The voltage-dependent step of the chloride transporter of *Valonia utricularis* encounters a Nernst–Planck and not an Eyring type of potential energy barrier

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ABSTRACT Voltage-clamp experiments were performed on cells of the giant marine alga *Valonia utricularis* to study the voltage dependence of the previously postulated chloride transporter (Wang, J., G. Wehner, R. Benz, and U. Zimmermann. 1991. *Biophys. J.* 59:235–248). Only one exponential current relaxation (apart from the capacitive spike) could be resolved up to a clamp voltage of ~120 mV within the time resolution of our experimental instrumentation (100 μs). This means that the rate constants of the heterogeneous complexation, k_R (association) and k_D (dissociation), were too fast to be resolved. Therefore, the "Läuger" model for carrier-mediated ion transport with equilibrium heterogeneous surface reaction was used to fit the experimental results. The voltage dependence of the initial membrane conductance was used for the evaluation of the voltage dependence of the translocation rate constant of the complexed carriers, k_{AS}. The initial conductance was found to be independent on the clamp voltage, which means that the translocation rate constant k_{AS} is a linear function of the applied voltage and that the voltage dependence of the translocation of charged carriers through the plasmalemma could be explained by a square-type Nernst–Planck barrier. The movement of the complexed form of the carrier through the membrane may be explained by a diffusion process rather than by simple first-order kinetic jump across an Eyring-type potential well. The current relaxation after a voltage clamp was studied as a function of the external chloride concentration. The results allowed an estimation of the stability constant, K, of the heterogeneous complexation reaction and a calculation of the translocation rate constants of the free and the complexed carriers, k_S and k_{AS}, respectively.

INTRODUCTION

Biological membranes separate solutions of different solute composition. To maintain this asymmetry, they contain transport systems for the transport of ions and neutral substrates. Many of these transport systems are electrophoretic or electrogenic, which means that the transport of substrates occurs with net charge movement across the membranes. Examples for these electrophoretic transport systems are the H^+- or Na^+-driven cotransport of sugar and amino acids in plant (Komor and Tanner, 1976; Felle, 1980) and animal (Läuger and Jauch, 1986) cells. These systems use the electrochemical gradients of H^+ and Na^+, respectively, for the accumulation of the substrates inside the cells. Subsequently, the addition of the substrates leads to a depolarization of the cell membrane (Slayman and Slayman, 1974; Felle, 1980; Overath and Wright, 1983; Bergman and Bergman, 1985). Similarly, adenosine triphosphate (ATP)[1]-driven electrogenic ion pumps transfer net charge through membranes and contribute to the membrane potential (Slayman and Slayman, 1974; Gradmann, 1975; Shimmen and Tazawa, 1980; Gradmann et al., 1982; Beilby, 1984, De Weer et al., 1988).

The voltage dependence of electrophoretic transport systems introduced into lipid bilayer membranes has been investigated in detail. The Born charging energy results in a very small solubility of ions in membranes because of their low dielectric constant (Parsegian, 1969). As a consequence, large organic ions ("lipophilic ions") (Benz, 1988), carriers (Läuger and Stark, 1970; Benz and Läuger, 1976), and channel-forming substances (Bamberg and Läuger, 1973) are needed to smear the charges over a large sphere and to overcome the electrostatic energy of ions within membranes. The voltage dependence of lipophilic ions and carriers is very steep, and an Eyring type of barrier (Zwolinsky et al., 1949; Johnson et al., 1974) has been used to explain the voltage dependence of these transport systems (Ketterer et al., 1971; Knoll and Stark, 1975; Benz et al., 1976). However, considerable deviations between predicted and observed current voltage curves have been found for voltages > 80–100 mV.

Hladky (1974) has presented evidence that a modified Nernst–Planck barrier is able to explain the current–voltage characteristics of carrier-mediated ion transport. This barrier allows the transition of a square Nernst–Planck barrier with a linear voltage dependence to a triangular Eyring barrier with an exponential dependence on the basis of a single parameter. In fact, a much better fit of the voltage dependence of lipophilic ions and carrier molecules is obtained using this formalism (Andersen and Fuchs, 1975; Benz and McLaughlin, 1983; Kasianowicz et al., 1987). In all these cases a trapezoidal barrier explains the voltage dependence best. However, the observed current–voltage characteristics are still more exponential than linear.

Such detailed study of the voltage dependence of biological transport systems are not frequent. Only the voltage dependence of the proton pump of *Neurospora crassa* (Slayman and Slayman, 1974; Gradmann et al., 1982), of the chloride pump of *Acetabularia* (Grad-

This work is dedicated to the memory of Peter Läuger, the originator of investigation into the quantitative analysis of carrier-mediated ion transport.

[1] *Abbreviations used in this paper:* ASW, artificial sea water; ATP, adenosine triphosphate; ATPase, adenosine triphosphatase. MES, 2 (N-morpholino)ethane sulfonate; NSW, natural sea water.

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mann, 1975; Gradmann et al., 1982; Tittor et al., 1983), of the Na^+-K^+-adenosine triphosphatase of mammalian cells (Kaplan, 1985; Fendler et al., 1985; De Weer et al., 1988), of the gating charge of the Na^+-channel in nerve (Armstrong and Bezanilla, 1973) and muscle membranes (Chandler et al., 1975; Almers, 1978), and of the proton-driven cotransport systems of *Riccia fluitans* (Felle, 1980; Felle and Bentrup, 1980; Bertl et al., 1984) have been studied in some detail. The voltage dependence of the stationary pump current of the proton pump of *N. crassa* (Gradmann et al., 1982) and that of the chloride pump of *Acetabularia* (Tittor et al., 1983) suggests that the potential energy profile within the membrane could be explained by an Eyring barrier. On the other hand, the voltage dependence of the proton-driven cotransport systems of *R. fluitans* (Felle, 1980; Felle and Bentrup, 1980) is very weak. Other electrogenic ion pumps such as the Na/K adenosine triphosphatase have a rather complicated mechanism that makes it very difficult to attribute the current relaxations in the case of the Na/Na exchange (in the absence of K^+) by a defined step within the reaction rates (De Weer et al., 1988; Rakowski, 1992). In any case, voltage-clamp experiments have suggested a steep voltage dependence of the corresponding rate constant (Rakowski, 1992). Similar studies (i.e., the evaluation of rate constants) of electrophoretic transport systems in cell membranes are not known. The reason for this is that it is rather difficult to perform time-resolved electrical measurements with small cells since two intracellular electrodes and a time resolution of ≤ 0.1–1 ms are needed for an exact description of the voltage dependence of carrier-mediated ion transport. The voltage dependence of the translocation rate constant of the charged form across a cell membrane has only been measured directly for very few cases.

Another well-studied electrophoretic system is the mobile charges in the cell membrane of the giant marine algae *Valonia utricularis* (Zimmermann et al., 1982; Benz and Zimmermann, 1983; Büchner et al., 1985) and *Halicystis parvula* (Benz et al., 1988). These mobile charges have been identified as part of a chloride transport system of *V. utricularis* with an extremely high surface concentration of the carriers (up to 7 pmol/cm^2) in the cell membrane (Wang et al., 1991). Because of the kinetics of the different steps involved in chloride transport (i.e., the rate-limiting step of the ion transport is one of the voltage-independent steps), the carriers contribute to the apparent specific capacitance of the cell similar as the chloride pump of *Acetabularia* (Benz and Zimmermann, 1983; Wang et al., 1991; Gradmann, 1975).

In this article we report results of voltage-clamp experiments performed with *V. utricularis*. For the analysis of the data, we used a previously proposed simple carrier model (Läuger and Stark, 1970; Läuger, 1972; Wang et al., 1991). The results suggested that the plasmalemma contains a transport system for chloride, which is always in equilibrium for the heterogeneous surface reaction between carrier and ion. The voltage dependence of the translocation of charged carriers through the plasmalemma could be explained by a square-type Nernst–Planck barrier. This means that the voltage dependence of the translocation rate constant of the charged carrier is very weak. The movement of the complexed form of the carrier through the membrane may be explained by a diffusion process rather than by a single jump over an Eyring type of potential well.

MATERIALS AND METHODS

Cells of *V. utricularis* originally collected from the Mediterranean Sea near Naples, Italy, were maintained as has been described previously (Benz and Zimmermann, 1983; Wang et al., 1991). Cells of nearly elliptical shape and a surface area between 50 and 110 mm^2 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 *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid/NaOH, and the temperature was 20°C throughout the experiments if not otherwise stated.

Experiments were started 1.5–2 h after insertion of the two electrodes: a current microelectrode consisting of a 10-μm-thick platinum wire and a potential microelectrode in the cell. 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 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). Current and potential electrodes (Ag/AgCl, 3 M KCl) were connected to the inputs of a voltage-clamp instrument, which previously had been devised for voltage-clamp experiments on squid giant axon (Moore and Cole, 1963; Benz and Conti, 1981). The current after a voltage clamp was measured with a digital oscilloscope (model 2090; Nicolet Instrument Corp., Madison, WI). Two large silver/silver chloride reference electrodes were used: one for the clamp current and one (3 M KCl, agar bridge) for the recording of the membrane potential. Both external electrodes were placed close to the surface of the alga.

The current relaxation patterns recorded with the digital oscilloscope contained 4,096 data points with 12-bit amplitude resolution. The waveforms were transferred to a PC/AT computer and analyzed with a multiple-exponential-fitting program. The current versus time curve could be fitted to one exponential relaxation and a stationary part with sufficient accuracy. The significance of the fit was checked with the Student's t test. At 599 mM chloride, $T(n-2)$ was ≥ 200 for 1,000–2,000 data points. At small chloride concentrations (0 or 54 mM), $T(n-2)$ was lowest with ~ 40 for 200 data points of the relaxation. This means that the fit was also in these cases significant ($P \ll 0.001$). Only in a very limited number of experiments could a second relaxation with a very small amplitude be resolved. In all of these cases, inspection of the current curves showed the existence of noise or extraneous perturbations that therefore seemed to cause this additional relaxation. It has to be noted that in 10 successive experiments, taken on the same algal cell at time intervals of 30 s, the time constant and current amplitude did not vary by >3%, which means that the results obtained from one single cell were highly reproducible but varied considerably from cell to cell.

THE KINETIC MODEL FOR CHLORIDE TRANSPORT UNDER VOLTAGE-CLAMP CONDITIONS

We have shown previously that the Läuger model (Läuger, 1972) provides an excellent description of car-

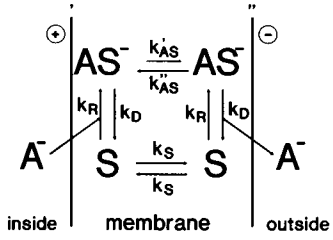

FIGURE 1 Diagram illustrating the mechanism by which the chloride carrier of *V. utricularis* transports chloride across the plasmalemma. The rate constants k_R and k_D refer to the heterogeneous reaction between carrier S and chloride A. The voltage-dependent rate constants k'_{AS} and k''_{AS} refer to the movement of AS from the left to the right interface and vice versa, respectively. The rate constant k_S refers to the movement for the free carrier between the two interfaces.

rier-mediated chloride transport across the plasmalemma of *V. utricularis* (Wang et al., 1991). Therefore, we will list here only the basic assumptions and implications of the model, which is illustrated in Fig. 1. It is assumed that a 1:1 carrier-ion complex is formed at the membrane-solution interface described by overall rate constants k_R (association) and k_D (dissociation). $K = k_R/k_D$ is the stability constant of the heterogeneous reaction between carrier S and anion A. The translocation of free and charged carriers through the membrane is given by the rate constants k_S and k_{AS}, respectively. The system is symmetrical with respect to the membrane, i.e., it is not distinguished between inward and outward movement of free and charged carriers. Heterogeneous surface reaction and diffusion of the free carrier through the membrane are assumed to be voltage independent. This means that 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 (Hladky, 1974; Benz and McLaughlin, 1983):

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

$$k''_{AS} = k_{AS}(bzu/2)\exp(-zu/2)/\sinh(bzu/2), \quad (2)$$

where $u = FV_m/RT$ is the reduced voltage; V_m is the membrane voltage; F, R, and T are standard symbols; and z is the valency of the carrier-ion complex. For $b = 0$, the carrier-ion complex encounters an Eyring barrier. For $b = 1$, k'_{AS} and k''_{AS} are approximately 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* separate(s) identical solutions of chloride (concentration c). The interfacial concentrations of free and complexed carriers on the two sides of the membrane change with time are given by the Eqs. A1 to A4 (see Appendix) (Läuger and Stark, 1970; Benz and Läuger, 1976). In the case of the chloride transport in *V. utricularis,* it is assumed that the total concentration of carriers within the membrane (complexed and uncomplexed) is constant (Eq. A5). In previous charge pulse studies with *V. utricularis* (Benz and Zimmermann, 1983; Büuchner et al., 1985; Wang et al., 1991), only two relaxation processes could be resolved with sufficient accuracy. Similarly, the voltage-clamp experiments reported here showed only one current relaxation (apart from the capacitive spike). These results suggest that one of the different reactions involved in a carrier-mediated chloride transport (see Fig. 1) is always in equilibrium because it is much faster than the others (Benz and Läuger, 1976; Benz and McLaughlin, 1983). For the two possibilities, we have demonstrated in a previous publication (Wang et al., 1991) that the heterogeneous surface reaction for the chloride transport in the plasmalemma of *V. utricularis* is always in equilibrium, i.e.,

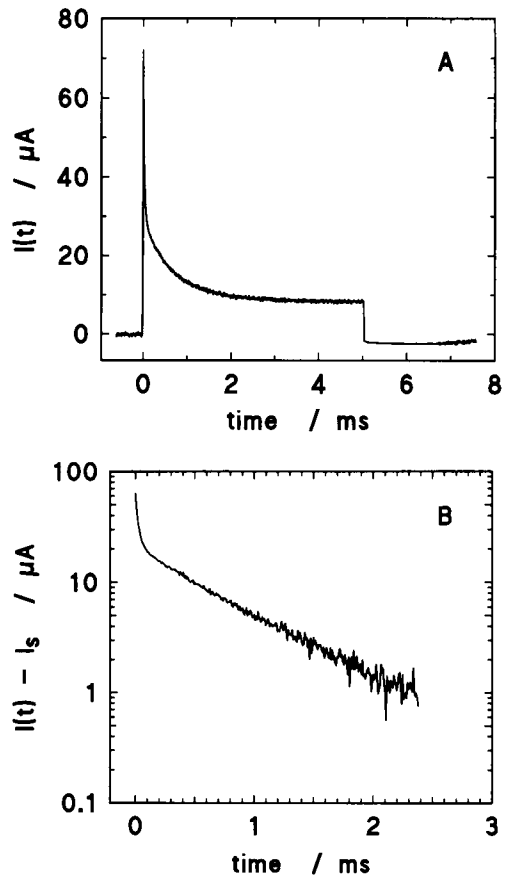

FIGURE 2 (*A*) Voltage-clamp experiment on application of 10.6 mV (resting potential 3.4 mV) to *V. utricularis* cell W57, bathed in ASW containing 599 mM Cl^-, pH 8.1; T = 20°C. Surface area A = 0.407 cm^2; volume V = 20.81 mm^3; turgor pressure 0.299 MPa. (*B*) Semilogarithmic plot of the current versus time curve. The current decay was fitted to one single exponential relaxation with the following relaxation parameters: $I_0 = I_s + I_d$ = 28.6 μA, I_s = 8.47 μA, τ = 708 μs. The corresponding conductances were G_0 = 6.61 mS/cm^2 and G_s = 1.96 mS/cm^2. The rate constants were calculated according to Eqs. A15 to A17: K_{AS} = 570 s^{-1}, K_S = 196 s^{-1}, N_0 = 6.62 pmol/cm^2; N_0 was calculated by assuming R_m = 6,000 Ωcm².

TABLE 1 **Results of voltage-clamp experiments (10.6-mV clamp potential) on four different *V. utricularis* cells measured at two different external chloride concentrations**

c	V_m	P	τ	G_0	G_s	K_{AS}	K_S	N_0
M	*mV*	*MPa*	*ms*	*mS/cm²*		*1/s*		*pmol/cm²*
Alga w63								
0.599	6	0.34	1.28	4.01	1.90	211	180	9.9
0	31	0.32	6.29	0.39	0.33	17	63	9.1
0.599	5	0.34	1.48	3.36	1.60	182	155	9.4
Alga w65								
0.599	1	0.33	0.92	6.66	0.83	488	57	7.0
0	23	0.24	2.43	1.15	0.79	76	131	7.0
0.599	1	0.31	0.93	6.02	1.48	416	124	7.4
Alga w67								
0.599	3	0.36	0.69	5.32	1.59	535	189	4.9
0	23	0.19	3.45	0.76	0.58	56	90	4.6
0.599	3	0.29	0.72	5.13	1.29	551	145	4.6
Alga w69								
0.599	4	0.32	0.81	4.40	1.04	507	109	4.2
0	−26	0.44	1.30	2.14	1.05	228	156	4.2
0.599	−17	0.29	0.83	4.67	0.42	586	15	3.9

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 2. The analysis of the experimental data was performed using Eqs. A15 to A17. P is the turgor pressure.

$$k_R c, \quad k_D \gg k_{AS}, k_S. \tag{3}$$

Under these conditions, the system of four differential equations is reduced to one single differential equation (see A11). The time course of the current density, $I_m(t)$, has under voltage-clamp conditions the following form (see Appendix):

$$I(t) = zFN_0\left(\frac{K'^2_{AS} - K''^2_{AS}}{2\beta}\exp(-\beta t) + \frac{K_S(K'_{AS} - K''_{AS})}{\beta}\right) + \frac{V_m}{R_m}. \tag{4}$$

N_0 is the total number of carrier molecules in the membrane, R_m is the specific membrane resistance caused by ion transport other than that of chloride, and $\tau = 1/\beta$ is the relaxation time constant with:

$$\beta = 2K_S + K'_{AS} + K''_{AS}. \tag{5}$$

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

$$K_S = k_S/(1 + Kc), \tag{6}$$

$$K'_{AS} = k'_{AS}Kc/(1 + Kc), \tag{7}$$

$$K''_{AS} = k''_{AS}Kc/(1 + Kc). \tag{8}$$

Eqs. 6 to 8 demonstrate that the rate constants k_S and k_{AS} cannot be derived from a single voltage-clamp experiment. Instead, K_S and K_{AS} have to be measured as a function of the chloride concentration to receive the rate constants k_{AS} and k_S (Wang et al., 1991; see also below).

The stationary current, I_s (i.e., the current density after many relaxation times), is given by:

$$I_s = zFN_0\frac{K_S(K'_{AS} - K''_{AS})}{2K_S + K'_{AS} + K''_{AS}} + \frac{V_m}{R_m}, \tag{9}$$

and the initial current, I_0, by:

$$I_0 = I_d + I_s = zFN_0(K'_{AS} - K''_{AS})/2 + V_m/R_m. \tag{10}$$

I_d is that part of the total current density that decays with the time constant τ to zero. The corresponding specific conductances G_S and G_0 can be calculated from Eqs. 9 and 10 by division of the corresponding current densities by the membrane voltage, V_m:

$$G_s = I_s/V_m = \frac{z^2F^2N_0}{RTzu}\frac{K_S(K'_{AS} - K''_{AS})}{2K_S + K'_{AS} + K''_{AS}} + \frac{1}{R_m}, \tag{11}$$

$$G_0 = I_0/V_m = z^2F^2N_0(K'_{AS} - K''_{AS})/(RTzu) + 1/R_m. \tag{12}$$

The current relaxation in voltage-clamp experiments could be fitted to just one exponential relaxation process, whereas the general case of the carrier model requires the knowledge of two exponential relaxations for complete description (Stark et al., 1971; Benz and McLaughlin, 1983). Thus, information on the rate constants of the heterogeneous reaction cannot be obtained. Furthermore, k'_{AS}, 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.

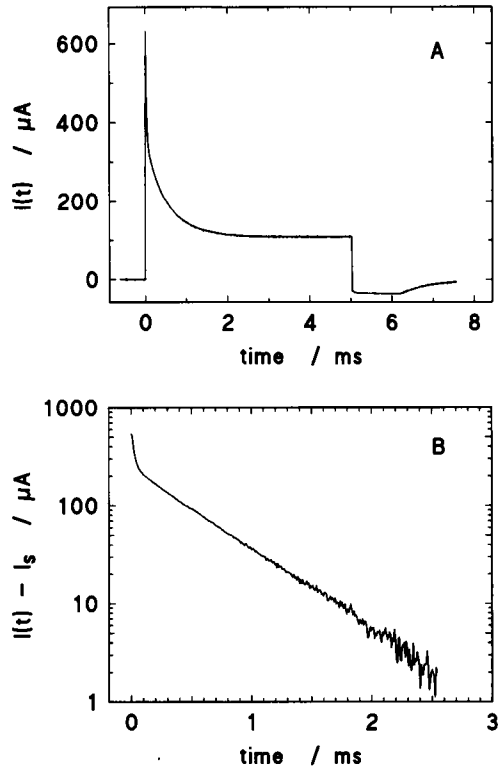

FIGURE 3 (*A*) Voltage-clamp experiment on application of 116.2 mV to the same *V. utricularis* cell as in Fig. 2 (resting potential, 3.4 mV). (*B*) Semilogarithmic plot of the current versus time curve. The current decay was fitted to one single exponential relaxation with the following relaxation parameters: I_0 = 346.5 μA, I_s = 108.9 μA, τ = 534 μs. The corresponding conductances were G_0 = 7.33 mS/cm² and G_s = 2.3 mS/cm². The turgor pressure was now 0.305 MPa. The rate constants calculated according to Eqs. A15 to A17: K_{AS} = 658 s⁻¹, K_S = 272 s⁻¹, N_0 = 5.7 pmol/cm²; N_0 was calculated by assuming R_m = 6,000 Ω cm².

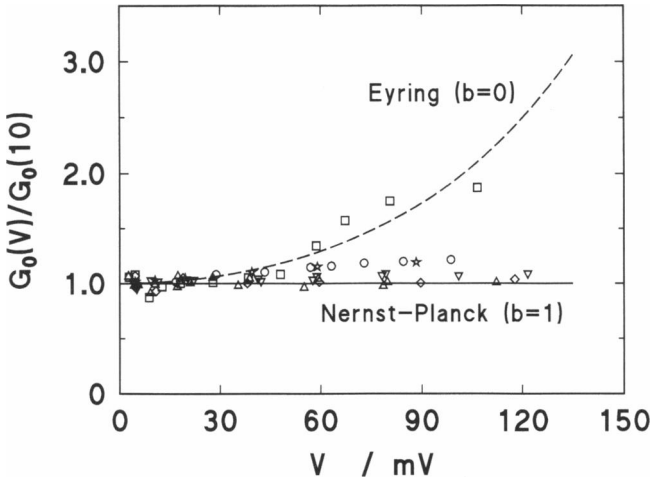

FIGURE 4 Conductance versus voltage curve for carrier-mediated chloride transport in *V. utricularis*. The initial conductance $G_0(V)$ was divided by the initial conductance, $G_0(10)$, measured on application of 10 mV and plotted against the applied voltage, V. Experiments of six different cells are shown by different symbols. The solid line shows the voltage dependence obtained by a Nernst–Planck type of barrier (b = 1; Eq. 13), and the dashed line is the prediction of Eq. 13 in the limit that b approaches 0, a single-barrier Eyring model.

RESULTS

Voltage dependence of carrier-mediated chloride transport in *V. utricularis*

Fig. 2 *A* illustrates a current record taken from a *V. utricularis* cell immersed in ASW containing 599 mM chloride at a clamp voltage of 10.6 mV (starting from a resting potential of 3.4 mV). The initial current decays with two clearly distinguishable relaxations. The fast relaxation with a time constant of ~20 μs reflects the capacitive spike caused by the charging process of the membrane capacitance by the feedback system of the voltage-clamp instrumentation (Fig. 2 *B*). The current relaxation due to carrier-mediated chloride transport was analyzed using the following formalism. Fig. 2 *B* illustrates the data after the analysis by the computer: the logarithm of $(I(t) - I_s)$, where $I(t)$ is the current at a time t and I_s is the steady-state current, is a linear function of the time. Only a single exponential (despite the capacitive spike) with a relaxation time constant of 708 μs was observed. This situation was comparable with that for charge-pulse experiments: in these experiments two voltage relaxations have been observed, since the membrane voltage represents an additional variable in these measurements (Benz et al., 1976; Benz and

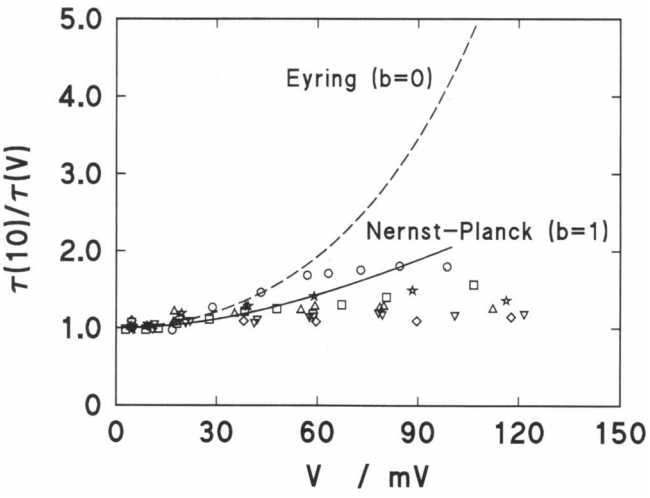

FIGURE 5 Voltage dependence of the relaxation time constant for carrier-mediated chloride transport in *V. utricularis*. The time constant, $\tau(10)$, measured on application of 10 mV, was divided by the time constant, $\tau(V)$, at a given membrane potential and plotted against the applied voltage, V. Experiments of six different cells are shown by different symbols. The solid line shows the voltage dependence obtained by a Nernst–Planck type of barrier (b = 1; Eq. 16), and the dashed line is the prediction of Eq. 15 in the limit that b approaches 0, a single-barrier Eyring model. The theoretical curves were drawn using k_{AS} = 4,800 1/s, k_S = 480 1/s, K = 0.34 1/M, and c = 599 mM.

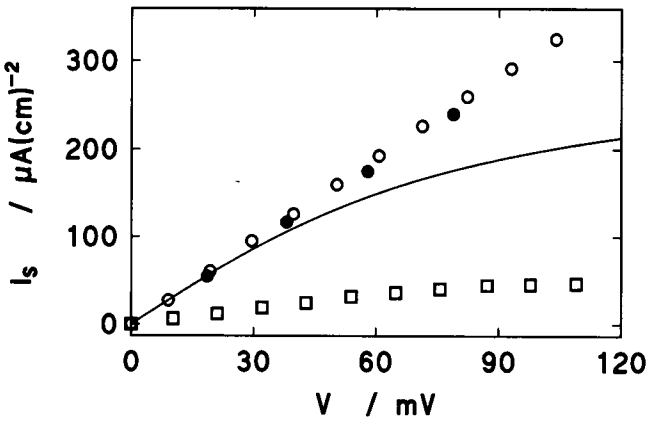

FIGURE 6 Voltage dependence of the stationary current, I_s, measured on *V. utricularis* cell s94 (resting potential −7 mV at 599 mM chloride and 20 mV at 0 mM chloride). The open circles, the open squares, and the closed circles represent the experimental results at 599 mM chloride, 0 external chloride, and back to 599 mM chloride, respectively. The solid line was drawn according to the predictions of Eq. 9 by using the parameters of carrier-mediated chloride transport calculated from the current relaxation (K_{AS} = 560 1/s, K_S = 450 1/s, N_0 = 6.0 pmol/cm^2, R_m = 6,000 Ωcm^2).

Läuger, 1976). One single exponential current relaxation out of two predicted by the Läuger model means that one of the different reactions involved in carrier-mediated chloride transport (see Fig. 1) is always in equilibrium, as has been demonstrated previously (Wang et al., 1991).

This means that only $K_S = k_S/(1 + Kc)$, $K_{AS} = k_{AS}Kc/(1 + Kc)$ and N_0 can be calculated from voltage-clamp experiments at a given chloride concentration. Table 1 shows the results of voltage-clamp experiments taken from four different algal cells at two external chloride concentrations (599, 0, and back to 599 mM). The turgor pressure of all cells was either constant or varied insignificantly during the duration of the experiments (~2 h). As can be seen from Table 1, the experimental parameters, τ, G_0, and G_s, the kinetic ones, K_{AS} and K_S, and the total surface concentration, N_0, of carrier sites varied considerably from cell to cell. The external chloride concentration also had a strong influence on these parameters. This is expected, in principle, for a chloride transporter and is investigated in more detail below.

The formalism for charge-pulse experiments allows only a closed solution for the time dependence of the membrane voltage when the initial voltage, V_0, is much below RT/F = 25 mV (Benz and Läuger, 1976; Benz and McLaughlin, 1983; Wang et al., 1991). The use of the voltage clamp allows a straightforward description of the voltage dependence of transport systems (Stark et al., 1971; see section Theory). Fig. 3 *A* shows a voltage-clamp experiment performed on the same algal cell as in Fig. 2, but the applied voltage was now 116 instead of 10.6 mV (starting from a resting potential of 3.4 mV). Again we observed only one exponential current relaxation apart from the capacitive spike (see Fig. 3 *B*). Interestingly, the time constant, τ, did not change much and was 534 μs at 116 mV. Simultaneously, the initial current, I_0, increased only by a factor of 12 as compared with that at 10.6 mV (28.6 as compared with 346.5 μA). It is interesting to note that the sign of the clamp potential had no influence on the relaxation data. This means that relaxation time constant and amplitude were virtually the same, irrespective of the direction of the applied electrical field.

Shape of the potential barrier for the voltage-dependent step of the chloride transporter

From all the different steps involved in carrier-mediated ion transport, only the translocation rate constant k_{AS} is assumed to be voltage dependent (see Eqs. 1 and 2). This means that the initial conductance, G_0, is voltage dependent and could be used for the evaluation of the barrier shape for the translocation rate constant k_{AS}. The voltage-clamp data were highly reproducible for experi-

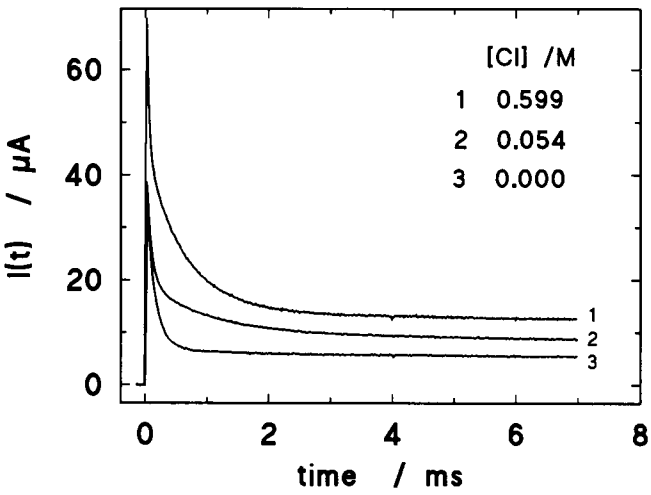

FIGURE 7 Voltage-clamp experiments on application of ~11.5 mV to *V. utricularis* cell W67, bathed in ASW containing (1) 599 mM (resting potential 3 mV), (2) 54 mM (resting potential 11.4 mV), and (3) 0 mM chloride (resting potential 21 mV), pH 8.1; T = 20°C; Surface area A = 70.7 mm^2; Volume V = 40.09 mm^3; turgor pressure 0.299 MPa. The anion concentration in the ASW was counterbalanced with MES to 599 mM. The current decays were fitted in all cases to one single exponential relaxation with the following relaxation parameters. The rate constants and N_0 were calculated according to Eqs. A15 to A17 and by assuming R_m = 3,600 Ωcm^2. (curve *1*) I_0 = 43.4 μA, I_s = 12.8 μA, τ = 684 μs. The corresponding conductances were G_0 = 5.38 mS/cm^2 and G_s = 1.59 mS/cm^2. The turgor pressure was 0.37 MPa. K_{AS} = 543 s^{-1}; K_S = 188 s^{-1}; N_0 = 4.92 pmol/cm^2. (curve *2*) I_0 = 19.06 μA, I_s = 8.23 μA, τ = 1,470 μs. The corresponding conductances were G_0 = 2.26 mS/cm^2 and G_s = 1.03 mS/cm^2. The turgor pressure was 0.15 MPa. K_{AS} = 221 s^{-1}; K_S = 129 s^{-1}; N_0 = 4.91 pmol/cm^2. (curve *3*) I_0 = 6.73 μA, I_s = 5.08 μA, τ = 3,520 μs. The corresponding conductances were G_0 = 0.768 mS/cm^2 and G_s = 0.579 mS/cm^2. The turgor pressure was 0.19 MPa. K_{AS} = 54.8 s^{-1}; K_S = 87.3 s^{-1}; N_0 = 4.68 pmol/cm^2.

TABLE 2 **Results of voltage-clamp experiments (clamp voltage, 11.5 mV) on *V. utricularis* cell w67 measured as a function of external chloride concentration**

c	V_m	P	τ	G_0	G_s	K_{AS}	K_S	N_0
M	*mV*	*MPa*	*ms*	*mS/cm²*		*1/s*		*pmol/cm²*
0.599	3	0.36	0.69	5.32	1.59	535	189	4.9
0.450	5	0.32	0.76	4.48	1.37	486	170	4.5
0.300	10	0.26	0.91	3.58	1.17	400	147	4.3
0.150	17	0.22	1.11	2.92	1.12	307	144	4.5
0.054	19	0.15	1.54	2.21	0.97	208	117	4.9
0	23	0.19	3.45	0.76	0.58	56	90	4.6
0.599	3	0.29	0.72	5.13	1.29	551	145	4.6

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. A15 to A17 by assuming R_m = 3,600 Ωcm². P is the turgor pressure and V_m is the resting potential of the algal cell.

ments on a given algal cell, but they varied considerably from cell to cell. Therefore, we analyzed the reduced initial conductance as a function of the applied membrane potential, i.e., $G_0(\dot{V})$ was normalized by division through the corresponding conductance at 10 mV, $G_0(10)$.

Assuming that R_m is sufficiently high to be neglected as compared with the (much lower) resistance of the chloride transporters (which indeed was valid for most algal cells), it is apparent from Eqs. 1, 2, and 12 that $G_0(V)/G_0(10)$ is given as a function of the membrane potential and the parameter b by (valency of the carrier-ion complex $z = -1$):

$$G_0(V)/G_0(10) = b \sinh(u/2)/\sinh(bu/2), \quad (13)$$

where b is the fraction of the membrane spanned by the minor base of the trapezoidal energy barrier. Fig. 4 illustrates the voltage dependence of the experimental results for $G_0(V)/G_0(10)$. It demonstrates also the predictions of two extremes. The dashed line shows the voltage dependence if the complex has to cross a single-jump Eyring barrier, equivalent to $b = 0$ (Hladky, 1974; Benz and McLaughlin, 1983). $G_0(V)/G_0(10)$ shows in this case an exponential dependence on applied voltage. The solid line represents the voltage dependence if the complex AS moves across a barrier of the Nernst–Planck type (b = 1). For such a barrier, $G_0(V)/G_0(10)$ is constant and independent on voltage. A comparison of these two possibilities with the experimental data given in Fig. 4 clearly shows that the carrier-ion complex AS encounters a square barrier within the membrane (i.e., b = 1). As a consequence, the initial current was linearly dependent on the voltage and the initial conductance $G_0(V)$ was independent on voltage.

Similar results were obtained for the voltage dependence of the relaxation time constants. According to Eqs. 1, 2, and 5, the voltage dependence of $\beta = 1/\tau$ is given by ($z = -1$):

$$\beta = 2(K_S + K_{AS}(bu/2)\cosh(u/2)/\sinh(bu/2)). \quad (14)$$

In the case of an Eyring barrier ($b = 0$), the inverse time constant, β, has the following voltage dependence:

$$\beta = 2K_S + 2K_{AS}\cosh(u/2). \quad (15)$$

The voltage dependence of β is, on the other hand, very shallow for a Nernst–Planck type of a square barrier:

$$\beta = 2K_S + K_{AS}\frac{u\cosh(u/2)}{\sinh(u/2)}. \quad (16)$$

Fig. 5 shows the ratio $\tau(10)/\tau(V)$ (which corresponds to the ratio $\beta(V)/\beta(10)$) as a function of the clamp voltage. Again, we obtained a much better fit of the data with a square Nernst–Planck barrier (*solid line*, b = 1) than with an Eyring barrier (*dashed line*, b = 0). This result is consistent with the voltage dependence of the normalized initial conductance $G_0(V)/G_0(10)$.

Voltage dependence of the stationary current

The rate-limiting step of carrier-mediated chloride transport in *V. utricularis* is the backtransport of the neutral form (Wang et al., 1991). This means that the stationary current, I_s, should saturate for increasing clamp potential according to Eq. 9. Fig. 6 demonstrates that I_s did not saturate with but showed a more or less linear dependence on voltage. This discrepancy may be explained by the assumption of another transport system in the plasmalemma of the cells, for instance, a channel for cations that conducts at voltages > 20–30 mV. To test this, we performed voltage-clamp experiments at zero external chloride. Surprisingly, the stationary current became very small (see Fig. 6), which means that the excess current of Fig. 6 was a chloride one. This means that the excess current is caused either by a chloride channel or an uncomplete description of the transport system by our theoretical treatment.

Replacement of external chloride by MES

Our experimental data suggested that the heterogeneous complexation reaction between free carrier and chloride

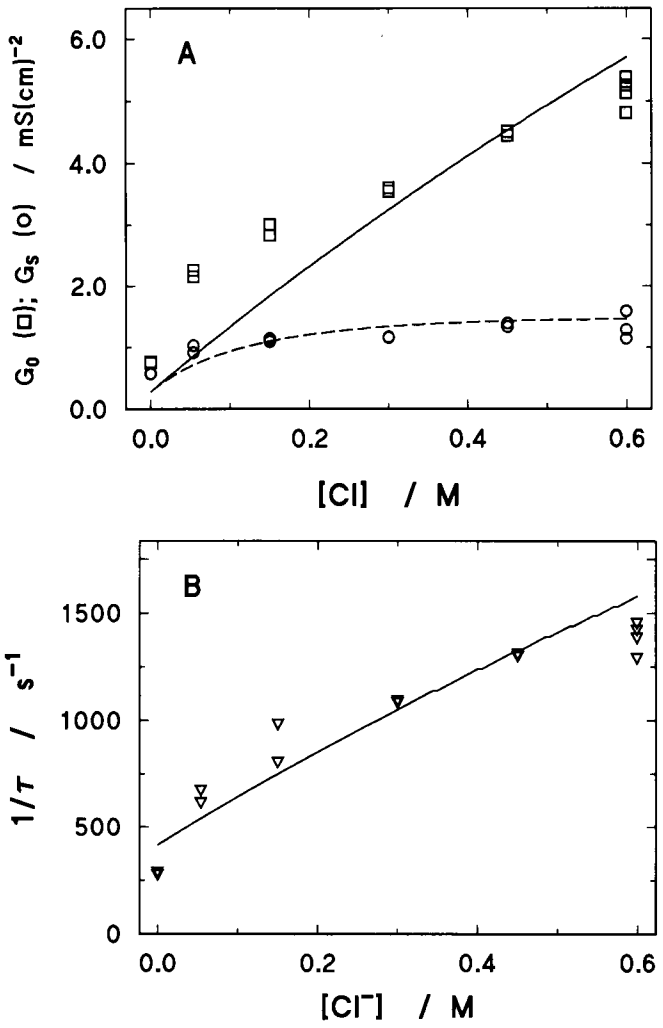

FIGURE 8 (*A*) Plot of the initial specific conductance $G_0(V)$ and the stationary specific conductance $G_s(V)$ as a function of the external chloride concentration for cell w67. The anion concentration of the ASW was balanced to 599 mM by the addition of increasing concentrations of MES; V = 11.5 mV; T = 20°C. The dashed line and the solid line represent the predictions of Eqs. 11 and 12, respectively, for G_s and G_0, by assuming N_0 4.4 pmol/cm^2, k_{AS} = 1,500 1/s, k_S = 250 1/s, K for chloride = 1.1 1/M, K for MES = 0.05 1/M, and the specific resistance, R_m = 3,600 Ωcm^2, of the cell membrane for transport systems other than chloride. (*B*) Plot of the relaxation time constant, $\tau(V)$, as a function of the external chloride concentration. The voltage-clamp experiments were performed on the same cell as in *A*. The decrease of chloride was counterbalanced by increasing the MES concentration from 0 to 599 mM; V = 11.5 mV; T = 20°C. The solid line was drawn according to Eq. 5 by assuming K = 1.1 1/M, k_{AS} = 1,500 1/s, and k_S = 250 1/s.

is always in equilibrium. This means that the translocation rate constants for the free and the complexed form of the chloride transporter cannot be derived from a single voltage-clamp experiment. Instead, K_{AS} and K_S can be calculated, which are functions of the stability constant, K, of the heterogeneous complexation reaction and the chloride concentration, c (see Eqs. 6 and A18). To derive K and calculate k_{AS} and k_S from voltage-clamp experiments, external chloride was reduced in many steps (599, 450, 299, 150, 54, and 0 mM) and replaced by the organic anion MES$^-$ (0, 149, 300, 449, 545, and 599 mM). After change of the external chloride concentration, the current relaxations always reached their final form within a few minutes. Simultaneously, the membrane potential changed when external chloride was replaced by MES$^-$. In general, *Valonia* cells had only a small membrane potential of 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 when the external solution did not contain any chloride. We would like to stress the point that the replacement of chloride by MES$^-$ did not kill the algal cells and that the MES$^-$-induced changes were reversible. The turgor pressure was more or less constant during the duration of the experiment (~3 h). Furthermore, the initial current relaxation was approximately obtained after the chloride-containing ASW had been reinstated for 30 min (see Table 1).

Fig. 7 shows current relaxations after voltage clamps of 11.5 mV performed on the same *V. utricularis* cell in different chloride concentrations (balanced always to 599 mM by the addition of MES$^-$). Curve *1* corresponds to the current relaxation in ASW (599 mM chloride). Curves *2* and *3* were obtained in 54 and 0 mM chloride, respectively. The decrease of the chloride concentration had a strong influence on the parameters of the current relaxations, and both the initial and the stationary conductances decreased by factors of 6.5 and 2.5, respectively. Simultaneously, the relaxation time constant increased from 684 to 3,520 μs. Both effects are expected from the theoretical predictions of our model. Table 2 presents the relaxation parameters derived from voltage-clamp experiments with the same algal cell as given in Fig. 7 at a variety of different chloride concentrations. As described above, the relaxation parameters showed a considerable dependence on the chloride concentration. Table 2 also presents the data for K_{AS}, K_S, and N_0, as calculated from the relaxation data by using Eqs. A15, A16, and A17. When external chloride was completely replaced by MES$^-$, K_{AS} decreased by a factor of ~10, whereas K_S decreased only a little, suggesting that MES$^-$ also binds to the carrier (Wang et al., 1991).

Fig. 8 *A* shows the plot of the initial and the stationary-specific conductances G_0 and G_s, and Fig. 8 *B* shows that of the relaxation time constant of voltage-clamp experiments as functions of the external chloride concentration taken from the same cell. The fit of the curves of Fig. 8, *A* and *B*, using Eqs. 6 and A15 to A18 (lines in Fig. 8) allowed the calculation of the parameters of the chloride transporter, k_{AS} = 1,500 1/s, k_S = 250 1/s, N_0 4.4 pmol/cm^2, and K = 1.1 1/M. The specific resistance of the membrane for systems other than the chloride transporter was 3,600 Ωcm^2. A somewhat better fit of the data

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

Cell	N_0	k_{AS}	k_S	K	K_a	R_m
	pmol/cm²	*1/(Ms)*	*1/s*	*1/M*		*Ωcm²*
w63	9.1	2,700	180	0.15	0.6	10,200
w65	7.1	3,100	120	0.32	<0.1	6,800
w67	4.61	2,800	220	0.47	0.8	3,600
w69	3.92	2,850	110	0.48	<0.1	3,200
Mean ± SD	6.0 ± 2.4	2,860 ± 170	158 ± 52	0.36 ± 0.16	0.36 ± 0.40	5,950 ± 3,260

The data were taken from the experimental data given in Tables I and II. 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. $T = 20°C$. R_m is the specific resistance of the cells derived by assuming that N_0 is independent on external chloride concentration.

(see Fig. 8) was achieved if the binding of MES^- to the chloride transporter was taken into account, which has been discussed previously in detail (Wang et al., 1991). In this case, the stability constant for the binding of MES^- to the transporter was ~0.05 1/M.

Table 3 presents the parameters of carrier-mediated chloride transport of the four different *V. utricularis* cells of Table 1. As pointed out above, there existed considerable variation of the data from cell to cell. The translocation rate constants k_S and k_{AS} and the total carrier concentration N_0 varied about two- to threefold. As pointed out earlier (Benz and Zimmermann, 1983; Wang et al., 1991), these variations reflect probably the different physiological growth states of the algal cells. Table 3 shows also the stability constants for the binding of chloride and MES^- to the binding sites. Again, we obtained same variations and both K for chloride varied little, whereas K_a for MES^- varied considerably. Besides the experiments described here, we also replaced chloride by bromide and nitrate. The results of these preliminary experiments were basically the same but suggested that these anions bind more strongly than does MES^- to the carrier (Wang, J., R. Benz, U. Zimmermann, unpublished observations).

DISCUSSION

In a previous study we presented evidence that the electrogenic transport system in the cell membrane of *V. utricularis* is a chloride carrier (Wang et al., 1991). In this investigation we studied the chloride transporter under voltage-clamp conditions. The reason for this was twofold since we were interested in (*a*) the shape of the barrier for the translocation of the charged form of the transporter and (*b*) the influence of chloride on the carrier kinetics under voltage-clamp conditions. Furthermore, we investigated the contribution of the tonoplast to the current relaxation of the whole cell under voltage-clamp conditions. The model shown in Fig. 1 represents the simplest possible mechanism by which chloride could be transported by a carrier system. In this study only one current relaxation could be resolved out of the two predicted by the Läuger model (Stark et al., 1971). This could mean that the time resolution of our experimental instrumentation is simply too poor to resolve the two current relaxations predicted by the model as has been argued previously (Stark et al., 1971). We believe instead that the amplitude of the other current relaxation was simply too small to be resolved. This is consistent with our charge-pulse data (Wang et al., 1991) and means that one of the reactions involved in carrier-mediated ion transport is always in equilibrium (Benz and Läuger, 1976; Benz and McLaughlin, 1983).

In the case of the electrogenic chloride transporter of *V. utricularis,* the heterogeneous surface reaction is always in equilibrium (i.e., the case $k_R c$, $k_D \gg k_S$, k_{AS}). The reasons for this have been presented in full detail previously (Wang et al., 1991) and are not discussed here. The on-rate k_R has to be $>10^4$ liter/(mol*s) (corresponding to $k_D > 3 \times 10^4$ 1/s) to explain our experimental results. Such association (and dissociation) rate constants are not unreasonable, since k_R and k_D for valinomycin-mediated cation transport could be as high as 10^5 liter/(mol*s) and 10^5 1/s, respectively (Knoll and Stark, 1975; Benz and Läuger, 1976; Benz et al., 1989). The heterogeneous reaction of the chloride pump of *Acetabularia* is also always in equilibrium, which requires similar values for k_R and k_D (Tittor et al., 1983). The fast electroneutral exchange of chloride and bicarbonate across the red cell membrane (band 3 protein; turnover number $> 10^4$ 1/s) probably has on- and off-rates that are of the same order of magnitude (Fröhlich, 1988).

The rate-limiting step for chloride transport is the diffusion of the neutral form. This step may include ATP hydrolysis if the system is a chloride pump, for which we do not have any evidence (Wang et al., 1991). This also means that the stationary current should saturate for increasing membrane potential, which we did not observe. There exist at least two possibilities to explain the discrepancy. One is the assumption of a voltage-dependent chloride channel since the excess conductance is chloride dependent. Another possibility is that k_S could be voltage dependent for unknown reasons. At this point we cannot

decide which one of these explanations is more likely, and further experiments are required to understand the voltage dependence of the stationary current.

Shape of the barrier for the voltage-dependent step

Two different formalisms have been proposed for the treatment of voltage dependence of charge transfer through membranes. One assumes a steep barrier in the center of the membrane across which the charge moves in a single step, i.e., its transport across the membrane, can be described by simple first-order kinetics (Zwolinsky et al., 1949; Johnson et al., 1974). The voltage dependence of the such-called Eyring barrier is given according to the theory of absolute reaction rates (Johnson et al., 1974). It is very steep and follows an exponential function of the applied voltage (see Eqs. 1 and 2). The other formalism uses the integration of the Nernst–Planck equation. The voltage dependence of the charge translocation is in this case very shallow since it represents a diffusion process, i.e., many steps (Neumcke and Läuger, 1969).

The voltage dependence of the initial conductance $G_0(V)$ has been used as a measure of the voltage dependence of the charge movement through the membrane (Ketterer et al., 1971; Hladky, 1974; Andersen and Fuchs, 1975; Knoll and Stark, 1975; Benz et al., 1976; Benz and McLaughlin, 1983; Kasianowicz et al., 1987). Hladky (1974) has presented evidence that a trapezoidal barrier (where the minor base spans the fraction b of the membrane) allows a better description of the barrier shape for charge transfer across membranes. In fact, Eqs. 1 and 2 provide a good description of the voltage dependence of transport of lipophilic ions and carrier-mediated ion transport across artificial lipid bilayer membranes in many investigations (Hladky, 1974; Andersen and Fuchs, 1975; Benz and McLaughlin, 1983; Kasianowicz et al., 1987), and b was found to be between 0.2 and 0.5, which means that the voltage dependence of the ion translocation was still more exponential than linear in these studies. The results presented here clearly demonstrate that a square barrier (i.e., $b = 1$) provides the best fit to our experimental data for $G_0(V)$ and τ, although we also found some deviations from the predictions of the Nernst–Planck type of barrier. This means that the movement of the charged form of the chloride transporter in *V. utricularis* can be explained by a diffusion process rather than by a simple first-order kinetics.

Although the shapes of the barrier wells have been studied well for model systems within artificial lipid bilayers, only a limited number of shapes are known for electrogenic transport systems in cell membranes. This is because it is very difficult to perform voltage-clamp experiments with sufficient time resolution in small cells. Very well-investigated systems are the gating currents of Na^+ channels in nerve (Armstrong and Bezanilla, 1973; Nonner et al., 1975) and muscle membranes (Chandler et al., 1975; Almers, 1978). The movement of gating particles could be described by two-state models and a steep Boltzmann function of the membrane potential (i.e. an Eyring barrier). The number of charges, z, moving through the entire membrane for channel gating was between 1.3 and 1.7 (corresponding to $RT/(zF)$ = 19–14 mV, respectively). An Eyring barrier also has been found for the charge transfer in the case of the chloride pumps of *Neurospora* (Gradmann et al., 1982) and *Acetabularia* (Tittor et al., 1983). However, the evidence for a steep barrier in *Acetabularia* (an organism that is related to *Valonia*) is somewhat weak in this case since the voltage dependence has been measured by the application of a sinusoidal potential to the cell and by the fit of the frequency dependences of phase shift and cell impedance with a three-state model (Tittor et al., 1983).

The voltage dependence of the Na/Na exchange mediated by the Na/K pump has been studied in guinea pig heart cells and in oocytes in some detail (Nakao and Gadsby, 1986; Rakowski, 1992). Although this exchange is in total electroneutral, current relaxations have been observed, which can be explained by a three-state model similar to that used here. One of the processes, possibly the binding of Na to the external face of the pump, is voltage dependent. Its dependence also can be explained by a Boltzmann expression by using $z = 1$ (i.e., $RT/(zF)$ = 25 mV) (Rakowski, 1992). This means that the known biological transport systems have barrier shapes that are more close to that of an Eyring barrier than to a square barrier. So far it is not clear if the chloride transporter of *V. utricularis* is in that respect an exception or if a weak voltage dependence (i.e., a square barrier) is more common.

Effect of chloride on the voltage-clamp data

The decrease of the external chloride concentration led to a decrease of the relaxation amplitude and to an increase of the relaxation time constant. Both effects are consistent with the proposed model. The 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 at zero external chloride. The residual chloride in the cytoplasm is caused presumably by chloride loss from the vacuole. The chloride concentration in the vacuole is more or less the same as in NSW (or ASW), and the loss is very slow (Wang et al., 1991). The chloride concentration dependence of the relaxation data allowed the estimation of the absolute rate constants, k_{AS} and k_S, and the stability constant for the binding of chloride to the binding site. The two rate constants are 2,900 1/s and 150 1/s, respectively. Especially the translocation rate constant of the charged form is

considerably higher than the rate constant of the corresponding step in the chloride pump of *Acetabularia* (Tittor et al., 1983) and also of the voltage-dependent step of the Na/Na exchange (Rakowski, 1992). The half saturation concentration of the binding of chloride to the binding site is ~2.8 M. This means that the site is not saturated at physiological conditions. Although it cannot be excluded, it is not likely that the plasmalemma of *V. utricularis* contains a chloride pump similar to that of *Acetabularia* (Gradmann, 1989).

Contribution of the tonoplast

Electrical measurements on the membranes of plant cells usually have the problem that tonoplast and plasmalemma cannot be separated in a simple way (Findlay and Hope, 1976; Bates et al., 1982) since the two internal electrodes are inserted normally into the vacuole. The same applies to the voltage-clamp experiments with *V. utricularis*. However, to explain the experimental data in terms of the chloride transport system, it is sufficient to assume that only one membrane (probably the plasmalemma) with a chloride-independent specific resistance of ~6,000 Ωcm^2 contains the carrier system and that the other membrane (the tonoplast) has a small specific resistance of <100 Ωcm^2. On the other hand, it is also possible (but extremely unlikely) that both membranes have identical electrical properties. Furthermore, the potential differences across the individual membrane are not known.

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 always have been consistent with the assumption that the experimental results reflect only one membrane (Benz and Zimmermann, 1983; Wang et al., 1991), which means that the tonoplast is high conducting. This seems to contradict the finding that chloride is retained within the vacuole at low external chloride (Wang et al., 1991). However, chloride will leak out of the vacuole only rapidly if the tonoplast is also highly permeable to the positively charged counterion, presumably potassium. Otherwise, a diffusion potential will hinder the chloride transport out of the vacuole. In fact, a potential of ~30 mV has been observed after replacement of external chloride (Wang et al., 1991). This would be sufficient to explain why the chloride concentration inside the vacuole is independent of the external chloride concentration.

APPENDIX

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 mol/cm^2), then the change of these quantities with time is given by the following four differential equations

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

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

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

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

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

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

The current density within the membrane is given by the flux of charges due to the carrier system and the contribution of the membrane resistance, R_m, which is not caused by the chloride transporters:

$$I(t) = zF(k'_{AS}N'_{AS} - k''_{AS}N''_{AS}) + V_m/R_m. \quad (A6)$$

Under voltage-clamp conditions, the solution for $I(t)$ in the common case is given by (Stark et al., 1971):

$$I(t) = a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2) + I_s. \quad (A7)$$

a_i, τ_i (i = 1, 2), and I_s are known functions of the four rate constants, N_0 and R_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 during the whole relaxation process the relations:

$$N'_{AS}/N'_S = N''_{AS}/N''_S = k_R c/k_D, \quad (A8)$$

and

$$N_{AS} = N'_{AS} + N''_{AS} = \frac{Kc}{1 + Kc} N_0 = \frac{k_R c}{k_D + k_R c} N_0, \quad (A9)$$

$$N_S = N'_S + N''_S = \frac{1}{1 + Kc} N_0 = \frac{k_D}{k_D + k_R c} N_0, \quad (A10)$$

hold. We assume that the valency of the carrier-anion complex is −1. Introducing Eqs. A8–A10 and 6–8 into the differential Eqs. 1–4 yields the following differential equation:

$$dN'_{AS}/dt = -(K_S + K'_{AS})N'_{AS} + (K_S + K''_{AS})N''_{AS}. \quad (A11)$$

Under voltage-clamp conditions, the solution of the differential Eq. A11 is given by (together with the boundary condition at 0 time $N'_{AS} = N_{AS}/2$):

$$N'_{AS}(t) = (k'_{AS} - K''_{AS})N_{AS} \exp(-\beta t)/(2\beta) + (K_S + K''_{AS})N_{AS}/\beta. \quad (A12)$$

$\beta = 2K_S + K'_{AS} + K''_{AS}$ is the inverse relaxation time constant τ.

In the limits of small voltages ($V_m \ll 25$ mV; $u \ll 1$) or in the case of a Nernst–Planck barrier, k'_{AS} and k''_{AS} are given by:

$$k'_{AS} = k_{AS}(1 + zu/2), \quad \text{(A13)}$$

$$k''_{AS} = k_{AS}(1 - zu/2). \quad \text{(A14)}$$

Introducing Eq. A13 and A14 into Eqs. 5, 11, and 12 gives:

$$\beta = 1/\tau = 2(K_S + K_{AS}), \quad \text{(A15)}$$

$$G_0 = z^2F^2N_0K_{AS}/(2RT) + 1/R_m, \quad \text{(A16)}$$

$$G_s = z^2F^2N_0K_{AS}K_S/(2RT(K_S + K_{AS})) + 1/R_m, \quad \text{(A17)}$$

with:

$$K_{AS} = k_{AS}Kc/(1 + Kc). \quad \text{(A18)}$$

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