Influence of external chloride concentration on the kinetics of mobile charges in the cell membrane of *Valonia utricularis*

Evidence for the existence of a chloride carrier

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ABSTRACT Charge pulse relaxation studies were performed on cells of the giant marine alga *Valonia utricularis*. Two exponential voltage relaxations were recorded as found previously (Benz, R., and U. Zimmermann. 1983. *Biophys. J.* 43:13–26.). The parameters of the two exponential voltage decays were studied as a function of the chloride concentration in the artificial sea water. Replacement of external chloride by 2(*N*-morpholino)ethanesulfonate (Mes^-) had a dramatic influence on the four relaxation parameters. This chloride dependence could not be satisfactorily explained by the simplified model used earlier. Accordingly, additional reaction steps had to be included in the model. Only two relaxation processes could be resolved under all experimental conditions. This means that the heterogeneous complexation reactions, k_R (association), and k_D (dissociation) were too fast to be resolved. Therefore a carrier model with equilibrium heterogeneous surface reactions was used to fit the experimental results. From the charge pulse data at different chloride concentrations the translocation rate constants of the free and complexed carriers, k_S and k_{AS}, through the membrane, as well as the total surface concentration of carrier systems, N_0, could be evaluated. The results described here indicate that the cell membrane of *Valonia utricularis* contains an electrogenic transport system for chloride.

INTRODUCTION

There exists some evidence that net charge transfer across the plasmalemma is associated with proton and chloride pumps as well as with proton-driven cotransport systems in plant cells (Slayman and Slayman, 1974; Komor and Tanner, 1976; Felle and Bentrup, 1977; Felle, 1980; Tittor et al., 1983; Gradmann, 1989). Evidence for the net movement of charge as part of an electrogenic transport systems was also obtained from charge-pulse experiments on the giant marine algal cells of *Valonia utricularis* and *Halicystis parvula* (Benz and Zimmermann, 1983; Zimmermann et al., 1982; Benz et al., 1988). These transport systems, known as mobile charges, are part of an electrogenic transport for ions and have an extremely large surface concentration of the order of 10 pmol/cm^2 (Benz and Zimmermann, 1983; Benz et al., 1988). This is similar to the total surface concentration of the chloride pump in the cell membrane of *Acetabularia mediterranea* (Tittor et al., 1983). The rate-limiting step of the ion transport is one of the voltage-independent steps, i.e., either the translocation of the uncharged carrier or the association-dissociation reaction between carrier and ion (Zimmermann et al., 1982; Benz and Zimmermann, 1983; Büchner et al., 1985). As a consequence the apparent specific capacity of *V. utricularis* is as large as 6 μF/cm^2 (Benz and Zimmermann, 1983; Zimmermann et al., 1982) and as large as 4 μF/cm^2 in *H. parvula* (Benz et al., 1988).

The pressure and temperature dependence of the translocation rate of the mobile charges, as well as the specific interactions of barium ions and of local anaesthetics with the same system in *V. utricularis* suggested that the mobile charges could be part of the K^+-transport system (Zimmermann, et al., 1982; Büchner et al., 1985, 1987; Walter et al., 1988). The K^+ influx and efflux are inversely pressure dependent and play an important role in maintainance of turgor pressure and regulation (Zimmermann et al., 1982). However, it is also possible that the mobile charges are part of a Cl^- transport system (Benz et al., 1988). There are indications that the Cl^- influx is also pressure dependent in *V. utricularis* (Zimmermann, U., K. H. Büchner, S. Wendler, unpublished results). In addition, there exists a very potent chloride pump in *H. parvula* (Graves and Gutknecht, 1977*a*, *b*) which has some similarities with the chloride pump in *Acetabularia mediterranea* (Gradmann, 1975; Tittor et al., 1983).

The disappearance of mobile charges in the cell membrane of both algal species upon decrease of aqueous pH further suggests that they are negatively charged. If the mobile charges are involved in Cl^- transport we have to assume that the negatively charged chloride-carrier complex binds protons with an apparent pK of ~6 (Zimmermann et al., 1982; Benz and Zimmermann, 1983). To explore the nature and the functional relationships between the mobile charges and ion transport in *V. utricularis* we changed the external chloride concentration. Brief charge pulses applied to the membrane elicited two voltage relaxations which were greatly influenced by the replacement of chloride by other anions. The mobile charge concept proposed earlier

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(Benz and Zimmermann, 1983) is too simple to explain these new experimental data. As a consequence we extended the earlier model so that it became a carrier system for chloride. We must assume that the interfacial reactions between anion and carrier are always in equilibrium. We confirmed the adequacy of the model by determining the rate constants of translocation of the free and complexed form, k_S and k_{AS}, respectively, and the total surface concentration, N_0, of carrier.

MATERIALS AND METHODS

Cells of *V. utricularis,* originally collected in Naples, Italy, were grown in natural seawater from the North Sea (supplied by Biomaris, Bremen, FRG) under a 12-h light/dark regime at 16°C. Before use the salinity of the North Sea water was adjusted to Mediterranean salinity (1.114 Osmol/kg, 3 MPa). Cells were used after a 1–4 mo adaptation to the salt-enriched water. Cells of nearly elliptical shape were selected (surface area between 50 to 110 mm^2, calculated from their dimensions measured microscopically). Single cells were fixed in a perspex chamber perfused with artificial sea water (ASW) containing 545 mM NaCl, 12 mM KCl, 11 mM $CaCl_2$, and 10 mM $MgCl_2$. The pH was maintained at 8,1 by inclusion of 10 mM Hepes (*N*-[2-Hydroxyethyl] piperazine-*N'*-[2-ethanesulfonic acid])/NaOH and the temperature was 20°C throughout the experiments if not otherwise stated.

Experiments were started 1.5 to 2 h after insertion of the microelectrodes. This time was sufficient to heal the punctured areas. The turgor pressure was recorded by insertion of a pressure probe (Zimmermann and Steudle, 1974). The charge pulse technique has been described in detail elsewhere (Benz and Conti, 1981; Benz and Zimmermann, 1983). Briefly, a current microelectrode consisting of a 10-μm thick platinum wire which was inserted deeply into the cell. The wire was connected to a fast, commercial pulse generator (model 214B, Hewlett Packard Co., Palo Alto, CA) through a diode with a reverse resistance larger than 10^{10} Ω. The cell was charged with a short, rectangular pulse of 1-μs duration. The injected charge Q was calculated from the voltage drop across a 10 Ω resistor connected in series with the current electrode. The shank of the microcapillary containing the current electrode was sealed by a rubber "O"-ring to an oil-filled perspex chamber in which a pressure transducer was mounted (Zimmermann and Steudle, 1974).

The potential electrode (Ag/AgCl, 3 M KCl) was connected to the input of a Nicolet (Frankfurt, FRG) 2090 digital oscilloscope through a fast, high-impedance voltage follower. In contrast to earlier investigations, two large silver/silver chloride reference electrodes were used, one (3 M KCl, agar bridge) for recording the voltage relaxation and one for the injection of the current pulses. Both external electrodes were placed close to the surface of the alga. The use of two separate reference electrodes allowed 50-Hz disturbances to be nearly eliminated and polarization effects at the recording electrodes induced by the injection of current pulses to be excluded. Such polarization effects interfered with the voltage relaxation measurements especially when the chloride concentration in the seawater was replaced by equivalent concentrations of 2(*N*-morpholino)ethanesulfonate (MES^-), while keeping the concentrations of the cations constant.

The voltage relaxation patterns recorded with the Nicolet 2090 digital oscilloscope contained 4096 data points with 12-bit amplitude resolution. The waveforms were transferred to a PC/AT computer and analyzed with a multiple-exponential-fitting program. This performed least squares fits between the semilogarithm of membrane voltage and time (Benz and Zimmermann, 1983). The voltage decays could be fitted to two exponential relaxations with sufficient accuracy. The significance of the fit was checked with the Student-*T*-test. At 599 mM chloride $T(n-2)$ was at least 500 for 1,000–2,000 data points. At small chloride concentrations (0 or 54 mM), $T(n-2)$ was lowest with ~50 for 200 data points of the slow relaxations. This means that the fit was also in these cases significant. Only in a very limited number of experiments an intermediate (third) relaxation with a very small relative amplitude (<0.05) could be resolved. In all of these cases inspection of the voltage curves showed the existence of noise or extraneous pertubations which therefore seemed to cause this "apparent" third relaxation. Furthermore, in 10 successive experiments, taken on the same algal cells at time intervals of 30 s, the time constants and voltage amplitudes did not vary by more than 3%, which means that the results obtained from one single cell were highly reproducible but varied considerably from cell to cell. To prove the accuracy of the charge pulse method in the range of low external chloride concentrations we also measured the specific membrane resistance, R_m^*, under current clamp conditions (20 μA, 10–20 ms) and compared the values of this parameter with those obtained by the charge pulse technique.

The internal chloride concentration of *V. utricularis* cells was measured as follows. Large cells were punctured with a fine cannula and the vacuolar sap from 30 algal cells was collected in a syringe. Such pooled samples were diluted 2,000-fold and the chloride concentration was measured photometrically with a chloride testkit (Spektroquant 14755; Merck, Darmstadt, FRG).

THE KINETIC MODEL FOR CHLORIDE TRANSPORT

Fig. 1 illustrates the kinetic model proposed here for the description of chloride transport through the membrane(s) of *V. utricularis.* This model is very similar to that used previously for the description of carrier-mediated ion transport in lipid bilayer membranes (Läuger, 1972; Benz and Läuger, 1976; Benz et al., 1989). Our model assumes a 1:1 carrier-ion complex which is formed at the membrane-solution interface. The heterogeneous complexation reaction is described by overall rate constants k_R (association) and k_D (dissociation). The stability constant of the carrier-anion complex is given by $K = k_R/k_D$. The translocation of free and charged carriers through the membrane are treated as

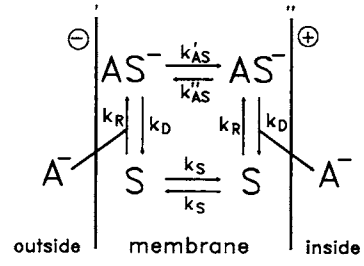

FIGURE 1 Kinetic scheme for carrier-mediated chloride transport in the cell membrane of *V. utricularis.* See text for further details.

simple first order reactions with rate constants k_S and k_{AS}, respectively. Of all the rate constants only k_{AS} is assumed to be voltage dependent. Its dependence is calculated on the basis of a single barrier of the Eyring or Nernst-Planck type (Benz and McLaughlin, 1983):

$$k'_{AS} = k_{AS}(bzu/2) \exp(zu/2)/\sin h(bzu/2) \quad (1)$$

$$k''_{AS} = k_{AS}(bzu/2) \exp(-zu/2)/\sin h(bzu/2). \quad (2)$$

$u = FV_m/RT$ is the reduced voltage. V_m is the membrane voltage, F, R, and T have the usual meaning, and z is the valency of the carrier-ion complex. For $b = 0$ the carrier-ion complex encounters an Eyring barrier. For $b = 1$ (and also for small voltages in the case of an Eyring barrier) k'_{AS} and k''_{AS} are proportional to $(1 + zu/2)$ and $(1 - zu/2)$, respectively, as predicted by a Nernst-Planck model with a square barrier (Benz and McLaughlin, 1983).

It is assumed that the membrane(s) of *V. utricularis* separates(s) identical solutions of the anion (concentration c). The interfacial concentrations of free and complexed carriers on the two sides of the membrane change with time as given by the Eqs. A1–A4 (see Appendix A; Benz and Läuger, 1976). The total concentration of carriers within the membrane (complexed and uncomplexed) is constant (Eq. A5). The rate of decay of the membrane voltage V_m after a brief charge pulse of 1-μs duration is determined by the specific membrane capacity C_m and by the current density I_m in the membrane given by the movement of the charged carrier molecules within the membrane and by the specific resistance, R_m, of the membrane (due to transport of ions other than chloride):

$$dV_m/dt = -I_m/C_m = zF(-k'_{AS}N'_{AS} + k''_{AS}N''_{AS})/C_m - V_m/(R_mC_m). \quad (3)$$

Eq. 3 represents together with Eqs. A1 and A5 a system of three coupled differential equations (Benz and Läuger, 1976). For small voltages ($u \ll 1$; $V_m \ll 25$ mV), this can be solved to give the time course of the membrane voltage (Benz and Läuger, 1976):

$$V_m(t) = V_0[a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2) + a_3 \exp(-t/\tau_3)] \quad (4)$$

$$a_1 + a_2 + a_3 = 1. \quad (5)$$

The relaxation times τ_i and the relative amplitudes a_i ($i = 1, 2, 3$) are known functions of the four rate constants, the total number of carrier molecules, N_0, the specific membrane capacity, C_m, and the specific membrane resistance, R_m (Benz and Läuger, 1976).

In this investigation and in previous charge pulse studies with *V. utricularis* (Benz and Zimmermann, 1983; Büchner et al., 1985, 1987; Walter et al., 1988) only two relaxation processes could be resolved with sufficient accuracy. Furthermore, preliminary voltage-clamp experiments with *V. utricularis* cells show only one current relaxation (apart from the capacitive spike; Wang, J., U. Zimmermann, and R. Benz, unpublished results). These results suggest that one of the different reactions involved in a carrier-mediated anion transport (see Fig. 1) is always in equilibrium because it is much faster than the others (Benz and Läuger, 1976; Benz and McLaughlin, 1983). This means either that $k_S \gg k_R c$, k_D, k_{AS}, or that $k_R c$, $k_D \gg k_{AS}$, k_S (the case $k_R c$, k_D, $k_S > k_{AS}$ is unlikely because only one relaxation is obtained in this case under charge pulse conditions [Benz and Läuger, 1976]). The second case was found to be consistent with the experimental results (see Discussion). In this case the differential Eqs. A1–A4 in Appendix *A* and Eq. 3 reduce to a system of two differential equations (see Appendix A). The solution for the membrane voltage after a charge pulse of short duration has the form (valency $z = -1$):

$$V_m(t) = V_0[a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2)] \quad (6)$$

$$a_1 + a_2 = 1. \quad (7)$$

Defining the quantities P_1, P_2, and P_3 (see Appendix A) as

$$P_1 = 1/\tau_1 + 1/\tau_2 \quad (8)$$

$$P_2 = 1/(\tau_1\tau_2) \quad (9)$$

$$P_3 = a_1/\tau_1 + a_2/\tau_2 \quad (10)$$

the rate constants $K_{AS} = k_{AS}Kc/(1 + Kc)$ and $K_S = k_S/(1 + Kc)$ and N_0 are given by, when $2BN_0K_{AS} \gg 1/(R_mC_m)$:

$$K_{AS} = (P_1 - P_3 - P_2/P_3)/2 \quad (11)$$

$$K_S = P_2/(2P_3) \quad (12)$$

$$N_0 = P_3/(2BK_{AS}) \quad (13)$$

with:

$$B = z^2F^2/(4RTC_m). \quad (14)$$

If the specific membrane conductance, R_m, caused by ion transport other than that of chloride cannot be neglected (especially in the case $c \leq 100$ mM) Eqs. 11–13 have the form:

$$K_{AS} = [(P_1 - P_3)P_3 - P_2]/[2[P_3 - 1/(R_mC_m)]] \quad (15)$$

$$K_S = (P_1 - P_3)/2 - K_{AS} \quad (16)$$

$$N_0 = [P_3 - 1/(R_mC_m)]^2/[B[(P_1 - P_3)P_3 - P_2]]. \quad (17)$$

The voltage relaxation after a brief charge pulse could be fitted to just two exponential relaxation processes, whereas the general case of the carrier model requires the knowledge of three relaxations for complete description (Benz and Läuger, 1976; Benz and McLaughlin, 1983). Thus, information on the rate constants of the heterogeneous reaction cannot be obtained. Furthermore, k_{AS}, k_S, and the stability constant, K, for the binding of chloride to the carrier cannot be obtained from experiments at only one chloride concentration. The parameters k_{AS} and k_S could be evaluated for a single cell by plotting K_{AS} and K_S as a function of the external chloride by assuming a value for $K = k_R/k_D$ that gives the best fit to the data. R_m could be estimated by assuming that N_0 is not dependent on the chloride concentration.

The results for K_{AS} and K_S as a function of external chloride concentration suggest that other anions may compete for the same binding site. If the transport of these anions can be neglected (see Appendix B), K_{AS} and K_S are also dependent on their concentration, c_a and the stability constant, K_a, for their binding to the chloride binding site (see Appendix B):

$$K_{AS} = k_{AS}Kc/(1 + Kc + K_a c_a) \quad (18)$$

$$K_S = k_S/(1 + Kc + K_a c_a). \quad (19)$$

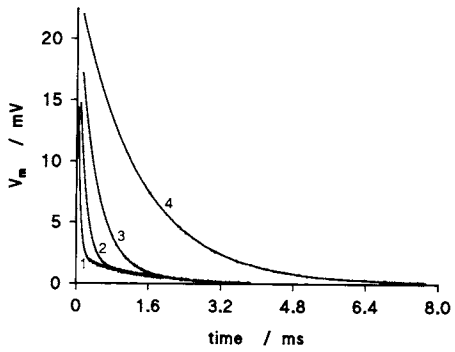

FIGURE 2 Oscillographic records of charge-pulse experiment performed on *V. utricularis* cell w2930, bathed in ASW containing different chloride concentrations (adjusted to 599 mM by the addition of MES^-). Curve 1: 599 mM; curve 2: 299 mM; curve 3: 54 mM; and curve 4: 0 mM chloride, pH 8.1; T = 20°. Charge pulses of 1-μs duration were applied to the cell (injected charge 14.8, 12.8, 11.4, and 10.45 nAs; initial voltage 34.3, 32.8, 28.7, and 25.2 mV, respectively). The voltage decay across the cell membranes was recorded using different intervals between the 4,096 data points. Surface area A = 0.746 cm^2; volume V = 42.5 mm^3. The turgor pressure was approximately constant during the time course of the experiment (see Table 1).

RESULTS

Replacement of external chloride by 2(*N*-morpholino)-ethanesulfonate (MES^-)

The replacement of chloride by MES^- had a strong influence on the decay of membrane voltage after a charge pulse. Fig. 2 shows experiments taken from the same *V. utricularis* cell in different chloride concentrations (balanced always to 599 mM by the addition of MES^-). Curve 1 corresponds to the voltage decay in ASW (599 mM chloride). Curves 2, 3, and 4 were obtained in 299, 54, and 0 mM chloride, respectively.

Fig. 3 *A* shows the semilogarithmic plot of voltage vs. time of curve 1 given in Fig. 2. The initial voltage V_0 decays with two clearly distinguishable exponential relaxations. The time constants of the fast and the slow voltage relaxations were 56 μs and 1.27 ms, respectively. The relative amplitude of the fast (first) relaxation process was approximately 10 times larger than that of the slow (second) process. These results were in agreement with data obtained previously from similar experiments on cells of this species (Zimmermann et al., 1982; Benz and Zimmermann, 1983; Büchner et al., 1985, 1987; Walter et al., 1988) and of *H. parvula* (Benz et al., 1988). The replacement of increasing concentrations of the external chloride by the organic anion MES^- which is commonly used as a buffer (pK_a 6.1) had a major influence on the relaxation parameters, especially on the time constant of the first (fast) relaxation. Fig. 3 *B* shows the semilogarithmic plot of curve 4 of Fig. 2, taken after complete replacement of external chloride by MES^-. The fast (first) process now had a time constant of 1.04 ms, whereas that of the slow (second) process was approximately constant with 1.75 ms. Similarly, the relative amplitudes changed and were now both close to 0.5. It has to be noted, however, that only the change of the time constant of the fast process was typical for the complete replacement of chloride by MES^-. In experiments with other cells we found only small changes of the amplitudes (see Table 2 for a comparison of a_2 at small chloride concentration).

This result indicates that chloride has a major influence on the electric properties of the cell membrane of *V. utricularis*. We would like to note that the change of the electrical properties was not caused by poisoning of the cell because the turgor pressure was more or less constant during the duration of the experiment (~3 h). Furthermore, the initial relaxation parameters were approximately reestablished after the chloride-containing artificial sea water had been reinstated for 30 min.

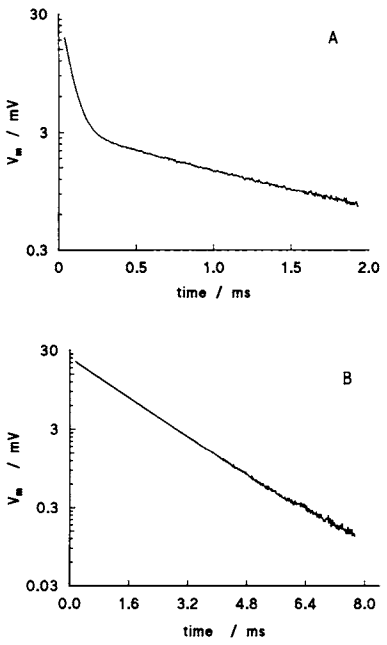

FIGURE 3 (*A*) Semilogarithmic plot of the voltage vs. time of curve 1 in Fig. 2 (599 mM chloride). The voltage decay across the cell membranes was fitted to the sum of two different voltage relaxations with the following relaxation parameters: V_1 = 31.3 mV, V_2 = 3.0 mV; τ_1 = 56 μs, τ_2 = 1.27 ms. The parameters of the first relaxation were obtained from the least-squares-fit to the first 1,000 points (r = 0.9995) and the parameters of the second relaxation were obtained from the last 2,000 points (r = −0.9950). The rate constants calculated according to Eqs. 15–17 were: K_{AS} = 744 s^{-1}; K_S = 410 s^{-1}; N_0 = 6.34 pmol/cm^2; C_m = 0.56 μF/cm^2. N_0 was calculated by assuming R_m = 7,500 Ωcm². (*B*) Semilogarithmic plot of the voltage vs. time of curve 4 in Fig. 2 (0 mM chloride and 599 mM MES^-) cell, ~7 min after change of the external chloride concentration from 54 mM to zero (corresponding to a change of MES^- concentration from 545 to 599 mM); T = 20°. Again the voltage decay across the cell membranes was fitted to two different voltage relaxations with the following relaxation parameters: V_1 = 13.6 mV, V_2 = 11.6 mV; τ_1 = 1042 μs, τ_2 = 1.75 ms. The parameters of the first relaxation were obtained from the least-squares-fit to the first 2,000 points (r = −0.9988) and the parameters of the second relaxation were obtained from the last 200 points (r = −0.8422). The rate constants were now: K_{AS} = 44 1/s; K_S = 336 1/s; N_0 = 4 pmol/cm^2.

Analysis of the experimental results

The analysis of the relaxation parameters of Figs. 2 and 3 with the earlier "mobile charge concept" (Zimmermann et al., 1982; Benz and Zimmermann, 1983) did not lead to satisfactory results because the rate constant of translocation, k, was dependent on the external chloride concentration. This is because the simple two-state model (Zimmermann et al., 1982) neglects the other reactions involved in carrier-mediated anion transport. The introduction of additional reaction steps into the model and the use of other simplifications (see The Kinetic Model For Chloride Transport section) allowed an appropriate description of the experimental data.

Nevertheless, we encountered several problems during the analysis of the experimental data. First, we neglected ion transport either by systems other than that for chloride or by unspecific leaks (i.e., $2BN_0K_{AS} \gg 1/[R_mC_m]$; see Theory). As a consequence the total apparent number of carriers in the membrane tended to increase as the external chloride concentration decreased. This was corrected by assuming that other transport systems contributed to the membrane resistance and Eqs. 15–17 were used to fit the data of Figs. 4–6 with R_m = 6,400 Ωcm². This made N_0 independent of chloride concentration, which seems reasonable because it is not expected that algal cells can incorporate or remove carriers during a 1-h experiment.

The second problem was the dependence of K_S on external chloride. Here we observed a small decrease of K_S with decreasing external chloride, although K_S should either be constant or increase with decreasing chloride concentrations depending on the magnitude of the stability constant, K, for chloride binding (see Theory). This discrepancy could be due to structural changes in the membrane caused by the low chloride concentration or to binding of MES^- to the carrier. For many cells, a reasonable fit of the data for K_S as a function of external chloride was only obtained by assuming that the stability constant, K_a, for the binding of MES^- to the carrier cannot be neglected. Table 1 presents the relaxation parameters derived from charge pulse experiments with the same algal cell as given in Figs. 2 and 3 at a variety of different chloride concentrations. As shown in Figs. 2 and 3, the relaxation parameters showed a considerable dependence on the chloride concentration. Table 1 also

TABLE 1 Results of charge-pulse experiments on *V. utricularis* cell w2930 measured as a function of external chloride solution

c	P	V_m	a_1	τ_1	τ_2	K_{AS}	K_S	N_0
M	*MPa*	*mV*		*μs*	*ms*	*1/s*	*1/s*	*pmol/cm²*
0.599	0.36	3	0.911	56	1.27	744	410	6.34
0.450	0.35	7	0.917	90	1.38	396	393	6.99
0.299	0.28	12	0.920	148	1.47	223	351	7.83
0.150	0.28	8	0.919	103	1.07	329	509	7.96
0.054	0.30	44	0.858	350	1.01	88	432	10.40
0	0.35	63	0.542	1036	1.75	44	336	4.00
0.599	0.36	18	0.914	62	0.92	657	610	6.69

The experiments were performed in ASW; pH 8.1; T = 20°C. The total anion concentration was held at 599 mM by addition of MES^-. The measurements were taken 7 min after change of the external solution. The analysis of the experimental data was performed using Eqs. 8–10 and 15–17 by assuming R_m = 7,500 Ωcm² and C_m = 0.56 μF/cm². P is the turgor pressure and V_m is the membrane potential.

presents the data for K_{AS}, K_S, and N_0, as calculated from the relaxation data by using Eqs. 15–17 and assuming R_m = 7,500 Ωcm². When external chloride was completely replaced by MES^-, K_{AS} decreased by a factor of more than 15, whereas K_S decreased only a little suggesting that MES^- also binds to the carrier (but is not transported in contrast to other anions).

The formalism described in Theory for the analysis of the charge-pulse experiments yields results for N_0, K_{AS}, and K_S. Table 2 shows the results of charge-pulse experiments taken from five different algal cells, each measured at two different external chloride concentrations (599 and 150 mM or 599 and 54 mM, balanced by MES^-). The turgor pressure of the cells did not vary significantly for the duration of the experiments. As can be seen from Table 2, the kinetic parameters K_{AS} and K_S varied considerably from cell to cell. Only the total surface concentration of carrier sites was fairly constant. The MES^--induced changes were reversible: the original relaxation parameters and rate constants were almost quantitatively reestablished after artificial sea water had been reinstated.

TABLE 2 Results of charge-pulse experiments on five different *V. utricularis* cells measured at two different external chloride concentrations

c	P	V_m	a_1	τ_1	τ_2	K_{AS}	K_S	N_0
M	*MPa*	*mV*		*μs*	*ms*	*1/s*	*1/s*	*pmol/cm²*
alga w14								
0.599	0.27	4	0.855	67	5.61	1095	70	4.83
0.054	0.28	39	0.901	448	7.24	126	36	5.36
0.599	0.22	13	0.863	61	8.85	1135	39	5.14
alga w1617								
0.599	0.19	2	0.895	62	4.29	824	124	4.84
0.054	0.23	4	0.913	218	2.58	171	206	6.71
0.599	0.30	0	0.891	62	5.20	799	118	5.13
alga w21								
0.599	0.24	0	0.840	43	0.88	1725	615	3.65
0.150	0.25	19	0.837	121	0.99	555	540	3.81
0.599	0.23	0	0.795	51	0.76	1790	742	2.80
alga w2526								
0.599	0.31	3	0.871	94	0.97	575	565	5.75
0.150	0.36	24	0.908	506	2.01	60	258	9.29
0.599	0.34	12	0.891	100	1.45	483	368	6.49
alga w2930								
0.599	0.36	3	0.911	56	1.30	744	410	6.34
0.054	0.30	44	0.858	350	1.01	71	433	10.40
0.599	0.33	18	0.914	64	0.94	647	597	6.69

The experiments were performed in ASW; pH 8.1; T = 20°C. The total anion concentration was held at 599 mM by addition of MES^-. The measurements were part of series of experiments similar to that shown in Table 1. The analysis of the experimental data was performed using Eqs. 8–10 and 15–17 and by using the values for R_m and C_m given in Table 3.
P is the turgor pressure and V_m is the membrane potential.

Fig. 4 shows the dependence of the normalized total surface concentration of carrier sites as a function of external chloride concentration (i.e., N_0/N_0^*, where N_0^* is the surface concentration in ASW). The open squares represent the results when N_0 is not corrected for R_m (Eqs. 11–13). The open circles show the corrected data. The correction seems essential only at external chloride concentrations below 0.1 M. As pointed out in the description of the model, k_{AS} and k_S can be evaluated by plotting K_{AS} and K_S as a function of the external chloride concentration and assuming a value for $K = k_R/k_D$ that gives the best fit to the data. Because we observed a considerable variation of K_{AS} and K_S from cell to cell this procedure can only be performed for single cells or for data normalized to the value at high chloride concentration (599 mM). Fig. 5 shows the dependence of the ratio K_{AS}/K_{AS}^*(K_{AS}^* is the value in ASW) for the eight cells represented in Table 3. A least squares fit of the data in Fig. 5 suggests that the stability constant, K, is ~0.34 L/mol and that for MES^-, K_a, was ~1.6 L/mol. Similar values for K and K_a also give a reasonable fit for K_S/K_S^* (K_S^* is the value in ASW) as a function of external chloride (see Fig. 6). Figs. 4–6 demonstrate that, at low external chloride concentration, a considerable variation of all data is observed. This may be due to the fact that the chloride concentrations on the two sides of the membrane (probably the plasmalemma, see Discussion) are not known exactly or that the assumption of identical solutions of chloride on the two sides of the membrane is not correct. Another problem is that the membrane(s) of *V. utricularis* probably contains more than one transport

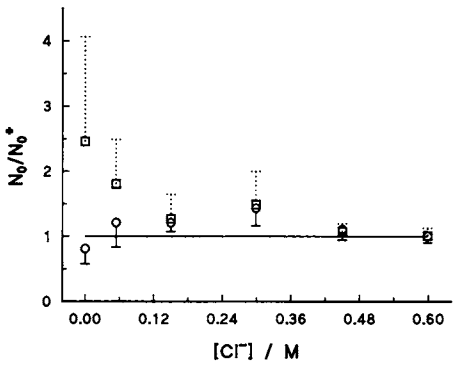

FIGURE 4 Plot of N_0/N_0^* as a function of the external chloride concentration. N_0^* is the total surface concentration of carriers in 599 mM chloride. The squares represent the data obtained from Eqs. 8–13 (not corrected for the passive membrane resistance R_m). The open circles represent the data for N_0 obtained by assuming a fixed value for R_m (Eqs. 15–17). The data points represent means of the eight cells (±SD) shown in Table 3.

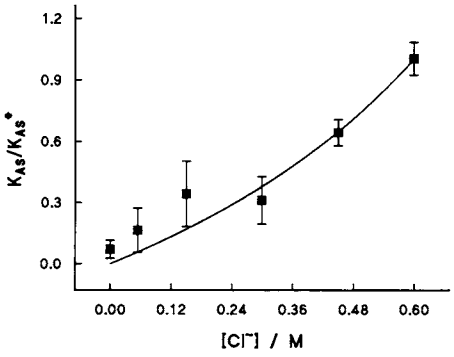

FIGURE 5 Plot of K_{AS}/K_{AS}^* as a function of the external chloride concentration. K_{AS}^* is the translocation rate constant K_{AS} in 599 mM chloride. The solid line was drawn according to Eq. 18 by assuming K = 0.34 1/M and K_a for MES^- = 1.6 1/M. The data points represent means of the eight cells (±SD) shown in Table 3.

system. Other systems could make a significant contribution to the voltage relaxations at low external chloride concentration when the amplitude of the chloride-carrier relaxation is reduced.

Table 3 shows the parameters of carrier-mediated chloride transport of eight different *V. utricularis* cells together with the electrical capacity, C_m, of the cells. It is interesting to note that for all cells there exists reasonable agreement between the specific resistance calculated from the kinetic data in Table 3 (given by $[a_1\tau_1 + a_2\tau_2]/C_m$) and the specific membrane conductance, R_m^* derived from current clamp experiments. Whereas the whole set of data was consistent for one single cell, we observed considerable variations of the specific resistance from cell to cell. These were also obtained for certain parameters of carrier-mediated chloride transport. The translocation rate constant k_S varied ~10-fold from 80 1/s to 920 1/s, whereas k_{AS} and N_0 varied only ~two- to threefold. As pointed out earlier (Zimmermann et al. 1982; Benz and Zimmermann, 1983), these variations reflect probably the different physiological growth states of the algal cells. Table 3 also shows the stability constants for the binding of chloride and MES^- to the binding sites. Again we obtained some variations and both K and K_a for MES^- varied about threefold. We have also replaced chloride by bromide and nitrate. The results of these preliminary experiments were basically the same but suggest that these anions bind more strongly than does MES^- to the carrier (Wang, J., R. Benz, and U. Zimmermann, unpublished results).

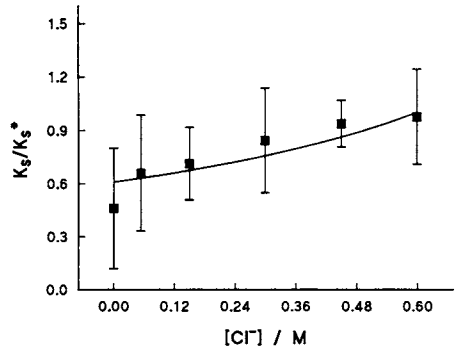

FIGURE 6 Plot of K_S/K_S^* as a function of the external chloride concentration. K_S^* is the translocation rate constant K_S in 599 mM chloride. The solid line was drawn according to Eq. 19 by assuming K = 0.34 1/M and K_a for MES^- = 1.6 1/M. The data points represent means of the eight cells (±SD) shown in Table 3.

TABLE 3 **Kinetic parameters of carrier-mediated chloride transport across the cell membranes of eight different *V. utricularis* cells**

Cell	C_m	R_m	N_0	k_{AS}	k_S	K	K_a
	$\mu F/cm^2$	Ωcm^2	$pmol/cm^2$	$1/(Ms)$	$1/s$	$1/M$	$1/M$
w13	0.62	3500	5.7	5200	630	0.29	2.4
w14	0.82	2600	5.3	5600	80	0.40	0.8
w1617	0.53	18000	5.5	5500	150	0.28	0.6
w21	0.63	2500	3.7	6100	920	0.65	1.4
w2526	0.69	4600	7.5	4400	550	0.22	2.5
w2627	0.53	7000	4.4	4900	650	0.30	2.5
w2930	0.56	7500	7.2	4500	600	0.26	0.8
w4344	0.55	5500	5.4	2500	280	0.30	2.0
mean	0.62	6400	5.6	4800	480	0.34	1.6
±SD	0.10	5000	1.3	1100	290	0.14	0.8

The data were taken in part from the experimental data given in Tables 1 and 3 by using the specific membrane capacity of each individual cell. N_0, k_{AS}, k_S, and the stability constants K and K_a for the binding of chloride and MES^- to the carrier, respectively, were derived as described in the text. T = 20°. R_m is the specific resistance of the cells derived by assuming that N_0 is independent on external chloride concentration. The last line contains mean values ± SD.

Time dependence of the voltage relaxation after the complete replacement of chloride

When the external chloride concentration was stepwise reduced (599, 450, 299, 150, and 54 mM), the voltage relaxation always reached its final form a few minutes after each reduction. We determined how much time has to pass until the voltage relaxation reaches its final form when all external chloride is replaced at once. The external chloride was replaced in one step and we measured the relaxation parameters, the cell turgor, and the membrane voltage as a function of time. Fig. 7 shows the time course of the relaxation amplitude a_1 (a_2 = $1 - a_1$) and the time constants after replacement of

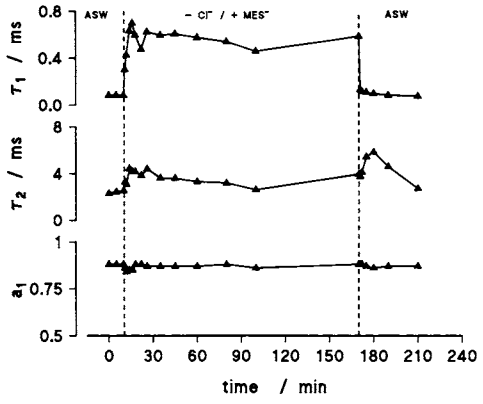

FIGURE 7 Effect of replacement of external chloride in the ASW by 599 mM MES^- on the relaxation parameters of the two voltage relaxations after a charge pulse of 1-μs duration. Cell w5859; T = 20°C.

external chloride. The values changed within 5 min and then stayed virtually constant for at least 160 min, except for some oscillations within the first 10 min. These oscillations may indicate some excitability of the cell membrane. When the chloride-containing medium was reinstated, approximately the same time was required for complete recovery of the initial relaxation parameters. Fig. 7 shows that the relaxation amplitudes are only slightly affected by the replacement of chloride, in contrast to the result of Fig. 3. This may be due to different concentrations of chloride remaining in the cytoplasm after replacement of external chloride (see Discussion). During the time course of Fig. 7 we also measured the cell turgor. This parameter showed only small variations during the experiments described in Fig. 7 (see Fig. 8).

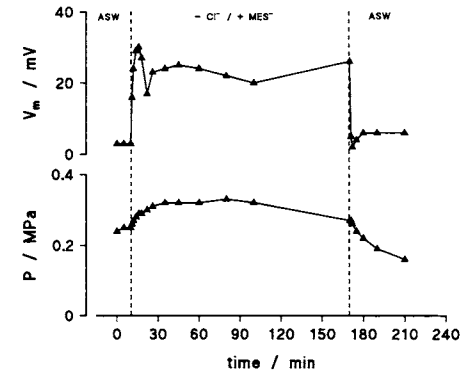

FIGURE 8 Effect of replacement of external chloride in the ASW by 599 mM MES^- on the potential of the cell (*upper trace*) and the cell turgor (*lower trace*). Same cell and same conditions as in Fig. 7.

Effect of external chloride on membrane potential

The electrical properties of *V. utricularis* are obviously limited by an electrogenic carrier system for chloride. If this is true, the membrane potential should also change when external chloride is replaced by MES^-. In general, the cells of *Valonia* have a membrane potential of only a few millivolts, in agreement with the published literature (Lainson and Field, 1976; Davis, 1981). The replacement of Cl^- by MES^- made the cell interior positive with a maximal value of ~30 mV (compare also Table 2) when the external solution did not contain any chloride (see Fig. 8). Besides an interesting oscillation (similar to that found for τ_1; see Fig. 7), the membrane potential reached a stable new value within 5 min after partial or complete replacement of chloride. This also occurred when the ASW, with its high chloride concentration, is reinstated. In general, the time course of the membrane potential is parallel to the changes of the relaxation parameters described above. Replacement of chloride by bromide or nitrate give comparatively small effects which do not exceed a few millivolts (positive or negative polarity). The relatively small effect of these anions on the membrane potential may be due to competition for the same transport system.

The chloride concentration in the vacuole is not affected by external MES^-

The results of relaxation measurements and the rapid change of the membrane potential after the replacement of external chloride suggest that the effects observed here may have their origin in the cytoplasmic membrane alone. The vacuole is a very large compartment of the cell (98% of the cell volume) with a chloride concentration of ~550 mM. Even very potent chloride transport systems would need a considerable time for the removal of all chloride from the vacuole. Furthermore, this would only be possible if corresponding amounts of cations could also leave the vacuole. To check this we measured the vacuolar chloride concentration in a large number of *Valonia* cells before and at various times after replacement of external chloride by MES^-. The results are summarized in Fig. 9. Replacement of external chloride for 1 h has virtually no effect on the vacuolar concentration. This result is consistent with the assumption that the plasmalemma contains a carrier system for chloride, which allows the rapid exchange of chloride between the cytoplasm and the external solution. The tonoplast, on the other hand, seems to be rather impermeable for chloride and/or for the corresponding counterion (potassium).

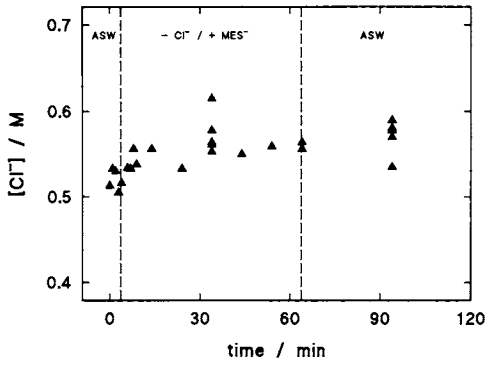

FIGURE 9 Effect of replacement of external chloride in the ASW by 599 mM MES^- on the vacuolar chloride concentration in the vacuoles. Each individual data point reflects the results taken from one cell.

DISCUSSION

In this study we have presented evidence that the system associated with the transport of mobile charges in the cell membrane of *V. utricularis* is a chloride carrier. The carrier model, its simplifications and the implications of the system for the physiology of the cell are discussed here. In previous publications (Zimmermann et al., 1982; Benz and Zimmermann, 1983) we have calculated from the relaxation data the translocation rate constant, k, and the total surface concentration of mobile charges, N_t. In the present theoretical treatment k is given by the sum of K_{AS} and K_S and N_t by $N_0(K_{AS}/[K_{AS} + K_S])^2$, which means that N_t is approximately half of N_0 at 599 mM chloride.

Which is limiting: k_S or the heterogeneous reaction rate?

The model shown in Fig. 1 represents the simplest possible mechanism by which chloride could be transported by a carrier system. Here and in previous publications (Zimmermann et al., 1982; Benz and Zimmermann, 1983; Büchner et al., 1985) only two relaxations are resolved under charge pulse conditions. This means that one reaction involved in carrier-mediated ion transport is always in equilibrium (Benz and Läuger, 1976; Benz and McLaughlin, 1983). We assume that this is the heterogeneous surface reaction, i.e., k_Rc, $k_D \gg k_{AS}$, k_S. We have shown that this assumption is consistent with the experimental data.

However, the case $k_S \gg k_{AS}$, k_Rc, k_D would also produce two voltage relaxations in our approach (or one current relaxation under voltage-clamp conditions; see Hladky, 1974, and Benz and Läuger, 1976). Therefore, we have solved Eqs. A1–A4 and Eq. 3 for the case $k_S \gg k_{AS}$, k_Rc, k_D, and calculated the rate constants k_{AS} and k_D and the surface concentration of complexed carriers, N_{AS}, from the parameters for the voltage relaxations. k_{AS} is a function of external chloride concentration (Fig. 10). It is impossible to explain the concentration dependence of the experimental data in the case $k_S \gg k_{AS}$, k_Rc, k_D. Thus it is very unlikely that the heterogeneous surface reaction, rather than the movement of the free carrier that is the rate-limiting step.

How fast is the heterogeneous surface reaction?

The experimental data can be explained when using the simplification k_Rc, $k_D \gg k_{AS}$, k_S. This allows the evaluation of a lower limit for the rate constants k_Rc and k_D by assuming a value for k_R (k_D is given by k_R/K) and by calculating the parameters of the three voltage relaxations from our model. We find that $k_R > 10^4$ L/(mol · s) (corresponding to $k_D > 3 \times 10^4$ 1/s). Smaller association rate constants lead to an amplitude for the third voltage relaxation, which is large enough to be resolved in our experiments. These rate constants of the heterogeneous surface reaction are not unreasonable because k_R and k_D for valinomycin-mediated cation transport are on the order of 10^5 L/(mol · s) and 10^5 1/s, respectively (Benz and Läuger, 1976; Benz et al., 1989). Similar values are found for the reaction rates of the heterogeneous reaction of the chloride pump of *Acetabularia* (Tittor et al., 1983). The fast electroneutral exchange of chloride and bicarbonate across the red cell membrane (turnover number > 10^4 1/s) probably has on and off rates which are of the same order of magnitude (Fröhlich, 1988). The same is true for the binding reaction between anion

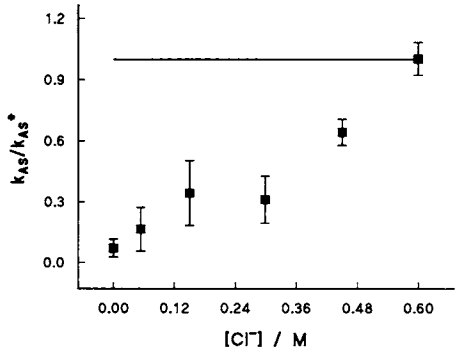

FIGURE 10 k_{AS}/k^*_{AS} as a function of the external chloride concentration. k^*_{AS} is the translocation rate constant of the carrier at 599 mM chloride. The data points were calculated from the relaxation parameters of the eight cells given in Table 3 by assuming $k_S \gg k_Rc$, k_D, k_{AS}. The solid line shows that k_{AS} should not be dependent on external chloride concentration.

and binding site in anion-specific channels (Benz and Hancock, 1987).

Is the transport system a chloride pump?

Gradmann and co-workers (Gradmann, 1975; Mummert et al., 1981; Tittor et al., 1983; Gerencser et al., 1988) have characterized a chloride pump in *Acetabularia mediterranea*. According to their results this algal cell contains a very active chloride pump as judged from potential measurements in the absence of external chloride or in the presence of metabolic inhibitors. As *Acetabularia* and *Valonia* are related species, it is of interest to know whether the carrier system investigated here is also an active chloride pump. The metabolic inhibitor carbonyl cyanide 3-chlorophenylhydrazone (CCCP) has no effect on the membrane potential (unpublished results). Furthermore, the removal of external chloride leads to only a small increase of the membrane potential. The chloride concentration inside the vacuole has more or less the same concentration as the external solution and there is no indication that the chloride concentration in the cytoplasm is larger than that of the vacuole or that of the external solution. Our relaxation data at low external chloride suggested that the cytoplasm was depleted of this ion and only contained a concentration between 20 and 50 mM presumably because chloride loss from the vacuole was very slow (see below). Otherwise we could not explain the charge pulse data. Thus, it is very difficult to believe that the plasmalemma of *V. utricularis* contains a chloride pump similar to that postulated for *Acetabularia* (Gradmann, 1989) or *H. parvula* (Graves and Gutknecht, 1977*a*, *b*).

One or two membranes?

In our experimental approach, both electrodes were inserted deeply into the cell. This means that their tips were located in the vacuole because this compartment comprises ~98% of the total cell volume. As a consequence the voltage relaxation investigated here as a function of external chloride should in principle be limited by the electrical properties of both membranes (plasmalemma and the tonoplast). According to the literature, it is an open question whether the specific resistance of the tonoplast of giant algal cells is high (Davis, 1981) or extremely low (Lainson and Field, 1976). Our own experimental data have always been consistent with the assumption that the experimental results reflect only one membrane (Zimmermann et al., 1982; Benz and Zimmermann, 1983; Büchner et al., 1985; Benz et al., 1988), although we cannot exclude the possibility of two identical membranes. The possibility that one or both electrodes were surrounded by the tonoplast could be excluded on the basis of the extremely good time resolution of our measurements (better than 10 μs; Benz and Zimmermann, 1983). The clogging of one or both electrodes by a membrane would lead to cable problems which would result in a resolution >100 μs, as has been found in charge pulse experiments with *Eremosphera viridis* (Wehner et al., 1990).

Electrical measurements on the membranes of algal cells usually have the problem that tonoplast and plasmalemma cannot be separated in a simple way (Findlay and Hope, 1976; Bates et al., 1982). To explain the charge-pulse experiments in terms of the chloride transport system, it is sufficient to assume that only one membrane (probably the plasmalemma) with a specific (geometrical) capacitance of ~ 0.6 μF/cm^2 and a chloride-independent specific resistance of $\sim 6{,}500$ Ωcm^2 contains the carrier system, and that the other membrane (the tonoplast) has a small specific resistance of <10 Ωcm^2. This is the case, either because the specific resistance of the tonoplast membrane is normally very small or because its integrity was destroyed by the insertion of the microelectrodes (Bates et al., 1982). Our instrumentation has a time resolution of a few microseconds, which means that the RC time constant of this membrane must be considerably below 10 μs, otherwise we should have been able to detect it. Such a small RC time constant is consistent with the results of Lainson and Fields (1976), but contradicts those of Davis (1981). Why the two voltage relaxation processes do not reflect the discharge of two membranes has been discussed in full detail in previous publications (Zimmermann et al., 1982; Benz and Zimmermann, 1983; Benz et al., 1988).

Is the tonoplast membrane highly conductive?

The analysis of our experimental data suggests that (*a*) after replacement of the external chloride, the chloride concentration in the cytoplasm was on the order of 20–50 mM, (*b*) the tonoplast has a small specific resistance, and (*c*) the chloride concentration in the vacuole is constant on the time scale of our experiments. The last two items seem to contradict one another and the published literature. Chloride should leak rapidly through a highly conductive membrane. However, this will only be possible if the tonoplast is also highly permeable to the positively charged counterion, potassium. Otherwise, a diffusion potential will hinder the chloride transport out of the vacuole. We observed an increase of

the membrane potential of ~30 mV after replacement of external chloride. We do not know whether this voltage corresponds to the potential across the tonoplast membrane, but such a potential would be sufficient to explain why the chloride concentration inside the vacuole is independent of the external chloride concentration.

A highly conductive tonoplast membrane of *Valonia* disagrees with Davis (1981). Furthermore, isolated vacuoles of various plant cells have a membrane potential and show specific uptake of substrates such as malate or potassium ions when they are energized (Hedrich et al., 1986). Secondly, the specific resistance of isolated vacuoles has recently been measured and a value of $\sim 10^4$ Ωcm^2 (similar to the plasmalemma) has been obtained (Bentrup et al., 1986). On the other hand, the vacuole of a marine alga may have a completely different function than that of higher plants, and this function may require a high conductivity for one type of ion but a small salt permeability.

Comparison with other transport systems

Our results indicate that the chloride transporting system of *V. utricularis* and other transport systems in plant cells show completely different features from those of animal cells. Electrogenic transport systems of animal cells do not contribute to the gating charge (Armstrong and Bezanilla, 1973; Almers, 1978), which means that for these transport systems the voltage-dependent step is rate limiting. Such systems do not contribute to the apparent specific capacity of the cell membranes, because a change in membrane potential causes little change in the asymmetry of the concentration of the carrier-ion complex (AS^-), as the other reactions (the two surface reactions and the free-carrier transport) are much faster than transport of AS^-. This means that apparent and geometrical capacitances are very similar (R. Benz, unpublished data).

The situation in plant cells is completely different. Here, the voltage-independent steps appear to be rate limiting (Felle and Bentrup, 1977; Felle, 1980; Gradmann, 1975, 1978; Benz and Zimmermann, 1983; Tittor et al., 1983). This means that the charge associated with the transport systems increases the apparent capacitance above that of the geometrical capacitance of the membranes (Gradmann, 1975, 1978; Benz and Zimmermann, 1983; Tittor et al., 1983). This may explain the small velocity of the action potential of plant cells compared with that of animal cells.

APPENDIX 1

Denoting the interfacial concentrations of the free and complexed carriers on the left side of the membrane by N'_S and N'_{AS} and the concentrations on the right side by N''_S and N''_{AS} (expressed in moles per square centimeters), then the change of these quantities with time is given by the following four differential equations:

$$\frac{dN'_{AS}}{dt} = (-k'_{AS}N'_{AS} + k''_{AS}N''_{AS}) + (-k_D N'_{AS} + k_R c N'_S) \quad \text{(A1)}$$

$$\frac{dN''_{AS}}{dt} = (k'_{AS}N'_{AS} - k''_{AS}N''_{AS}) + (-k_D N''_{AS} + k_R c N''_S) \quad \text{(A2)}$$

$$\frac{dN'_S}{dt} = (-k_S N'_S + k_S N''_S) + (k_D N'_{AS} - k_R c N'_S) \quad \text{(A3)}$$

$$\frac{dN''_S}{dt} = (k_S N'_S - k_S N''_S) + (k_D N''_{AS} - k_R c N''_S). \quad \text{(A4)}$$

It is assumed that the total surface concentration of carriers, N_0, either complexed or uncomplexed, is constant during an experiment:

$$N_0 = N'_{AS} + N''_{AS} + N'_S + N''_S = \text{const.} \quad \text{(A5)}$$

The solution of the five differential equations (Eqs. A1–A4 and Eq. 3) may be obtained by introducing new variables (see Benz and Läuger, 1976):

$$r = N'_S + N''_S \quad \text{and} \quad s = N'_{AS} + N''_{AS}, \quad \text{and}$$

$$x = N'_S - N''_S \quad \text{and} \quad y = N'_{AS} - N''_{AS},$$

where r and s are independent of time. The solution of the differential equations is given by Eq. 4 (Benz and Läuger, 1976). The parameters a_i and τ_i ($i = 1, 2, 3$) are known functions of the four rate constants, N_0, R_m, and C_m.

If the rate constants of association and dissociation of the carrier-anion complex are much larger than the translocation rate constants (i.e., k_D, $k_R c \gg k'_{AS}$, k''_{AS}, k_S), then the following relations hold during the whole relaxation process

$$N'_{AS}/N'_S = N''_{AS}/N''_S = k_R c/k_D \quad \text{(A6)}$$

and

$$N_{AS} = N'_{AS} + N''_{AS} = \frac{Kc}{1 + Kc} N_0 \quad \text{(A7)}$$

$$N_S = N'_S + N''_S = \frac{1}{1 + Kc} N_0. \quad \text{(A8)}$$

We assume that the valency of the carrier-anion complex is (a) introducing the new variable

$$v = N'_{AS}/N_{AS} \quad \text{(A9)}$$

and using $V_m < 25$ mV (corresponding to $u = V_m F/[RT] < 1$) as proposed by Benz and Läuger (1976) and Benz and McLaughlin (1983) (see also Eqs. 1 and 2). The differential equations (Eqs. 3 and Eqs. A1–A4) reduce to a system of two differential equations:

$$\frac{dv}{dt} = -2(K_S + K_{AS})v - (zK_{AS}/2)u + (K_S + K_{AS}) \quad \text{(A10)}$$

$$\frac{du}{dt} = -\frac{8BN_0K_{AS}}{z}v - \left(\frac{1}{R_mC_m} + 2BN_0K_{AS}\right)u + \frac{4BN_0K_{AS}}{z} \quad \text{(A11)}$$

with:

$$K_S = \frac{1}{1 + Kc}k_S \quad \text{(A12)}$$

$$K_{AS} = \frac{Kc}{1 + Kc}k_{AS}. \quad \text{(A13)}$$

The solution of Eqs. A10 and A11 has the form:

$$V_m(t) = V_0[a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2)] \quad \text{(A14)}$$

with:

$$a_1 + a_2 = 1. \quad \text{(A15)}$$

The time constants $\tau_1 = 1/L_1$ and $\tau_2 = 1/L_2$ are given by the roots of the characteristic equation:

$$L^2 - P_1L + P_2 = 0 \quad \text{(A16)}$$

with:

$$P_1 = L_1 + L_2 = 2(K_S + K_{AS}) + 2BN_0K_{AS} + 1/(R_mC_m) \quad \text{(A17)}$$

$$P_2 = L_1L_2 = 2(K_S + K_{AS})[2BN_0K_{AS} + 1/(R_mC_m)] - 4BN_0K_{AS}^2. \quad \text{(A18)}$$

The functions $v(t)$ and $u(t)$ have to fulfill the boundary conditions:

$$v(0) = v(\infty) = \tfrac{1}{2} \quad \text{(A19)}$$

$$u(0) = V_0F/(RT), u(\infty) = 0 \quad \text{(A20)}$$

which is equivalent to:

$$P_3 = a_1L_1 + a_2L_2 = 2BN_0K_{AS} + 1/(R_mC_m). \quad \text{(A21)}$$

Eqs. 11–13 and 15–17 were derived from Eqs. A17, A18, and A21.

The case $k_S \gg k_D, k_RC, k'_{AS}, k''_{AS}$

For a large translocation rate constant of the free carrier the surface concentrations of the free carrier on both sides of the membrane are always identical:

$$N'_S = N''_S. \quad \text{(A22)}$$

Then the system of differential equations has the form:

$$\frac{dv}{dt} = -(2k_{AS} + k_D)v - (zk_{AS}/2)u + (k_{AS} + k_D/2) \quad \text{(A23)}$$

$$\frac{du}{dt} = -\frac{8BN_{AS}k_{AS}}{z}v - \left(\frac{1}{R_mC_m} + 2BN_{AS}k_{AS}\right)u + \frac{4BN_{AS}k_{AS}}{z}. \quad \text{(A24)}$$

The solution of Eqs. A23 and A24 has the same form as the solutions of the Eqs. A10 and A11. The time constants $\tau_1 = 1/L_1$ and $\tau_2 = 1/L_2$ are given by the roots of the characteristic equation:

$$L^2 - P_1L + P_2 = 0 \quad \text{(A25)}$$

with:

$$P_1 = L_1 + L_2 = 2k_{AS} + k_D + 2BN_{AS}k_{AS} + 1/(R_mC_m) \quad \text{(A26)}$$

$$P_2 = L_1L_2 = (2k_{AS} + k_D)(2BN_{AS}k_{AS} + 1/[R_mC_m]) - 4BN_{AS}(k_{AS})2. \quad \text{(A27)}$$

The functions $v(t)$ and $u(t)$ have to fulfill the same boundary conditions as given above (Eqs. A19 and A20), which is equivalent to:

$$P_3 = a_1L_1 + a_2L_2 = 2BN_{AS}k_{AS} + 1/(R_mC_m). \quad \text{(A28)}$$

For low passive resistance of the membrane (i.e., for $2BN_{AS}k_{AS} \gg 1/[R_mC_m]$), k_D, k_{AS}, and the surface concentration of complexed carriers, N_{AS}, are given by:

$$k_{AS} = (P_1 - P_3 - P_2/P_3)/2 \quad \text{(A29)}$$

$$k_D = P_2/P_3 \quad \text{(A30)}$$

$$N_{AS} = P_3/(2Bk_{AS}). \quad \text{(A31)}$$

If the specific membrane conductance, R_m, caused by ion transport other than chloride, cannot be neglected (especially in the case $c < 100$ mM) Eqs. A29–A31 have the form:

$$k_{AS} = [(P_1 - P_3)P_3 - P_2]/[2[P_3 - 1/(R_mC_m)]] \quad \text{(A32)}$$

$$k_D = (P_1 - P_3) - 2k_{AS} \quad \text{(A33)}$$

$$N_{AS} = [P_3 - 1/(R_mC_m)]^2/\{B[(P_1 - P_3)P_3 - P_2]\} \quad \text{(A34)}$$

APPENDIX 2

Derivation of Eqs. 18 and 19

Chloride was replaced in the experiments described in this study by MES^-. This and other anions may also bind to the carrier system and may also be transported. In the following we give a complete description of the carrier-mediated anion transport in the presence of two different anions, A and B, and under the assumption that the heterogeneous surface reactions are always in equilibrium (see Fig. 11). The symbols "A" and "B" denote the association rate constants, the dissociation rate constants, and the translocation rate constants with respect to the two different anions. Their binding constants to the carrier are given by $K_A = k_{RA}/k_{DA}$ and $K_B = k_{RB}/k_{DB}$, respectively. Denoting the surface concentrations of S, AS, and BS in the left interface by N'_S, N'_{AS}, and N'_{BS} and the concentrations in the right interface by N''_S, N''_{AS}, and N''_{BS}, respectively, then the rate of change of these quantities with time is given by:

$$\frac{dN'_{AS}}{dt} = (-k'_{AS}N'_{AS} + k''_{AS}N''_{AS}) + (-k_{DA}N'_{AS} + k_{RA}c_AN'_S) \quad \text{(B1)}$$

$$\frac{dN''_{AS}}{dt} = (k'_{AS}N'_{AS} - k''_{AS}N''_{AS}) + (-k_{DA}N''_{AS} + k_{RA}c_AN''_S) \quad \text{(B2)}$$

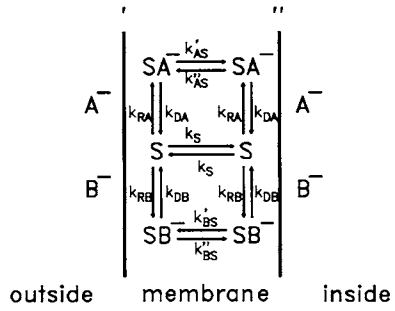

FIGURE 11 Kinetic scheme for carrier-mediated chloride transport valid under the condition that the replacement anion binds also to the carrier and is also transported.

$$\frac{dN'_S}{dt} = (-k_S N'_S + k_S N''_S) + (k_{DA} N'_{AS} - k_{RA} c_A N'_S) + (k_{DB} N'_{BS} - k_{RB} c_B N'_S) \quad \text{(B3)}$$

$$\frac{dN''_S}{dt} = (k_S N'_S - k_S N''_S) + (k_{DA} N''_{AS} - k_{RA} c_A N''_S) + (k_{DB} N''_{BS} - k_{RB} c_B N''_S) \quad \text{(B4)}$$

$$\frac{dN'_{BS}}{dt} = (-k'_{BS} N'_{BS} + k''_{BS} N''_{BS}) + (-k_{DB} N'_{BS} + k_{RB} c_B N'_S) \quad \text{(B5)}$$

$$\frac{dN''_{BS}}{dt} = (k'_{BS} N'_{BS} - k''_{BS} N''_{BS}) + (-k_{DB} N''_{BS} + k_{RB} c_B N''_S). \quad \text{(B6)}$$

The number of carrier molecules in the different states is independent of time which means:

$$N_0 = N'_{AS} + N''_{AS} + N'_{BS} + N''_{BS} + N'_S + N''_S = \text{const.} \quad \text{(B7)}$$

In the following we assume again that the interfacial reactions for the anions A and B are always in equilibrium:

$$k_{DA}, k_{RA} c_A \gg k'_{AS}, k''_{AS}, k_S \quad \text{(B8)}$$

$$k_{DB}, k_{RB} c_B \gg k'_{BS}, k''_{BS}, k_S. \quad \text{(B9)}$$

Under the conditions (Eqs. B8 and B9) the following equations hold (see Appendix 1):

$$\frac{N'_{AS}}{N'_S} = \frac{N''_{AS}}{N''_S} = \frac{k_{RA} c_A}{k_{DA}} \quad \text{(B10)}$$

$$\frac{N'_{BS}}{N'_S} = \frac{N''_{BS}}{N''_S} = \frac{k_{RB} c_B}{k_{DB}} \quad \text{(B11)}$$

The introduction of Eqs. B10 and B11 into the differential Eqs. B1–B6 reduces them to the following differential equation:

$$\frac{dN'_{AS}}{dt} = -(K_S + K'_{AS} + K'_{BS}) N'_{AS} + (K_S + K''_{AS} + K''_{BS}) N''_{AS} \quad \text{(B12)}$$

in which K_S, K'_{AS}, K''_{AS}, K'_{BS}, and K''_{BS} are given by:

$$K_S = \frac{1}{1 + K_A c_A + K_B c_B} k_S \quad \text{(B13)}$$

$$K'_{AS} = \frac{K_A c_A}{1 + K_A c_A + K_B c_B} k'_{AS} \quad \text{(B14)}$$

$$K''_{AS} = \frac{K_A c_A}{1 + K_A c_A + K_B c_B} k''_{AS} \quad \text{(B15)}$$

$$K'_{BS} = \frac{K_B c_B}{1 + K_A c_A + K_B c_B} k'_{BS} \quad \text{(B16)}$$

$$K''_{BS} = \frac{K_B c_B}{1 + K_A c_A + K_B c_B} k''_{BS}. \quad \text{(B17)}$$

The experimental results in the case of the replacement anion MES^- are consistent with $k'_{BS} = k''_{BS} = 0$, which means that this anion is not transported by the carrier system. The solution of the differential Eqs. 3 and B13 is identical to that derived in Appendix A with the use of Eqs. B13 and B18 instead of Eqs. A12 and A13

$$K_{AS} = \frac{K_A c_A}{1 + K_A c_A + K_B c_B} k_{AS}. \quad \text{(B18)}$$

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REFERENCES

Almers, W. 1978. Gating currents and charge movements in biological membranes. *Rev. Physiol. Biochem. Pharmacol.* 82:96–110.

Armstrong, C. M., and F. Bezanilla. 1973. Current related to the movement of the gating particles of the sodium channels. *Nature (Lond.).* 242:459–461.

Bates, G. W., M. H. M. Goldsmith, and T. H. Goldsmith. 1982. Separation of tonoplast and plasma membrane potential and resistance in cells of oat coleoptiles. *J. Membr. Biol.* 66:15–23.

Bentrup, F.-W., M. Gogarten-Boekels, B. Hoffmann, J. P. Gogarten, and C. Baumann. 1986. ATP-dependent acidification and tonoplast hyperpolarization in isolated vacuoles from green suspension cells of *Chenopodium rubrum* L. *Proc. Natl. Acad. Sci. USA.* 83:2431–2433.

Benz, R., and F. Conti. 1981. Structure of the squid axon membrane as derived from charge pulse relaxation studies in the presence of absorbed lipophilic ions. *J. Membr. Biol.* 59:91–104.

Benz, R., and R. E. W. Hancock. 1987. Mechanism of ion transport through the anion-selective channel of *Pseudomonas aeruginosa* outer membrane. *J. Gen. Physiol.* 89:275–295.

Benz, R., and P. Läuger. 1976. Kinetic analysis of carrier-mediated ion transport by the charge pulse technique. *J. Membr. Biol.* 27:171–191.

Benz, R., and S. McLaughlin. 1983. The molecular mechanism of action of the proton ionophore FCCP (carbonylcyanide *p*-trifluoromethoxyphenylhydrazone). *Biophys. J.* 41:381–398.

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

Benz, R., K.-H. Büchner, and U. Zimmermann. 1988. Mobile charges in the cell membranes of *Halicystis parvula. Planta.* 174:479–487.

Benz, R., H.-A. Kolb, P. Läuger, and G. Stark. 1989. Ion carriers in plantar bilayers: relaxation techniques and noise analysis. *Methods Enzymol.* 171:274–286.

Büchner, K.-H., K. Rosenheck, and U. Zimmermann. 1985. Characterization of mobile charges in the membrane of *Valonia utricularis. J. Membr. Biol.* 88:131–137.

Büchner, K.-H., L. Walter, and U. Zimmermann. 1987. Influence of anesthetics on the movement of the mobile charges in the algal cell membranes of *Valonia utricularis. Biochim. Biophys. Acta.* 903:241–247.

Davis, R. F. 1981. Electrical properties of the plasmalemma and tonoplast in *Valonia ventricosa. Plant Physiol. (Bethesda).* 67:825–831.

Felle, H. 1980. Amine transport at the plasma membrane of *Riccia fluitans. Biochim. Biophys. Acta.* 602:181–195.

Felle, H., and F. W. Bentrup. 1977. A study of the primary effect of the uncoupler CCCP on membrane potential and conductance in *Riccia fluitans. Biochim. Biophys. Acta.* 464:179–187.

Findlay, G. P., and A. B. Hope. 1976. Electrical properties of plant cells: methods and findings. *In* Encyclopedia of Plant Physiology, New Series. Transport in Plants II. U. Lüttge and M. G. Pitman, editors. Springer Verlag, New York. 2:53–92.

Fröhlich, O. 1988. The "tunneling" mode of biological carrier-mediated transport. *J. Membr. Biol.* 101:189–198.

Gerencser, G. A., J. F. White, D. Gradmann, and S. L. Bonting. 1988. Is there a Cl^- pump? *Am. J. Physiol.* 255:R677–R692.

Gradmann, D. 1975. Analog circuit of the *Acetabularia* membrane. *J. Membr. Biol.* 25:183–208.

Gradmann, D. 1978. Green light (550 nm) inhibits electrogenic Cl-pump in the *Acetabularia* membrane by permeability increase for the carrier ion. *J. Membr. Biol.* 44:1–24.

Gradmann, D. 1989. ATP-Driven chloride Pump in giant alga *Acetabularia. Methods Enzymol.* 174:490–504.

Graves, J. S., and J. Gutknecht. 1977*a*. Chloride transport and the membrane potential in the marine alga, *Halicystis parvula. J. Membr. Biol.* 36:65–81.

Graves, J. S., and J. Gutknecht. 1977*b*. Current-voltage relationships and voltage sensitivity of the Cl^- pump in *Halicystis parvula. J. Membr. Biol.* 36:83–91.

Hedrich, R., U. I. Flügge, and J. M. Fernandez. 1986. Patch-clamp studies of ion transport in isolated plant vacuoles. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 204:228–232.

Hladky, S. B. 1974. The energy barriers to ion transport by nonactin across the lipid bilayer membranes. *Biochim. Biophys. Acta.* 352:71–85.

Komor, E., and W. Tanner. 1976. The determination of the membrane potential of *Chlorella vulgaris.* Evidence for electrogenic sugar transport. *Eur. J. Biochem.* 70:197–204.

Lainson, R., and C. P. Field. 1976. Electrical properties of *Valonia ventricosa. J. Membr. Biol.* 29:81–94.

Läuger, P. 1972. Carrier-mediated ion transport. *Science (Wash. DC).* 178:24–30.

Mummert, H., U. P. Hansen, and D. Gradmann. 1981. Current voltage curve of electrogenic Cl^- pump predicts voltage-dependent Cl^- efflux in *Acetabularia. J. Membr. Biol.* 62:139–148.

Slayman, C. L., and C. W. Slayman. 1974. Depolarization of the plasma membrane of *Neurospora* during active transport of glucose: evidence for a proton dependent cotransport system. *Proc. Natl. Acad. Sci. USA.* 71:1935–1939.

Tittor, J., U.-P. Hansen, and D. Gradmann. 1983. Impedance of the electrogenic Cl^- pump in *Acetabularia:* electrical frequency entrainements, voltage-sensitivity, and reaction kinetic interpretation. *J. Membr. Biol.* 75:129–139.

Walter, L., K.-H. Büchner, and U. Zimmermann. 1988. Effect of alcaline earth ions on the movements of mobile charges in *Valonia utricularis. Biochim. Biophys. Acta.* 939:1–7.

Wehner, G., B. Friedmann, and U. Zimmermann. 1990. Biphasic voltage relaxation in cells of *Eremosphaera viridis* after injection of charge pulse of short duretion: detection of tip clogging of intracellular microelectrodes by charge pulse technique. *Biochim. Biophys. Acta.* 1027:105–115.

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

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