the two membranes independently of each other, provided that the membranes contain sterol. In the course of the experiments it turned out that the above polyene antibiotics were effective in *V. utricularis.* They are, therefore, promising tools for studying the electrical properties of the tonoplast and plasmalemma separately and elucidating their contributions to the charge pulse relaxation spectrum.

The results of these and the FCCP studies give strong and consistent evidence that the two exponentials of the charge pulse relaxation spectrum originate from the two membranes. This has two consequences: (i) The area-specific tonoplast resistance is high and, presumably, higher than the area-specific resistance of the plasmalemma. (ii) The vacuolar membrane must be multiply folded or contain an equivalent amount of mobile charges of an unknown ion carrier system.

Materials and Methods

Culture Conditions

Cells of *Valonia utricularis* (*Siphonocladales, Chlorophyceae*) were collected from the rocky coast at Ischia (Gulf of Naples, Italy) and cultivated in 40-liter tanks of natural sea water (NSW, 1124 mosmol/kg, pH 8.1). The sea water was aerated continuously and illuminated for 12 hr per day (2 × 36 W Fluora lamps, Osram, München, Germany). The temperature was maintained at 19 ± 1°C.

Experimental Conditions

For the experiments, mature cells were selected according to regularity of shape and size (about 1 mm in diameter). After removal of encrustations, the cells were bathed in artificial sea water (ASW, 1124 mosmol/kg) containing (in mM): 545 NaCl, 12 KCl, 11 $CaCl_2$ and 10 $MgCl_2$. The pH of ASW was adjusted to 8.1 by the addition of 10 mM N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)/NaOH (HEPES/NaOH).

For vacuolar perfusion, artificial vacuolar sap (AVS, pH 6.3, 1340 mosmol/kg) was used containing (in mM): 220 NaCl, 440 KCl, 3 $CaCl_2$, 3 $MgCl_2$ and 3 phosphate buffer. The concentrations of the major ions of AVS corresponded closely (but not completely) to those of the normal vacuolar sap of *V. utricularis* (in mM): (222 Na^+, 450 K^+, 610 Cl^-, 6 Ca^{2+}, 6 Mg^{2+}, pH 6.3).

For the experiments in which the effect of FCCP (carbonyl cyanide *p*-trifluoromethoxyphenyhydrazone) on the charge pulse relaxation spectrum was studied, stock solutions were used which contained 10 mM FCCP dissolved in acetone. Control experiments showed that 0.2% acetone had no significant effects on the electrical properties of the membranes.

Separate permeabilization of the tonoplast and the plasmalemma was achieved by addition of nystatin or amphotericin B. The antibiotics were predissolved in dimethyl sulfoxide (DMSO). A portion of this 5 mM stock solution was added to artificial cytoplasm sap (ACS; pH 7.3, 1116 mosmol) containing (in mM): 40 NaCl, 80 KCl, 408 K_2SO_4, 5 $CaCl_2$, 10 $MgCl_2$, 10 EGTA and 10 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 electrical properties of the membranes of *V. utricularis.*

All salts were obtained from Merck (Darmstadt, Germany). The buffers, nystatin, amphotericin B, FCCP, acetone and DMSO were purchased from Sigma (Deisenhofen, Germany).

A fiber-optic light source (model KL 150B, Schott, Mainz, Germany) containing 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 if not otherwise stated.

Microelectrode Measurements under Vacuolar Perfusion Conditions

The perfusion assembly and the integrated charge pulse relaxation setup is shown in Fig. 1. A giant cell (C) was fixed in a plexiglass™ chamber (MC) containing slowly flowing seawater (S, inflow; O, outflow). Two sharp borosilicate glass pipettes (P1, outflow; P2, inflow) of about 30 μm outer tip diameter were inserted through the wall and protoplast into the center of the vacuole using micromanipulators. Both pipettes were completely filled with perfusion solution prior to insertion. The shanks of the pipettes were sealed pressure-tight by rubber 'O'-rings to two small plexiglass™ chambers (PC1, PC2) filled with perfusion solution. These chambers contained transducers (PT1 and PT2) for recording the turgor pressure (*see* pressure probe principle, Zimmermann & Steudle, 1974). The inflow chamber PC2 was connected with teflon™ tubing (1.8 mm o.d. and 0.5 mm i.d.; Pharmacia, Uppsala, Sweden) through a valve (V2) to a plexiglass™ pressure chamber (PC3) equipped with a pressure transducer (PT3). The pressure transducer registered the pressure exerted by the syringe reservoir (SY) connected to the pressure chamber (PC3). The perfusion solution in the syringe was forced into the vacuole of the cell by a perfusion pump (PP, PERFUSOR™ SECURA FT, B. Braun Melsungen AG, Melsungen, Germany). Perfusion flow rates of <50 μl min^{-1} were used throughout the experiments. The outflow chamber (PC1) was connected with teflon™ tubings through a valve (V1) to the pressure chamber (PC) in which a pressure regulator (PR) was mounted to adjust the pressure in the cell and in the other part of the hydraulic system as required. In the absence of tip clogging (*see* below) the difference between the hydrostatic pressure values measured at the various locations by means of PT1, PT2, PT3 and PR was smaller than 0.005 MPa. Thus, the turgor pressure of the cell equalled the pressure in PC for practical purposes.

For injection of charge pulses, a 300 μm-thick platinum wire (PW, tip diameter 10 μm) was moved through the outflow pipette (P1) deeply into the vacuole. The reference current-electrode (RE1) in the bathing sea water consisted of a rectangular steel plate of 28 × 8 mm. For recording the resting and generated potential across the membrane, a pipette-microelectrode (PE) was used which was filled with 3 M KCl and contained a silver/silver chloride wire. The potential-measuring electrode was integrated into the inflow pipette. The external potential-recording reference electrode (RE2) consisted of Ag/AgCl and a 3 M KCl agar bridge.

For charge pulse experiments in perfused cells, the membranes were charged with a rectangular pulse of 1 μsec duration (generated by a fast, commercial pulse generator, PG, model 214B, Hewlett Packard,

Palo Alto, CA) through a diode (D, reverse resistance larger than 10^{10} Ω). The injected charge Q was calculated from the voltage drop (observed on a digital oscilloscope, DO2, model 2440, Tektronix, Beaverton, OR) across a 10 Ω resistor (R, connected in series within the charging circuit). The voltage relaxation across the membranes after a charge-pulse was recorded by a fast, differential amplifier (A) of high input impedance connected to the potential-measuring electrodes (PE and RE2) and stored on a digital oscilloscope (DO1, Nicolet 490, 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., 1994). The whole setup was shielded against electromagnetic waves by a Faraday cage.

Analysis of the Voltage Relaxation Data

The semilogarithmic plot of the voltage relaxation *vs.* time recorded on cells of *V. utricularis* after the administration of a charge-pulse could be fitted by the sum of two exponential decays as a first approximation (Benz & Zimmermann, 1983; Wang et al., 1991, 1995; Spieß et al., 1993, but see also below), using a multiple-exponential-fitting program:

$$V_m(t) = V_1\exp(-t/\tau_1) + V_2\exp(-t/\tau_2) = V_0(a_1\exp(-t/\tau_1) + a_2\exp(-t/\tau_2)) \quad (1)$$

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 and $V_{1,2}$ the initial absolute amplitudes of the rapidly and slowly decaying voltages, respectively (for details, see Zimmermann et al., 1982; Wang et al., 1991). The fit to the experimental data was very significant under all circumstances ($P < 0.001$, checked by using Student's *t*-test).

In the case of the ''mobile charge'' concept (Zimmermann et al., 1982; Benz & Zimmermann, 1983; Wang et al., 1991), the area-specific membrane capacitance (C_m) and the area-specific membrane resistance (R_m) of the total membrane barrier are given by:

$$C_m = Q/(AV_0) \quad (2)$$

and

$$R_m = 1/G_m = (\tau_1 a_1 + \tau_2 a_2)/C_m \quad (3)$$

where Q is the injected charge, A the geometric surface area of the alga and G_m the area-specific membrane conductance.

In the case of the ''two membrane'' model, the area-specific membrane capacitances (C_p) and (C_t) and the area-specific membrane resistances (R_p) and (R_t) of the plasmalemma and the tonoplast respectively, are given by the following equations (provided that the surface areas of the plasmalemma and the tonoplast are equal to the geometric surface area of the alga; Benz & Zimmermann, 1983):

$$C_p = Q/(A_pV_1) = Q/(AV_1);\ R_p = 1/G_p = \tau_1/C_p \quad (4)$$

and

$$C_t = Q/(A_tV_2) = Q/(AV_2);\ R_t = 1/G_t = \tau_2/C_t \quad (5)$$

where G_p and G_t are the area-specific membrane conductances of the plasmalemma and the tonoplast, respectively.

The area-specific capacitance (C_m) and resistance (R_m) of the total membrane barrier are given by[1]

$$C_m = C_pC_t/(C_p + C_t) = Q/(AV_0) \quad (6)$$

and

$$R_m = 1/G_m = R_p + R_t = (\tau_1 a_1 + \tau_2 a_2)/C_m \quad (7)$$

For the case of $C_p = C_t$ (*see below*), the area-specific membrane resistance (R_t^*) and the surface area of the tonoplast (A_t^*) are given by:

$$R_t^* = \tau_2/C_t = \tau_2/C_p;\ A_t^* = A_pV_1/V_2 = AV_1V_2 \quad (8)$$

Results

Perfusion Procedure

Screening experiments showed that the use of pipettes with tip diameters of larger than 100 μm irreversibly damaged the cells of *V. utricularis*, particularly if perfusion was performed above turgor pressures of 0.2 MPa. Therefore, tip diameters of 30 μm were used throughout the experiments. However, perfusion of the vacuole of *Valonia* with pipettes of this diameter was difficult to perform because of frequent clogging of the tip of the outflow pipette with gelatinous material present in the vacuoles of most of the cells. This may be one of the reasons why other authors (e.g., Gutknecht, 1967) have used pipettes with tip diameters of about 200 μm. With the present setup (Fig. 1), clogging could be easily eliminated due to the inserted platinum wire (PW). The wire was moved gently by appropriate turning of the micrometer screw to remove clogging material in the tip.

At the beginning of an experiment, the valves (V1,

[1] Note that C_m and R_m in Eqs. 6 and 7 have a different meaning than in Eqs. 2 and 3 despite the identical form of the equations. In Eqs. 2 and 3 it is assumed that the tonoplast is highly conductive. Thus, the parameters reflect predominantly the properties of the plasmalemma, whereas in Eqs. 6 and 7 both membranes contribute to C_m and R_m.

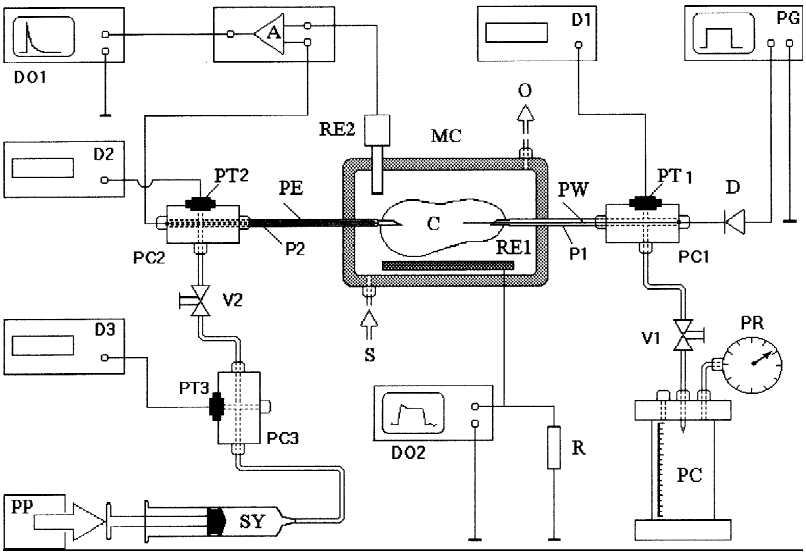

Fig. 1. Schematic diagram of the setup for perfusion of the vacuole of *V. utricularis. A:* differential amplifier; *C:* alga cell; *D:* diode, *D1, D2* and *D3:* pressure display; *DO1* and *DO2:* digital storage oscilloscope; *MC:* plexiglass™ chamber; *O:* outflow of the bath solution, *P1, P2:* outflow, inflow perfusion pipettes; *PC, PC1, PC2, PC3:* pressure chambers; *PE:* potential-measuring electrode; *PG:* pulse generator; *PP:* perfusion pump; *PR:* pressure regulator; *PT1, PT2, PT3:* pressure transducers; *PW:* platinum wire (internal current electrode); *R:* 10 Ω resistance; *RE1:* current reference electrode; *RE2:* potential-measuring reference electrode; *S:* inflow of the bath solution; *SY:* syringe; *V1, V2:* valves. For further explanation, *see* text.

V2) were shut. Then, the in- and outflow pipettes were inserted into the vacuole. Successful puncture of the vacuole (without significant loss of water) was indicated by a pressure of >0.1 MPa (recorded by the pressure transducers PT1 and PT2). This pressure value corresponded to the normal turgor pressure measured in cells of *V. utricularis* (Zimmermann & Steudle, 1974). Next, the pressure in the chamber PC was adjusted to the turgor pressure of the cell with the regulator PR. After adjustment, the valve V1 was opened. The flow rate was selected and the perfusion pump (PP) was started. The hydrostatic pressure in the chamber PC3 increased continuously as recorded by the transducer PT3. When this pressure reached the turgor pressure of the impaled cell, the valve V2 was opened for perfusion. During perfusion, the flow rate and the pressure could be independently changed by the perfusion pump (PP) and the pressure regulator (PR). Build up of a pressure difference of more than 0.01 MPa between the two transducers PT1 and PT2 was a clear-cut indication of clogging in the tip of the outflow pipette. Clogging occurred usually within the first 20 min after onset of perfusion and was eliminated by movement of the platinum wire as described above. To change the perfusion solution, the valve V2 was first shut. After replacing the syringe with another containing vacuolar sap of different composition, perfusion was started again in the manner described above. In this way, exchange of the perfusion solutions could be performed without exposure of the cell to a pressure shock.

In the first set of experiments, the vacuole of a cell of *V. utricularis* was perfused with AVS. The flow rate was adjusted to 20 μl min^{-1} and the turgor pressure to 0.15 MPa. Charge-pulse experiments were performed

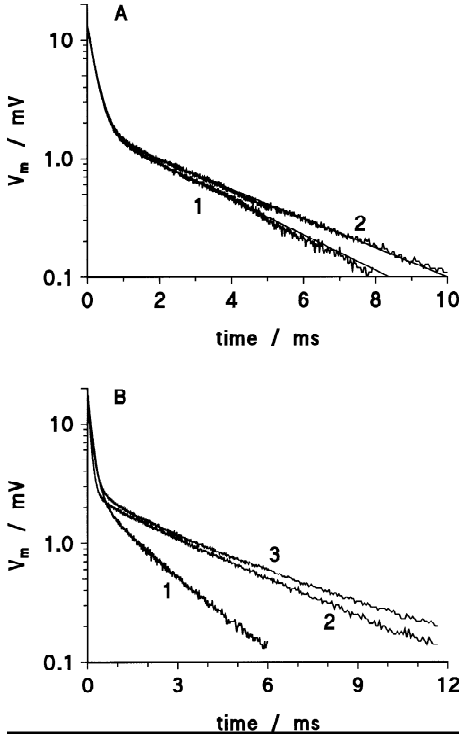

Fig. 2. Semilogarithmic plots of charge pulse relaxation spectra recorded on AVS-perfused cells of *V. utricularis* (bathed in ASW) at various turgor pressures. (*A*) Control experiment at a turgor pressure of 0.15 MPa; cell parameters: surface area $A = 71$ mm^2 and volume $V = 51$ mm^3. Curve 1: no perfusion, measurement 1.5 hr after insertion of the two pipettes *P1* and *P2* into the vacuole; curve 2: perfusion rate of 20 μl min^{-1}, measurement 2 hr after the onset of perfusion with AVS. (*B*) Recordings at 0.16 MPa (curve 1), 0.02 MPa (curve 2), and 0.32 MPa (curve 3); cell parameters: $A = 77$ mm^2 and $V = 48$ mm^3. Measurements were performed about 2 hr after the onset of perfusion.

Table 1. Electrical properties of the plasmalemma and the tonoplast of cells of *Valonia utricularis*

Cell	A mm^2	Q nAs	V_{m0} mV	P_0 MPa	τ_1 μsec	V_1 mV	τ_2 msec	V_2 mV	C_p 10^{-2}F/m^2	R_p $10^{-2}\Omega$m^2	C_t 10^{-2}F/m^2	R_t $10^{-2}\Omega$m^2	R_t^* $10^{-2}\Omega$m^2	A_t^* mm^2
1	62	4.9	−1	0.39	125	10.2	1.95	0.90	0.77	1.61	8.75	2.23	25.3	705
2	63	6.5	2	0.15	100	9.0	1.47	2.00	1.16	0.86	5.17	2.84	12.7	281
3	99	7.3	−1	0.15	149	11.6	2.13	2.12	0.64	2.34	3.48	6.11	33.2	538
4	76	8.4	3	0.16	138	15.6	1.28	2.03	0.71	1.95	5.42	2.36	18.0	580
5	79	7.2	2	0.22	116	11.3	2.18	1.81	0.80	1.45	5.01	4.35	27.2	495
6	41	3.1	−5	0.30	131	12.8	1.69	0.81	0.59	2.21	9.38	1.80	28.5	644
7	42	3.8	0	0.14	116	10.8	1.58	1.63	0.85	1.37	5.64	2.79	18.6	277
8	36	2.6	−1	0.28	126	9.7	1.49	0.74	0.73	1.71	9.65	1.55	20.3	479
9	60	5.6	2	0.30	158	11.0	1.62	2.31	0.86	1.85	4.06	3.99	19.0	286
10	74	6.1	−3	0.35	227	13.1	4.00	1.49	0.63	3.59	5.53	7.23	63.3	648
Mean	57	5.6	0	0.24	139	11.5	1.94	1.58	0.77	1.89	6.21	3.53	26.6	493
± SE	26	1.9	3	0.09	35	1.9	0.78	0.58	0.16	0.73	2.22	1.89	14.2	162

Charge pulse experiments were performed on cells bathed in ASW under nonperfusion (cell 1-5) and perfusion (cell 6-10, AVS, 20 μl/min) conditions. 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 exponential decays (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-squares-fits. Q is the injected charge, A is the surface area of the alga, V_{m0} is the vacuolar resting membrane potential and P is the turgor pressure. The area-specific membrane capacitances and resistances of the plasmalemma (C_p and R_p) and that of the tonoplast (C_t and R_t) respectively, were calculated by using Eqs. 4 and 5 for the boundary condition of $A_p = A_t = A$. The area-specific membrane resistance (R_p^*) and the surface area of the tonoplast (A_t^*) were calculated by using Eq. 8 under the assumption of $C_t^* = C_p$. For further details, see text.

before (curve 1) and during (curve 2) perfusion (Fig. 2*A*). The voltage relaxation spectrum could be fitted to the sum of two exponential relaxations according to Eq. 1. The following parameters were obtained from the least square fitting of the curve 1: $V_1 = 11.6$ mV, $\tau_1 = 208$ μsec, $V_2 = 1.8$ mV, and $\tau_2 = 2.9$ msec; of the curve 2: $V_1 = 12.4$ mV, $\tau_1 = 225$ μsec, $V_2 = 1.9$ mV, and $\tau_2 = 3.5$ msec. The corresponding results obtained for five other perfused (and unperfused) cells are given in Table 1. It is obvious that the data agree quite well indicating that the perfusion of the vacuole with AVS did not significantly change the electrical parameters of the membrane barrier. Long-term perfusion resulted in a relatively small increase of the time constants of the two exponential voltage decays. The average time constant of the fast relaxation process, τ_1, changed by <15% and that of the slow process, τ_2, by <30% after 6 hr perfusion of the alga with AVS at physiological turgor pressure. The area-specific conductance of the membrane barrier decreased by <25%.

Turgor Pressure Effects on the Charge Pulse Relaxation Spectrum

When the vacuole was perfused under turgorless conditions (or when the turgor pressure was lost by inappropriate puncture of the alga with the pipettes) the time constant of the slow relaxation (τ_2) and the membrane resistance (R_m) increased significantly (Fig. 2*B* and Fig. 3). Stepwise adjustment of the turgor pressure between zero and 0.32 MPa revealed that both parameters decreased up to the physiological turgor pressure of about 0.15 MPa. Beyond this pressure, a significant increase was observed (Fig. 3). The relationship between turgor pressure and τ_2 or R_m did not depend on the direction of the pressure change. Upward and downward pressure changes led to the same results. In contrast, the time constant of the fast relaxation (τ_1) showed strong hysteresis effects (Fig. 3*B*). τ_1 generally increased when pressure was raised from zero to 0.3 MPa. This was particularly noticeable at high pressures. However, τ_1 remained high when the pressure was lowered again (Fig. 3*B*). The membrane capacitance (C_m), the relative amplitudes of the fast and slow voltage relaxations (a_1 and a_2) and the resting vacuole potential V_{m0} remained nearly constant over the pressure range of 0 to 0.3 MPa.

Effect of Vacuolar FCCP on the Charge Pulse Relaxation Spectrum

The experiments were carried out at the physiological turgor pressure. Addition of 16 μM of FCCP to the perfusion solution had very strong effects on vacuolar potential (V_{m0}), the membrane resistance (R_m) and the two time constants of the charge pulse relaxation spectrum (Table 2). Compared to the controls V_{m0} dropped from slightly positive values of 4 to fairly high negative values of about −22 mV, whereas R_m increased from 6.8×10^{-2} to 40.6×10^{-2} Ωm^2, τ_1 increased from 146 μsec to 425 μsec and τ_2 from 2.5 msec to 18.3 msec. Lower con-

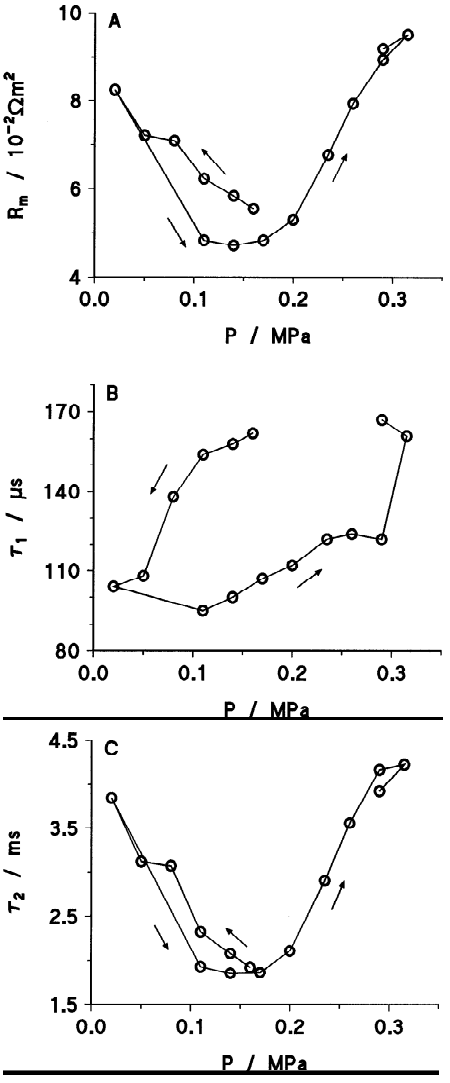

Fig. 3. The dependence of the area-specific resistance of the total membrane barrier (R_m, *A*) the time constants of the fast (τ_1, *B*) and the slow (τ_2, *C*) relaxations on turgor pressure. Data were obtained from charge pulse relaxation spectra recorded on the cell shown in Fig. 2*B*. Measurements were started at the physiological turgor pressure value of about 0.15 MPa 1.5 hr after the onset of perfusion with AVS. Then the turgor pressure was lowered stepwise down to 0.02 MPa followed by a stepwise increase to 0.32 MPa (*see* arrows). The time constants τ_1 and τ_2 were obtained from the least-squares-fits of the voltage decays (*see* Fig. 2) by using Eq. 1. The resistance R_m was calculated by using either Eq. 3 or Eq. 7 (both equations yielded the same results, for explanation, *see* text). Note that hysteresis effects occurred.

centrations of 4 µM FCCP were still effective. However, the changes in the parameters mentioned above were slightly smaller (*see* Table 2).

Effects of Nystatin and Amphotericin B on the Charge Pulse Relaxation Spectrum

In the following set of experiments the effects of vacuolar and external nystatin on the charge pulse relaxation spectrum were investigated. The turgor pressure was adjusted to the physiological value.

Screening experiments in which different concentrations of nystatin (5–50 µM) were added to the ASW or to the AVS showed strong effects on both relaxations. The two relaxations disappeared nearly simultaneously within a very short time (*data not shown*). Apparently, the diffusion of high concentrations of Cl^--ions into the cytoplasm after permeabilization of one of the membranes leads to lethal effects (Wyn Jones, Brady & Speirs, 1978). Indeed, when the sea water or the vacuolar perfusion solution were replaced by an artificial solution, which corresponded, presumably, more closely to the ionic composition of the cytoplasm (ACS, *see* Materials and Methods) than AVS and ASW, the cells survived the treatment with nystatin. ACS contains only 140 mM Cl^- much less than AVS and ASW. Lowering of the Cl^- concentration resulted (in agreement with previous results, Wang et al., 1991) in an increase of the time constants of the two relaxations (the transient increase of τ_1 and τ_2, *see* Fig. 5*A* and *B*). Under these conditions, the effects of nystatin clearly depended on the site of addition. This was particularly the case if a concentration of 50 µM was used. Upon external addition of nystatin (to both perfused or nonperfused cells) τ_1 decreased dramatically (compare curve 1 with curve 2 in Fig. 4*A*). After 1 hr the fast relaxation process could no longer be resolved (Fig. 5*A*). Interestingly, τ_2 did not change significantly over a period of 10 hr (part of the data are given in Fig. 5*A*, see also curve 2 in Fig. 4*A*). The opposite results were found when nystatin was added to the perfusion solution. Under these conditions, the time constant of the slow voltage relaxation (τ_2) decreased rapidly and the relaxation disappeared after about 0.5 hr (compare curve 1 with curve 2 in Fig. 4*B* and see also Fig. 5*B*), while τ_1 remained nearly constant for more than 5 hr (*see* curve 2 in Fig. 4*B* and Fig. 5*B*).[2] Replacement of nystatin by equivalent concentrations of amphotericin B led to the same results (*data not shown*).

Discussion

The results presented here demonstrate that perfusion of the vacuole of algae cells under turgescent conditions is a very promising way to get insights into the electrical (and transport) properties of the tonoplast and the plas-

[2] Note that in the presence of external or vacuolar nystatin an intermediate relaxation with a very small amplitude of about 0.6 mV and a time constant of 500 µsec occurred regularly. Such an intermediate relaxation could also sporadically be resolved under control conditions (*see* also Zimmermann et al., 1982). The origin of this relaxation process is unknown, but there are some indications that it is linked with the structure and/or with transport processes within the tonoplast.

Table 2. Effect of FCCP on the vacuolar resting membrane potential (V_{m0}), on the area-specific membrane resistance of the total membrane barrier (R_m), and on the two time constants (τ_2 and τ_2) of the relaxation spectrum.

[FCCP] μM	V_{m0} mV	R_m $10^{-2}\Omega m^2$	τ_1 μsec	τ_2 msec
0*	4 ± 1	4.58 ± 0.22	134 ± 15	1.61 ± 0.18
0**	5 ± 1	6.77 ± 0.32	146 ± 10	5.50 ± 0.22
4	−7 ± 2	25.42 ± 3.47	306 ± 20	10.97 ± 1.65
16	−22 ± 2	40.64 ± 0.89	425 ± 60	18.29 ± 0.70

The cell of *V. utricularis* was bathed in ASW and perfused with AVS (surface area A = 140 mm^2, volume V = 113 mm^3). The vacuolar perfusion was started 1.5 hr after inserting the two pipettes at the physiological turgor pressure P_0 (= 0.15 Mpa) with a flow of 20 μl/min. The data represent the means ± SD_{n-1} of four charge-pulse experiments performed at intervals of 10 min *: control measurements 1 hr after the insertion of the two pipettes and before the perfusion; **: control experiments 30 min after the start of vacuolar perfusion of AVS without FCCP; Measurements were performed 30 min after addition of 4 μM (0.04% acetone) and 16 μM FCCP (0.16% acetone) in AVS, respectively. The similar results were also obtained from the experiments carried out on other two different cells. For further details, see the legend of Table 1 and text.

malemma. The possibility of changing flow rate and turgor pressure independently of each other (while maintaining the internal and external ion concentration) and of adding agents to the perfusion solution allows controlled manipulation of the cell system from the vacuolar side. Artificial changes introduced by impalement of the cell with two relatively large pipettes could be ruled out for two reasons. Firstly, comparison of the charge pulse relaxation spectra obtained under perfusion and nonperfusion conditions at the same turgor pressure did not reveal any significant changes in the time constants of the two exponentials. Secondly, the change in the area-specific resistance of the total membrane barrier with turgor pressure recorded here (Fig. 3*A*) is consistent with results that were obtained on cells of *V. utricularis* under nonperfusion conditions by Zimmermann & Steudle (1974). These authors have shown that the area-specific membrane resistance assumed a minimum value of about 5×10^{-2} Ωm^2 in the pressure range between 0.05 MPa and 0.2 MPa. These data correlate nicely with the findings reported here. Under nonperfusion conditions however, the area-specific membrane resistance reached maximum (about 20×10^{-2} Ωm^2) at somewhat higher turgor pressures (0.15 to 0.35 MPa, depending on cell size). Beyond this value, the resistance decreased again. Under perfusion conditions a maximum in the area-specific membrane resistance could not be resolved up to 0.3 MPa. A possible cause for this may be that under nonperfusion conditions the turgor pressure is adjusted by changing the osmolality of the external sea water, whereas in perfused cells the internal and external osmotic pressures were kept constant as already mentioned.

The experiments in which the effect of nystatin (and amphotericin B) on the charge pulse relaxation spectrum were investigated tend to confirm the conclusion that the two exponentials of the charge pulse relaxation spectrum originate from the electrical properties of the tonoplast and the plasmalemma. The ''two membrane'' hypothesis predicts that the antibiotics should affect exclusively either the slow or the fast relaxation depending on the site of addition (vacuolarly or externally). This prediction is in agreement with the experimental results. By contrast, the ''mobile charge concept'' postulates that an effect of these agents on the charge pulse spectrum should only occur in response to external addition, not to vacuolar (Zimmermann et al., 1982; Benz & Zimmermann, 1983; Wang et al., 1991, Spieß et al., 1993). This prediction could not be verified. In the light of this clear-cut experiment, it is therefore justified to analyze the data of the charge pulse relaxation spectrum in terms of the ''two membrane'' model.

To this end, we assume that the surface area of the plasmalemma is equal to the geometric surface area of the alga. The assumption seems to be reasonable because the hydrostatic pressure gradient must drop across this membrane due to the stabilization of the wall (Hastings & Gutknecht, 1974; Wendler & Zimmermann, 1978). The area-specific membrane capacitance and resistance of the plasmalemma is then calculated[3] according to Eq. 4 on the basis of the data in Table 1 to be $C_p = 0.77 \times 10^{-2} \pm 0.16 \times 10^{-2}$ Fm^{-2} and $R_p = 1.89 \times 10^{-2} \pm 0.73 \times 10^{-2}$ Ωm^2. For the tonoplast the corresponding values are $C_t = 6.21 \times 10^{-2} \pm 2.22 \times 10^{-2}$ Fm^{-2} and $R_t = 3.53 \times 10^{-2} \pm 1.89 \times 10^{-2}$ Ωm^2, assuming a planar vacuolar membrane (Eq. 5). We are faced with the problem, as in the past (Zimmermann et al., 1982; Benz & Zimmermann, 1983; Wang et al., 1991), that the area-

[3] In the analysis of the data, the intermediate relaxation is neglected for a first approximation because of its small amplitude (*see* Footnote 2).

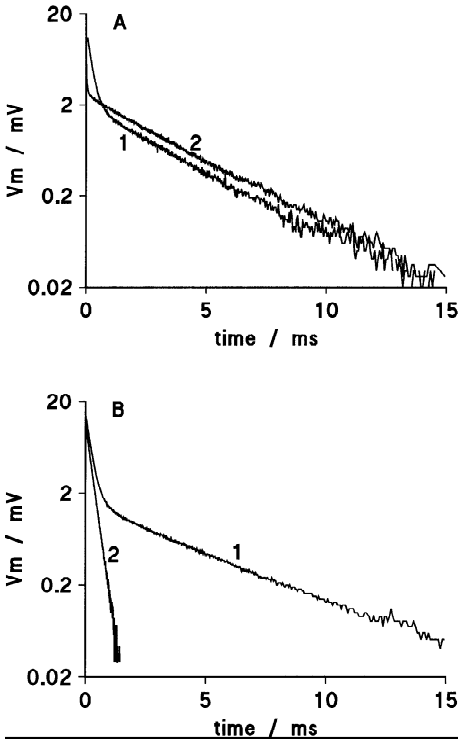

Fig. 4. Effects of 50 μM nystatin on the charge pulse relaxation spectrum of *V. utricularis* after external (*A*) and vacuolar (*B*) addition. The turgor pressure was adjusted to 0.15 MPa prior to the addition of nystatin. Cell parameters: (*A*) surface area $A = 100$ mm^2 and volume $V = 57$ mm^3; (*B*) $A = 74$ mm^2 and $V = 48$ mm^3. Curves marked 1 represent the control experiments: (*A*) bathed in flowing ASW, no perfusion; (*B*) perfused with AVS. Curves marked 2 represent charge pulse experiments performed 1 hr after exchange of ASW (*A*) and 0.5 hr after exchange of AVS (*B*) against nystatin-containing ACS. Note the disappearance of the fast relaxation in Fig. 4*A* and of the slow one in Fig. 4*B*.

specific capacitance value of one of the membranes is unusually high compared to the literature data for a biological membrane with smooth surface ($0.5 \times 10^{-2} - 1.3 \times 10^{-2}$ Fm^{-2}; Pauly, 1962; Cole, 1968; Walker, 1976; Pethig, 1979; Arnold & Zimmermann, 1988; Fuhr et al., 1996).

Following the line of previous arguments for the analysis of the spectra in terms of mobile charges embedded in the plasmalemma (Zimmermann et al., 1982; Benz & Zimmermann, 1983; Wang et al., 1991, 1994) we can postulate the presence of high area-specific concentrations of mobile charges within the tonoplast. This assumption would explain straightforwardly the apparent increase of the area-specific membrane capacitance of the tonoplast and would also be consistent with the bulk of evidence obtained from measurements on lipid bilayer membranes and biomembranes doted with artificial mobile charges (Läuger et al., 1981; Wang et al., 1994; Sukhorukov & Zimmermann, 1996). Alternatively, we would have to assume that the tonoplast surface in *V. utricularis* is multiply folded or exhibits a spongelike structure. For $C_t = C_p$, it can be easily shown (*see* Table 1) by using Eq. 8 that the surface area of the tonoplast must be by a factor of about 8.7 larger than that of the plasmalemma to explain the data. Such area enlargement which leads to an apparent increase of the area-specific capacitance is reported for the plasmalemma in the literature (Kanno & Loewenstein, 1963; Kottra & Frömter, 1984; Clausen & Dixon, 1986; Arnold & Zimmermann, 1988; Fuhr et al., 1996). Therefore, an enlargement of the tonoplast is not *a priori* unreasonable because it would facilitate solute exchange between the vacuole and the cytoplasma. Hints for a spongelike structure of the vacuole in *V. utricularis* were presented recently by Shihira-Ishikawa and Nawata (1992). The electronmicroscopy studies of these authors suggest the

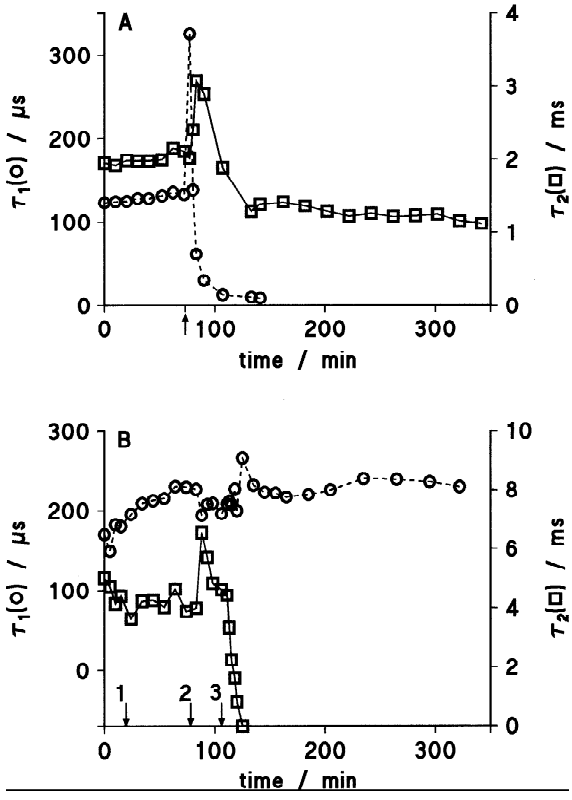

Fig. 5. Time course of the effect of nystatin on the time constants of the fast (τ_1) and the slow relaxation (τ_2) after external (*A*) and vacuolar (*B*) addition. Data were taken from charge pulse relaxation studies performed on the cells shown in Figs. 4*A* and *B*, respectively. The arrow in Fig. 5*A* marked the change of external medium from ASW to nystatin-containing ACS. The arrow 1 in Fig. 5*B* indicates the start of perfusion with AVS, arrow 2 the replacement of AVS by ACS and the arrow 3 the exchange of ACS with nystatin-containing ACS. Note that (1) charge pulse relaxation spectra were recorded approximately every two minutes. For clarity, only part of the data are shown. (2) External as well as vacuolar perfusion of ACS increased the two time constants of the relaxation process significantly because of the relatively low Cl^- concentration (*see* text).

presence of such structures in *V. ventricaria* although artifacts cannot be excluded with certainty because of the large vacuole which prevents rapid fixing of the cell material. However, confocal laser scan microscopy has also not revealed any clear boundary between the cytoplasma and the vacuole for *V. utricularis* in contrast to *Characeen* where the tonoplast was clearly visible (Wang & Zimmermann, *unpublished data*). Taking this together, we can conclude that membrane folding is an important alternative explanation for the unusually high area-specific capacitance value of the tonoplast.

The above calculations show independently that the area-specific resistance of the tonoplast must be higher than that of the plasmalemma. This contradicts the general belief that the tonoplast of giant algal cells is substantially more conductive than the plasmalemma (Spanswick & Costerton, 1967; Spanswick, 1972; Lainson & Field, 1976; Findlay & Hope, 1976; Coster & Smith, 1977; Smith, 1983; Beilby, 1989; Wang et al., 1991). If our results can be generalized, this calls for a reconsideration of much of the electrophysiological data obtained with vacuole-placed microelectrodes, because for data analysis it was assumed that the two membranes in series were thought to reflect primarily the properties of the plasmalemma. A high resistance is also suggested by the FCCP experiment. The strong effect of FCCP on the total membrane potential suggests that a H^+-ATPase and/or a pyrophosphatase (PP_iase) is operating in the tonoplast of *V. utricularis.* This is found in other plant cells (Hedrich & Schroeder, 1989; Martinoia, 1992; Barkla & Pantoja, 1996). FCCP apparently abolishes the vacuole-directed H^+-gradient leading to a depolarization of the tonoplast and an increase in resistance. It appears that a H^+-ATPase and/or a PP_iase is responsible for the slightly positive vacuolar potential (in sea water) in *V. utricularis* and that the transport and electrical properties of the vacuole of giant algal cells resemble those of vacuoles of higher plants.[4]

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