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Effects of light and inhibitors of ATP-synthesis on the chloride carrier of the alga *Valonia utricularis*: is the carrier a chloride pump?

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Abstract

The effect of metabolic inhibitors, such as cyanide, antimycin A and azide was studied on the chloride transport system of the giant marine alga *Valonia utricularis* by using the charge pulse relaxation method. Two clearly defined voltage relaxations were resolved. The addition of 10–30 μM cyanide to the artificial sea water (ASW) bathing the algal cells increased the time constants of the slow voltage relaxation, τ_2, significantly when the algal cells were kept in the dark. The cyanide-effect reached a plateau value at 100–300 μM and was fully reversible when cyanide was removed from the ASW. Analysis of the charge pulse data in terms of the Läuger-model demonstrated that the translocation rates of the free, k_S, and the charged carrier, k_{AS}, decreased. The decrease of k_S was more pronounced than that of k_{AS}. 10 μM antimycin A and 3 mM azide had similar effects on the rate constants when the light was switched off. Upon illumination the cyanide- and antimycin A-, but not the azide-mediated effects disappeared. At concentrations higher than 1 mM cyanide caused a further, dramatic decrease of k_S and k_{AS}, while the surface concentration of the carrier molecules, N_0, was not affected. This cyanide-effect was also reversible, but not light-dependent. Measurements of the ATP level showed that 3 mM cyanide reduced the ATP level by about 70% both under light and dark conditions. In the presence of 30 μM cyanide (or 10 μM antimycin A) the ATP level decreased by about 50%, but only in the dark. These results suggest two different effects of cyanide on the Cl^--carrier system: in the micromolar concentration range cyanide (and antimycin A) reduced predominantly the translocation of the free carrier by inhibition of ATP synthesis by oxidative phosphorylation, whereas in the millimolar concentration range cyanide apparently inhibits the translocation rates of both the free and charged carriers by its binding to the carrier. The results provide some evidence that the chloride transport of *V. utricularis* could be coupled to metabolic energy but it is an open question whether it is a pump or not.

Keywords: Electrogenic ion transport; Metabolic inhibition; Cyanide; Antimycin A; Azide; Turgor pressure regulation

1. Introduction

Maintenance of a rather constant turgor pressure enables growing plant cells – among other things – a constant rate of growth provided that other environmental factors are favorable [1–7]. Cells subjected to changing salinities would especially benefit by having a negative feedback mechanism for controlling their turgor pressures. In the giant marine alga *Valonia* turgor pressure and, in turn, vacuolar osmotic regulation is achieved by adjusting the uptake and release of K^+-ion in response to hydrostatic pressure changes [8,9]. The Cl^--fluxes are presumably also regulated directly by turgor pressure (unpublished data). The question is by what mechanism pressure signals are transformed into transport processes. Elucidation of the turgor pressure sensor in *Valonia* will certainly also contribute to a better understanding of turgor-, volume- and osmoregulation phenomena observed in other walled cells [10–13].

There is strong evidence from charge-pulse and voltage-clamp experiments on cells of *V. utricularis* that a Cl^--transport system is localized within the plasmalemma [14–17]. The transporter is electrogenic and contributes to the electrical properties of the cell membrane, which means that charge transfer occurs with chloride transport. The replacement of chloride by the large organic anion Mes strongly decreased membrane conductance and retarded the relaxations of charge-pulse and voltage-clamp experiments. The tonoplast does not contribute to the voltage

Abbreviations: ASW, artificial sea water; CCCP, carbonyl cyanide M-chlorophenylhydrazone; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; DNP, 2,4-dinitrophenol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); Mes, 2-(*N*-morpholino)ethanesulfonate.

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relaxations under charge-pulse [14] and to the current relaxations at voltage-clamp conditions [15]. Furthermore, harmonic system analysis of the chloride transport system has provided strong evidence that the vacuolar membrane has a low resistance [16]. The chloride transport system could reasonably well be explained by a Läuger-model [18] with some minor modifications [14]. It is possible that the transport system is the transformation site of turgor pressure signals. The translocation rate of the mobile, negatively charged carrier molecules exhibited a similar turgor pressure-dependence to the active (energy-dependent) K^+-influx [19]. Recent studies have also shown (unpublished data) that the Cl^--carrier seems to be linked to the K^+-transport system.

The electrogenic Cl^--carrier system in *V. utricularis* has very similar kinetic properties to the chloride pump in *Acetabularia mediterranea* [20–22] and in *Halicystis parvula* [23,24]. The translocation rate of the free carrier in *V. utricularis* was rate-limiting, but voltage-independent, whereas the translocation rate of the charged Cl^--carrier system was voltage-dependent [14]. The carrier increased the apparent capacitance of the membrane and contributed about 90% of the membrane conductance [16,25,26]. Since *Acetabularia, Halicystis and Valonia* are related species, it can be speculated whether the electrogenic Cl^- transport system in the plasmalemma of *V. utricularis* is also an active chloride pump.

However, despite much effort there is still no consistent evidence whether or not the Cl^- transport in *V. utricularis* is an active process [14,17,27,28]. The reason for this is that the cytosol of *V. utricularis* is not accessible for the direct determination of the electrochemical potential of chloride. Furthermore, in contrast to the electrogenic ion pumps in other plant cells (which hyperpolarize the membrane potential beyond the limit of the diffusion potential, see, e.g., [20,29–31]), the electrogenic Cl^--carrier system of *V. utricularis* does not apparently contribute significantly to the vacuolar membrane potential.

Therefore, for a first approach it is necessary to answer the question whether the electrogenic Cl^--carrier in *V. utricularis* is ATP-dependent or not. In this communication we therefore investigated the effects of cyanide, antimycin A and azide on the kinetic parameters of the chloride carrier in the presence and absence of light by using the charge pulse technique. The results show (in combination with measurements of the ATP level) that the translocation of the free carrier is strongly ATP-dependent. In addition, higher (millimolar) concentrations of CN^- bind to the carrier and dramatically reduce its mobility.

2. Materials and methods

2.1. Plant material

Cells of the giant marine alga *Valonia utricularis* were collected during the summer months from the coast at Ischia (Gulf of Naples, Italy) and kept in Mediterraneansea water at 18–19°C under a 12 h light/dark period (25 W Fluora lamps from Osram, München, Germany). Single cells of nearly elliptical shape (adapted for 2–4 months to laboratory conditions) were clamped in a small plexiglas™ chamber and perfused with *a*rtificial *s*ea *w*ater (ASW) containing 545 mM NaCl, 12 mM KCl, 11 mM $CaCl_2$ and 10 mM $MgCl_2$. The pH was buffered at 8.1 by addition of 10 mM Hepes/NaOH. The surface area of the cells was between 31 to 69 mm^2, as calculated from their dimensions under the microscope.

2.2. Extraction and assay of ATP

The ATP content of single cells of *V. utricularis* was determined by the luciferin-luciferase assay. After incubation in ASW or after treatment with the inhibitors for 30 min, a given cell was transferred to 0.5 ml of a 6% perchloric acid ($HClO_4$) solution, mashed with tweezers and then extracted for 30 min at 0°C. After neutralization of the sample with about 0.5 ml of 1 M KOH, the cell wall and $KClO_4$ were removed by centrifugation of the sample at $13\,000 \times g$ for 5 min. The pH of the sample was adjusted to 7.8 by addition of 50 mM Tricine/KOH buffer. Luciferin-luciferase assays were carried out by using the ATP Bioluminescent Assay Kit (Sigma, 82041 Deisenhofen, Germany). The ATP-dependent luminescence was measured with a microprocessor-controlled photon counter (Lumac™/3M Biocounter, Model 2010A, Perstorp Analytical GmbH, 63085 Rodgau, Germany). The ATP concentration in a given sample was determined and corrected by means of an internal ATP standard (10–100 pM).

2.3. Electrical set-up

The charge pulse technique has been described in detail elsewhere [19,26]. Briefly, the intracellular current electrode consisted of a 10 μm thick platinum wire. The wire was moved a considerable distance into the vacuole through a microcapillary (outer tip diameter 30 μm) after impalement of the cell. The shank of the microcapillary was sealed by a rubber 'O'-ring to an oil-filled plexiglas™ chamber in which a pressure transducer was mounted (for recording the turgor-pressure, see [9,32–34]. The platinum wire was connected to a commercial, fast pulse generator (model 214 B, Hewlett Packard, Palo Alto, CA, USA) through a diode with a reverse resistance larger than 10^{10} Ω. The membrane was charged with a short, rectangular pulse of 1 μs duration. The injected charge was calculated from the voltage drop (observed on a digital oscilloscope, model 2440, Tektronix, Beaverton, OR) across a 10 Ω resistor connected in series with the platinum wire. The intracellular potential-recording electrode (3 M KCl, Ag/AgCl) was connected to a fast, differential amplifier of high input impedance. The data points were stored on a

Nicolet 2090 digital oscilloscope (6000 Frankfurt, Germany), then transferred to a PC/AT computer and analysed with a multiple-exponential-fitting program.

Two external reference electrodes of extremely large surface area were used, one for injection of the current pulses (rectangular steel plate, dimensions 28 mm × 8 mm) and one for recording of the membrane potential (Ag/AgCl, 3 M KCl, agar bridge). The electrodes were placed close to the surface of the alga.

A fiber-optic light source (model KL 150B, Schott, Mainz, Germany) containing a halogen lamp (15 V, 150 W; Osram, München, Germany), a heat protecting filter KG 1 and a fiber-optic bundle (Schott, Mainz, Germany) was used to illuminate the cells during the experiments with white light (40 W m^{-2}).

2.4. Experimental procedure

Experiments were started 1.5–2 h after insertion of the two intracellular electrodes. This time was sufficient to heal the punctured areas as shown by the reproducibility of the charge pulse relaxation pattern.

If not stated otherwise, the effects of the metabolic inhibitors on the voltage relaxation pattern were investigated at 20°C in the dark in order to eliminate ATP synthesis by photophosphorylation. The light was turned off about 1 h before the experiment. Cyanide was added to the perfusion medium (ASW) by using appropriate amounts of a 1 or 3 M aqueous stock solution of KCN (depending on the desired final concentration). The pH was re-adjusted when necessary. As the pK_a of HCN is 9.2, the fraction of cyanide present as CN^- is about 8% at pH 8.1. Antimycin A was pre-dissolved in acetone. A portion of a stock solution of 10 mM antimycin A was added to the ASW to give a concentration of 10 μM. The final concentration of acetone was 0.1%. Control experiments showed that the addition of 10 mM K^+-ions or of 0.1% acetone to the ASW-medium had no significant effects on the membrane electrical properties. It is important to note that in 10 successive experiments taken on the same algal cell at time intervals of 30 s under given conditions, the time constants and the amplitudes of the membrane voltage decay did not vary by more than 3%. This indicates that the results obtained on a single cell were highly reproducible. However, large variations were observed from cell to cell, as is usual for *V. utricularis* [14,19].

2.5. Analysis of the voltage relaxation data

We used here the Läuger-model [18,35] for the description of the chloride transport in *V. utricularis*. This model has been described in detail in previous publications [36,37,14]. The interested reader is referred to the review by Läuger et al. [38] for a complete description. Here only the basic assumptions of the model and the basic equations are listed, which are needed for the calculation of the kinetics of carrier-mediated chloride transport in the plasmalemma of *V. utricularis*. The model assumes a 1:1 carrier-chloride 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 $K = k_R/k_D$. In a previous study we have demonstrated that the interfacial complexation is always much faster than all the other reactions involved in carrier-mediated chloride transport. The translocation of the free and charged carriers, k_S and k_{AS}, respectively, are symmetrical and follow first order kinetics. The voltage decay after a charge pulse was fitted to a double-exponential decay function. By this means the initial voltage, V_0, the time constants τ_1 and τ_2, and the relative amplitudes a_1 and a_2, were obtained [14,26]:

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

where V_0 is the initial voltage to which the membrane was charged. The sum of the relative amplitudes of the two relaxation curves are given by:

$$a_1 + a_2 = 1 \quad (2)$$

The significance of the fit to the experimental data was checked using Student's t-test. Under all experimental conditions, the fit was very significant ($P < 0.001$).

The specific membrane capacity, C_m, was obtained from the injected charge, Q and the initial voltage V_0:

$$C_m = Q/(V_0 \cdot A) \quad (3)$$

where A is the surface area of the alga.

The specific membrane resistance, R_m (or the specific membrane conductance, G_m) can be calculated from the specific membrane capacitance, the amplitudes and time constants as shown by Benz and Zimmermann [26]:

$$R_m = 1/G_m = (\tau_1 a_1 + \tau_2 a_2)/C_m \quad (4)$$

The kinetic analysis of the biphasic voltage relaxation pattern (Eq. (1)) in terms of the Cl^--carrier model in Fig. 1 is based on the assumptions that (a) the interfacial reaction

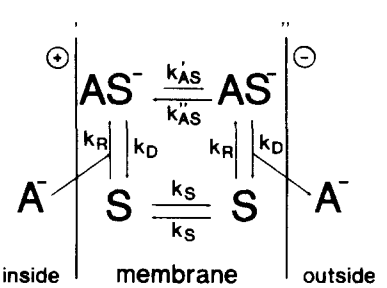

Fig. 1. Diagram illustrating the mechanism by which the Cl^--carrier system transports anions across the plasmalemma of *Valonia utricularis* (the Läuger-model). The symbols have the following meaning: The stability constant of the Cl^--carrier complex, AS, is given by $K = k_R/k_D$ where the rate constants k_R (association) and k_D (dissociation) refer to the heterogeneous complexation reaction between the anion (Cl^-), A, and the carrier, S. The rate constant k_S refers to the movement of the free carrier, whereas the voltage-dependent rate constants k'_{AS} and k''_{AS} refer to the translocation of the Cl^--carrier complex within the membrane.

between the anion and the carrier molecules is so fast compared to the other reaction steps that equilibrium for the binding reaction can always be assumed, (b) that the tonoplast membrane is highly conductive, and (c) that the majority of the membrane conductance, as well as the biphasic decay of the initial voltage, was attributable to the Cl^--carrier system in the plasmalemma (for experimental proof and details, see [14–17]).

As shown previously [14] the real rate constants k_{AS}, k_S and the stability constant, K, for the binding of Cl^- to the carrier cannot be obtained from a single charge-pulse experiment. However, they can be calculated by measuring the reduced rate constants of the charged, K_{AS}, and free carrier molecules, K_S, as functions of the Cl^--concentration, c. K_{AS} and K_S are defined by the following equations:

$$K_{AS} = k_{AS} K \cdot c/(1 + K \cdot c) \quad (5)$$

and

$$K_S = k_s/(1 + K \cdot c) \quad (6)$$

The validity of Eqs. (5) and (6) has been checked in a previous study [14].

These kinetic parameters and the total surface concentration of carrier molecules, N_0, are related to the relaxation parameters as follows:

$$K_{AS} = (P_1 - P_3 - P_2/P_3)/2 \quad (7)$$

$$K_S = P_2/(2 \cdot P_3) \quad (8)$$

$$N_0 = P_3/(2 \cdot B \cdot K_{AS}) \quad (9)$$

where

$$P_1 = 1/\tau_1 + 1/\tau_2 \quad (10)$$

$$P_2 = 1/(\tau_1 \cdot \tau_2) \quad (11)$$

$$P_3 = a_1/\tau_1 + a_2/\tau_2 \quad (12)$$

$$B = z^2 \cdot F^2/(4R \cdot T \cdot C_m) \quad (13)$$

and where $z(= -1)$ is the valency of the Cl^--carrier complex; F, R and T have their usual meanings.

3. Results

3.1. Effect of cyanide on the voltage relaxation pattern

Curve 1 in Fig. 2 shows the typical biphasic voltage decay recorded on a cell of *V. utricularis* under standard conditions (ASW, pH = 8.1, T = 20°C). After charging the membrane capacitance (from a vacuolar resting potential of inside −9 mV) by injection of a current pulse of 5.3 nAs amplitude and 1 μs duration, the initially induced voltage (V_0 = 23.2 mV) relaxed in two exponential processes (see the semi-logarithmic plot in the inset of Fig. 2,

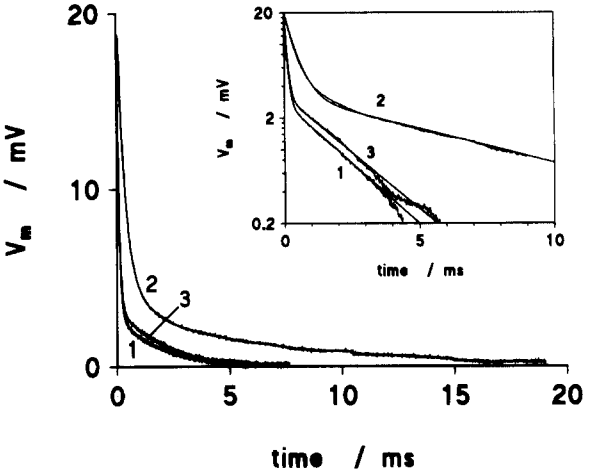

Fig. 2. Effect of 3 mM KCN on the voltage relaxation pattern of a cell of *V. utricularis* (surface area A = 33 mm^2, volume V = 12 mm^3 and turgor pressure P = about 0.2 MPa) after injection of a charge pulse (1 μs duration). Curve 1: control experiment (initial voltage V_0 = 23.2 mV) performed in ASW 1 h after the light was switched off and 1 min prior to the addition of KCN. Curve 2: voltage relaxation pattern (V_0 = 16.9 mV) recorded 15 min after exposure of the cell to KCN. Curve 3: voltage relaxation pattern (V_0 = 22.2 mV) measured 150 min after removal of KCN. Inset: semilogarithmic plots of curves 1 to 3. The voltage decay across the membranes of the cell was fitted to the sum of two voltage relaxations by using the least-squares method and the following parameters for the time constants, τ_1 and τ_2, and for the amplitudes, V_1 and V_2: curve 1: V_1 = 20.0 mV, τ_1 = 88 μs, V_2 = 3.3 mV, τ_2 = 1.89 ms; curve 2: V_1 = 14.1 mV, τ_1 = 395 μs, V_2 = 2.8 mV, τ_2 = 6.72 ms; curve 3: V_1 = 18.6 mV, τ_1 = 91 μs, V_2 = 3.6 mV, τ_2 = 2.04 ms.

curve 1). The fast relaxation had a time constant, τ_1, of 88 μs and a relative amplitude, a_1, of 0.86. The corresponding amplitude, a_2, and the time constant, τ_2, of the slow relaxation were 0.14 and 1.89 ms, respectively. These results are in agreement with previously published data [14,17,19,26,28,39].

Curve 2 in Fig. 2 was obtained on the same *Valonia* cell 15 min after addition of KCN to the perfusion medium (final concentration 3 mM). Comparison of curves 1 and 2 shows that cyanide had a strong influence on the membrane voltage relaxation pattern. Both the fast and the slow relaxation processes were significantly retarded. The time constant, τ_1, increased from 88 μs to 395 μs whilst τ_2 increased from 1.89 ms to 6.72 ms. However, no significant effect of cyanide on the relative amplitude, a_1, could be observed within the limits of experimental accuracy (3%, see Materials and methods), even when the duration of the cyanide treatment was extended to 50 min.

The effect of cyanide on the voltage decay pattern was almost completely reversible upon removal of KCN from the medium. Curve 3 in Fig. 2 was measured on the same *Valonia* cell 150 min after replacement of the KCN solution by KCN-free ASW. Evaluation of the time constants from the logarithmic plot (inset in Fig. 2, curve 3) revealed approximately the original values for the time constants (τ_1 = 91 μs, τ_2 = 2.04 ms). However, compared to the

rapid response of the charge pulse relaxation to the addition of KCN (within 15 min), the recovery process was much slower and required about 120 min.

Similar effects of cyanide on the charge pulse relaxation parameters were also obtained for other cells of *V. utricularis* (Table 1). After the addition of KCN τ_1 increased on average by a factor of 4.4 from 95 ± 32 μs to 417 ± 114 μs and τ_2 by a factor of 3.3 from 2.10 ± 0.65 ms to 6.92 ± 2.02 ms. The corresponding average data after removal of KCN were $\tau_1 = 99 \pm 29$ μs and $\tau_2 = 1.80 \pm 0.30$ ms. The average relative amplitude, a_1, did not show a significant change (in ASW: 0.84 ± 0.03; in cyanide: 0.84 ± 0.03; in ASW again: 0.83 ± 0.02).

As indicated in Table 1, KCN decreased the specific membrane conductance, G_m, by about 65% from 1.82 ± 0.32 to 0.62 ± 0.16 mS cm^{-2}. Replacement of the cyanide containing medium by ASW resulted in the restoration of the original G_m value within the limits of accuracy (2.04 ± 0.50 mS cm^{-2}). In contrast to G_m, the specific membrane capacitance, C_m, was not affected by cyanide (ASW: 0.73 ± 0.05 μF cm^{-2}, cyanide: 0.82 ± 0.04 μF cm^{-2} and ASW again: 0.77 ± 0.09 μF cm^{-2}).

The membrane potential, V_m, showed a large scatter. However, the average values after addition ($+7.3 \pm 8.8$ mV) and removal ($+4.4 \pm 7.5$ mV) of KCN were not significantly different from the average control value ($+2.4 \pm 6.4$ mV). Variations in the membrane potential are not unusual for *V. utricularis* as shown previously [19,26]. In all investigated cells the turgor pressure, P remained constant during the experimental period of up to 3 h (in ASW: 0.18 ± 0.02 MPa; in cyanide: 0.19 ± 0.02 MPa; in ASW again: 0.16 ± 0.4 MPa).

Table 1
Effect of 3 mM KCN on the charge pulse relaxation parameters, membrane conductance, capacity and potential of algal cells of *Valonia utricularis*

CPE	[KCN] (mM)	τ_1 (μs)	τ_2 (ms)	a_1	G_m (mS cm^{-2})	C_m (μF cm^{-2})	V_m (mV)	P (bar)
Cell 1								
A	0	77	2.88	0.85	1.34	0.7	−3	1.6
B	3	443	3.49	0.84	0.92	0.8	5	1.7
C	0	78	2.03	0.82	1.89	0.8	2	1.3
Cell 2								
A	0	176	1.40	0.84	1.73	0.7	9	1.5
B	3	696	7.22	0.85	0.46	0.8	13	1.6
C	0	172	1.53	0.82	1.72	0.7	14	1.5
Cell 3								
A	0	86	2.92	0.87	1.75	0.8	12	2.0
B	3	434	6.27	0.90	0.81	0.8	20	2.0
C	0	73	1.20	0.86	3.14	0.7	17	1.7
Cell 4								
A	0	88	1.89	0.86	2.03	0.7	−9	1.9
B	3	395	6.72	0.83	0.65	0.9	4	2.0
C	0	91	2.04	0.84	1.79	0.7	−8	1.7
Cell 5								
A	0	77	1.74	0.86	2.27	0.7	7	1.8
B	3	560	8.42	0.85	0.50	0.9	22	1.9
C	0	85	1.87	0.84	2.61	1.0	6	1.6
Cell 6								
A	0	101	1.42	0.77	2.07	0.8	3	1.8
B	3	311	4.10	0.77	0.68	0.8	1	1.6
C	0	100	1.85	0.78	1.62	0.8	5	0.4
Cell 7								
A	0	101	1.62	0.82	2.08	0.8	0	1.9
B	3	401	9.05	0.86	0.57	0.8	−1	1.9
C	0	105	1.62	0.83	1.97	0.7	0	1.8
Cell 8								
A	0	66	2.97	0.84	1.39	0.7	2	2.0
B	3	309	9.15	0.85	0.48	0.8	2	2.1
C	0	99	2.17	0.84	1.74	0.8	2	2.4
Cell 9								
A	0	86	2.02	0.84	1.72	0.7	1	1.7
B	3	211	7.87	0.84	0.53	0.8	0	1.9
C	0	91	1.92	0.83	1.84	0.7	2	2.0

Charge pulse experiments (CPE) were carried out on nine different cells in ASW shortly before (A), 15 min after addition of cyanide (B) and 120 min after its removal (C). Experiments were performed in the dark. The time constants of the fast (τ_1) and the slow relaxations (τ_2) and the relative amplitude of the fast relaxation (a_1) were obtained from the least-squares-fit of the voltage decay to two exponential relaxations; the specific membrane capacitance C_m and the specific conductance G_m were calculated according to Eqs. (3) and (4) (for more details, see text). P is the turgor pressure in the cell and V_m is the membrane potential.

3.2. Evaluation of the kinetic parameters of the electrogenic Cl^- transport in the presence of cyanide

The semi-logarithmic plots of the biphasic relaxation curves (inset in Fig. 2) show that the relaxation pattern of the cell could always be fitted to the sum of two exponential relaxations (Eq. (1)). A third relaxation could not be resolved before or after addition of KCN. The absence of a third relaxation, therefore, confirmed the adequacy of the model illustrated in Fig. 1 and justified the evaluation of the kinetic parameters of the Cl^--transport from the voltage relaxation pattern of cyanide-treated cells of *V. utricularis* according to the Eqs. (7)–(13).

As indicated by Table 2, cyanide significantly affected the translocation rates of the carrier. On average, the reduced translocation rate, K_{AS}, of the charged carrier decreased by 77% from 799 ± 216 s^{-1} to 184 ± 93 s^{-1}, and that of the free carrier, K_S, by 69% from 307 ± 94 s^{-1} to 94 ± 39 s^{-1}. After removal of KCN, K_{AS} and K_S increased again to 882 ± 278 s^{-1} and 325 ± 100 s^{-1} respectively. The real rate constants, k_{AS} and k_S, (given by $k_{AS} = K_{AS}(1 + K \cdot c)/(K \cdot c)$ and by $k_S = K_S(1 + K \cdot c)$, respectively) decreased correspondingly when KCN was added. The values in Table 2 were calculated by using Eqs. (5) and (6) and by assuming a binding constant of $K = 0.34$ M^{-1} and a Cl^--concentration of $c = 0.599$ M [14]. In contrast, the surface concentration of the carrier, N_0, remained unaltered within the limits of accuracy (in ASW: 4.6 ± 0.7 pmol cm^{-2}; in cyanide: 5.7 ± 1.6 pmol cm^{-2}; in ASW again: 4.5 ± 0.7 pmol cm^{-2}).

3.3. Time-dependence of the time constants and the translocation rates of cyanide-treated cells

Because of the large scatter of the values of the time constants of the individual cells under control conditions

Table 2
Effect of 3 mM KCN on the kinetic parameters of the electrogenic Cl^--carrier system in the plasmalemma of *Valonia utricularis*

CPE	[KCN] (mM)	K_{AS} (10^3 s^{-1})	K_S (s^{-1})	N_0 (pmol cm^{-2})	k_{AS} (10^3 s^{-1})	k_S (s^{-1})
Cell 1						
A	0	0.89	203	4.3	5.26	244
B	3	0.13	166	6.6	0.77	200
C	0	1.05	297	4.3	6.21	357
Cell 2						
A	0	0.34	415	4.8	2.01	500
B	3	0.09	80	5.9	0.53	96
C	0	0.41	392	4.5	2.42	472
Cell 3						
A	0	0.69	195	6.0	4.08	235
B	3	0.10	88	9.2	0.59	106
C	0	0.84	480	5.4	4.96	578
Cell 4						
A	0	0.72	305	5.0	4.26	367
B	3	0.19	88	5.7	1.12	106
C	0	0.80	290	4.4	4.73	349
Cell 5						
A	0	0.84	333	5.0	4.96	401
B	3	0.11	69	6.1	0.65	83
C	0	0.87	317	5.9	5.14	382
Cell 6						
A	0	0.96	448	3.5	5.67	539
B	3	0.31	156	3.4	1.83	188
C	0	0.95	339	3.4	5.61	408
Cell 7						
A	0	0.76	370	4.4	4.49	445
B	3	0.16	64	5.3	0.95	77
C	0	0.71	368	4.3	4.20	443
Cell 8						
A	0	1.12	199	4.3	6.62	239
B	3	0.22	64	5.0	1.30	77
C	0	1.45	108	4.2	8.57	130
Cell 9						
A	0	0.87	294	4.1	5.14	354
B	3	0.35	75	4.5	2.07	90
C	0	0.86	330	4.2	5.08	397

The kinetic parameters of the *Valonia* Cl^--transporter were derived by analysis of the experimental relaxation data given in Table 1, using the carrier-mediated transport model of Fig. 1. The reduced rate constants of the translocation of the charged, K_{AS}, and of the free carriers, K_S, as well as the total surface concentration of the carriers, N_0, were calculated by using Eqs. (7)–(13). Eqs. (5) and (6) were used to calculate the real rate constants of the translocation of the charged, k_{AS}, and free carriers, k_S (thereby assuming a binding constant of $K = 0.34$ M^{-1} and a Cl^--concentration of $c = 0.599$ M [14]. The symbols CPE, A, B and C have the same meaning as shown in Table 1.

(Table 1) the effect of cyanide as a function of time was quantitatively evaluated by normalising τ_1 and τ_2 to the control values (τ_1^* and τ_2^*) for a given cell. The mean values of τ_1/τ_1^* and τ_2/τ_2^* were plotted as a function of time (obtained on 6 cells, data not shown). Fig. 3 shows the corresponding normalized (reduced) translocation rates of the charged Cl^--carrier complexes (K_{AS}/K_{AS}^*) and that of the free carrier (K_S/K_S^*) as calculated from the experimental data. It is obvious that an effect of cyanide on the normalised time constants and kinetic parameters could be recorded within 1 min after addition of KCN. The half-time of the cyanide-effect was about 150 s. The final stationary values were reached within 15 min.

3.4. Concentration-dependence of the cyanide-effect

Voltage relaxation measurements in micro- and millimolar concentrations of cyanide revealed that this metabolic inhibitor apparently interacts in two distinct ways with the Cl^--carrier system. Cyanide-concentrations as low as 10 μM measurably influenced τ_2 and slightly higher values (about 30 μM) also affected τ_1. Both time constants increased as rectangular hyperbolic functions of cyanide-concentration until plateau values were reached (at about 300 μM for τ_2 and at about 1 mM for τ_1, data not shown). Above 1 mM for τ_1 and 300 μM for τ_2 the time constants increased again with increasing cyanide-concentration. This response pattern of the time constants shows that the cyanide-interaction with the Cl^--carrier could be separated into two different components, i.e., a high-affinity effect at relatively low and a superimposed low-affinity effect at higher concentrations. This is also supported by the corresponding data of the translocation rates, K_{AS} and K_S, plotted in Fig. 4A and B. For the 'high-affinity' concentration range (shown in the insets of Fig. 4 A and B) the apparent Michaelis constant, K_m, (i.e., the concentration at which 50% inhibition of the translocation rates is observed) is about 10 μM for K_S and 50 μM for K_{AS}. The corresponding values for the millimolar concentration range are obviously much higher. However, their exact values cannot be given because even 10 mM cyanide did not reach a plateau value.

A similar dependence of the time constants and the translocation rates were also found for other *V. utricularis* cells, except that the inhibitory influence of micromolar cyanide-concentrations on K_{AS} (and also on τ_1) was not always so pronounced as indicated in Fig. 4. However, in all cells investigated a pronounced effect of the inhibitor on τ_2 and K_S was found. No effect of cyanide on the relative amplitude, a_1, or on the surface concentration of the carrier molecules, N_0, could be detected over the whole concentration range.

3.5. The light-dependence of the 'high- and low-affinity' cyanide-effects

When the cells were bathed in ASW no significant change of τ_1 and K_{AS} occurred upon illumination (Fig. 5, columns I). However, τ_2 decreased slightly from 2.10 ± 0.65 ms to 1.50 ± 0.50 ms and correspondingly K_S increased from 307 ± 95 s^{-1} to 428 ± 133 s^{-1}. Illumination had no effect on the inhibition of K_{AS} and K_S exerted by 3 mM cyanide (see Fig. 5, columns III and also Tables 1 and 2). τ_1 increased from 103 ± 27 μs to 427 ± 198 μs and τ_2 from 1.50 ± 0.50 ms to 5.58 ± 1.98 ms. Correspondingly, K_{AS} and K_S decreased by about 70%. On the other hand, the strong inhibition of K_S exerted by 30 μM cyanide in the dark could be essentially reversed by illumination (Fig. 5, columns II).

3.6. Effect of antimycin A and azide

The dark-dependence of the high-affinity effect of cyanide suggest that metabolic inhibition is the primary

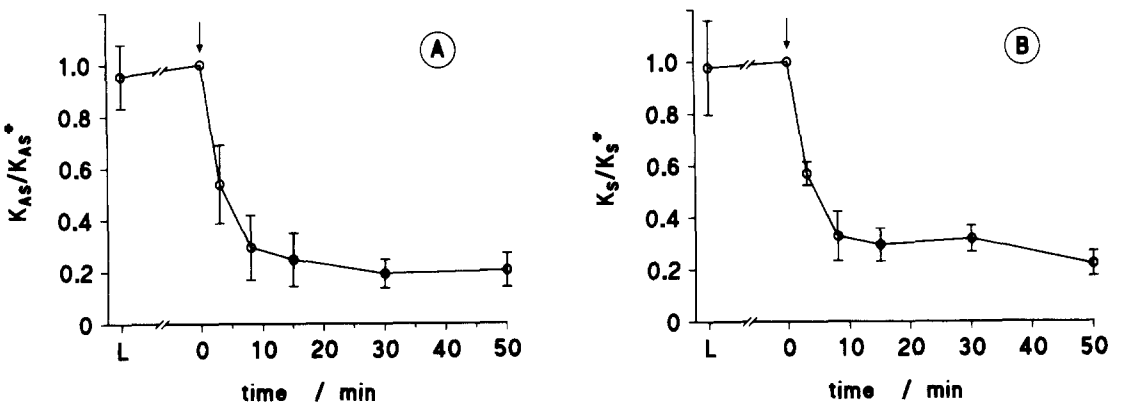

Fig. 3. Time-course of cyanide-inhibition of the normalized, reduced translocation rates of the charged Cl^--carrier complex, K_{AS}/K_{AS}^* (A) and of the free carrier, K_S/K_S^* (B). For definitions of the reduced translocations rates, see Materials and methods. Data were calculated from the experimental data by using Eqs. (7)–(13). The data points represent the means $\pm$ S.D.$_{n-1}$ of 6 experiments performed on different cells. The rate constants measured in ASW with 3 mM KCN were normalised to the rate constants (*) recorded for the respective cell in ASW without KCN 1 h after the light was switched off and shortly before addition of KCN (arrow). The data points marked by L represent the experiments recorded 1.5–2 h after impalement of the cells shortly before the light was switched off.

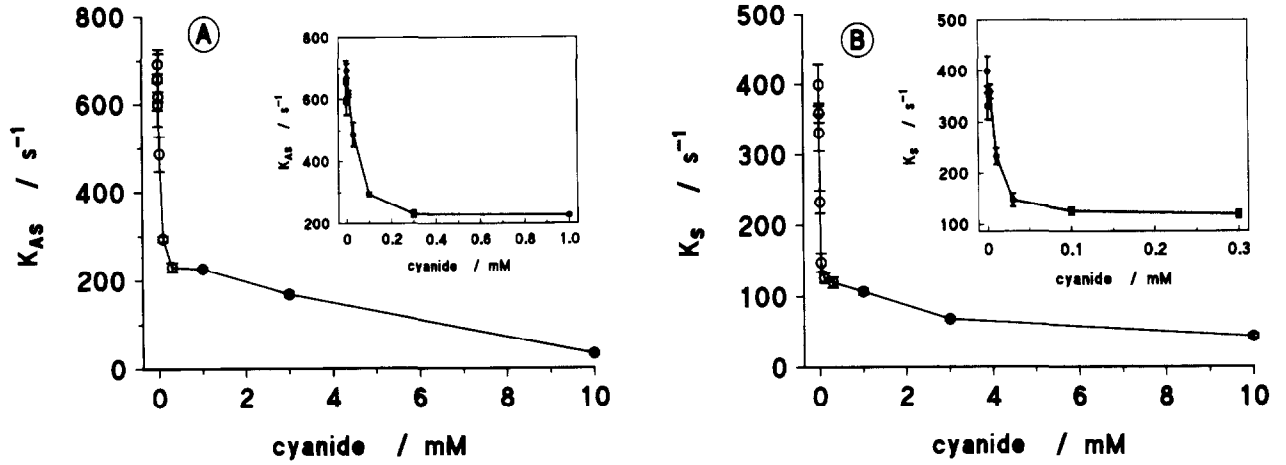

Fig. 4. Concentration-dependence of the cyanide-inhibition of the translocation rates of the charged Cl^--carrier complex, K_{AS} (A) and of the free carrier, K_S (B). The kinetic parameters were calculated by using Eqs. (7)–(13) from the experimental data measured on an algal cell ($A = 37$ mm^2, $V = 15$ mm^3 and $P = 0.2$ MPa). Experimental conditions: 2 h after impalement of the cells with the microelectrodes the light was turned off. 1 h later and immediately before the first addition of KCN, 4 charge pulse relaxations were performed at intervals of 1 min as controls. The concentration of cyanide was increased stepwise by addition of appropriate amounts of KCN to the perfusion medium. Each data point represents the mean $\pm$ S.D.$_{n-1}$ of 4 charge pulse experiments performed 10 min after each increase of the KCN concentration (the interval between the measurements was 1 min). Insets: magnified plots of the low cyanide-concentration range (A: cyanide < 1 mM; B: cyanide < 0.3 mM).

mechanism of the changes in the translocation rates of the carrier system. Therefore, it can be expected that the metabolic inhibitor antimycin A, which is not expected to bind the Cl^--carrier, should have a comparable effect on the relaxation pattern to that of 30 μM cyanide. Fig. 6 shows that indeed antimycin A inhibited the transport system only in the dark. τ_2 increased on average from 1.56 ± 0.55 ms to 3.36 ± 0.87 ms and K_S correspondingly decreased from 413 ± 168 s^{-1} to 181 ± 51 s^{-1} (about 50%) when 10 μM antimycin A was added in the dark. τ_1 and K_{AS}, which were only affected slightly by low concentrations of cyanide, did also not change significantly in the presence of antimycin A. The similarity of the effects of the two inhibitors support the hypothesis that the translocation rate constants of the carrier depend on the ATP level.

In addition to experiments with cyanide, which may interact with the chloride transporter and iron-containing proteins, azide is also used to block the ATP-synthesis. The addition of 3 mM azide to the ASW led to a specific increase of the slow relaxation time constant, τ_2, by a factor of about three. Simultaneously, the rate constant K_S decreased to 35% of its initial value. This effect was reversible when NaN_3 was removed from the ASW. In general, the effects of different concentrations of azide (1–10 mM) were similar to those observed with antimycin A and small concentrations of cyanide (< 300 μM). It is noteworthy, however, that the effect of azide on the chlo-

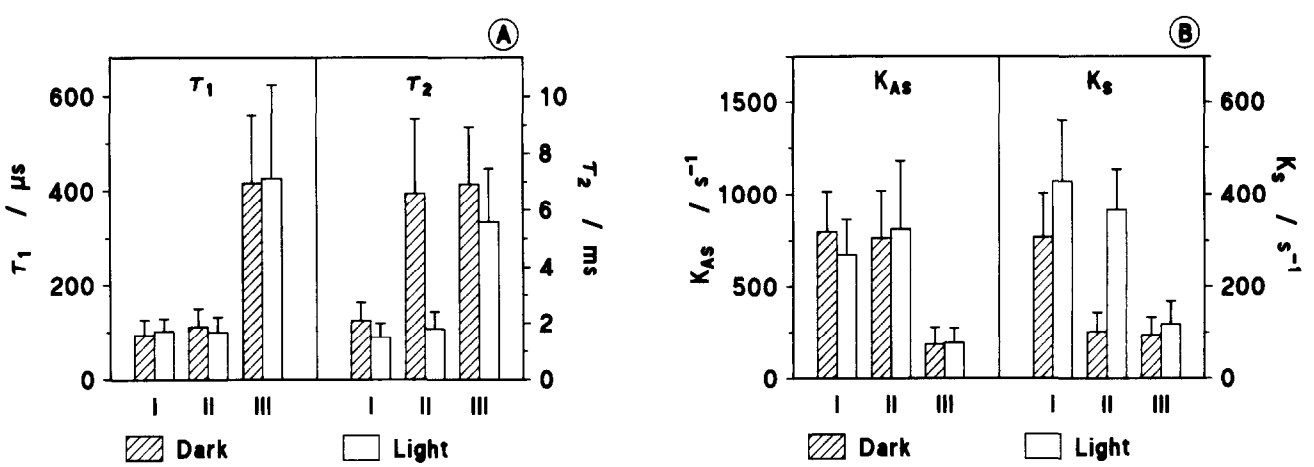

Fig. 5. Light-dependence of the cyanide-effects on the time constants, τ_1 and τ_2, (A) and on the translocations rates, K_{AS} and K_S, (B). The columns represent the means $\pm$ S.D.$_{n-1}$ of 3–9 experiments on different cells. I: control measurements in ASW without cyanide were performed immediately before addition of KCN; II and III: measurements in the presence of 30 μM and 3 mM KCN, respectively 15 min after addition of the inhibitor. The open columns represent experiments conducted under illumination, the hatched columns experiments conducted in the dark 1 h after turning off the light.

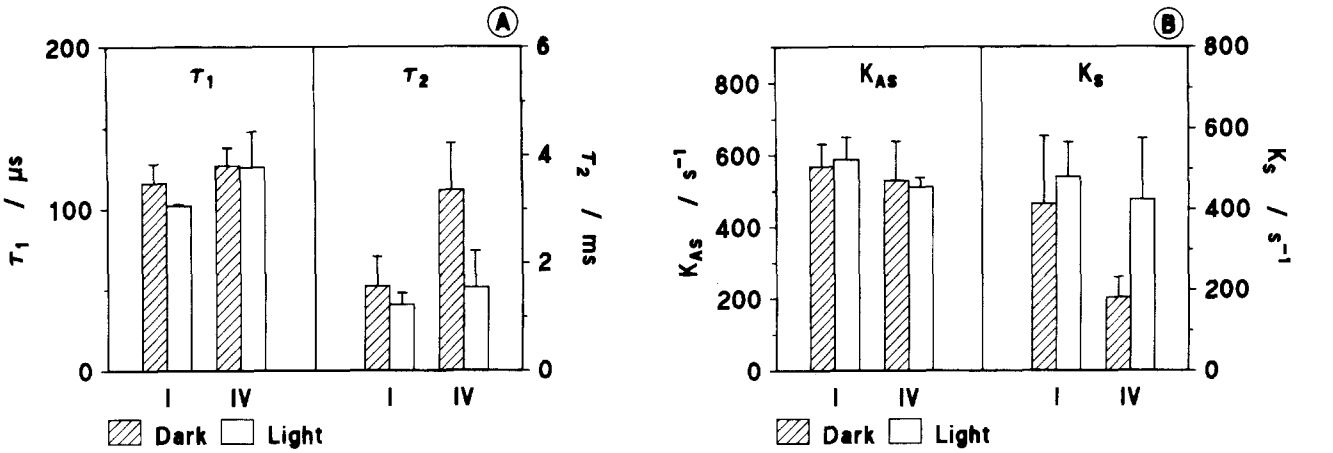

Fig. 6. Effect of antimycin A on the time constants (A) and on the translocations rates (B) in the light (open columns) and in the dark (hatched). The data represent the means ± S.D.$_{n-1}$ of 3 experiments using different cells. I: control measurements performed immediately before addition of antimycin; IV: data obtained after 15 min incubation in ASW containing 10 µM antimycin A.

ride transporter were light-independent, which indicated that azide inhibited in *V. utricularis* both photo- and oxidative phosphorylation.

3.7. ATP level in the presence of metabolic inhibitors

The average ATP content in algal cells bathed in ASW was determined to be 0.6 ± 0.2 mM (n = 10; calculated by assuming the cytoplasmic to be 8 µm thick, see [40]) both under light and dark regime (Fig. 7, column I). The value of the ATP level was comparable to that measured, e.g., in *Characeen* (0.7 mM, [41]), in guard cells of *Vicia faba* (1.3 mM, [42]) and in *Neurospora crassa* (2.6 mM, [29]). Addition of 30 µM cyanide (Fig. 7, columns II) markedly lowered the ATP level in the dark (0.3 ± 0.1 mM, n = 8), but not in light (0.6 ± 0.2 mM, n = 6). This result agreed with the charge pulse measurements, which demonstrated

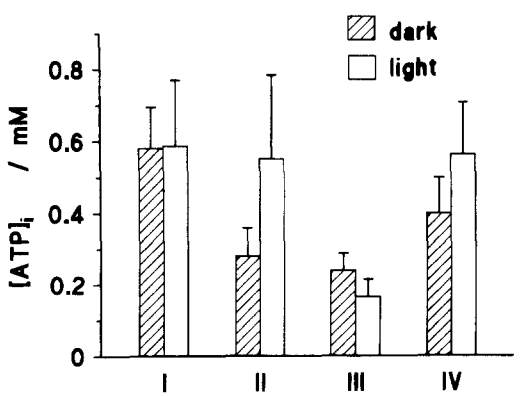

Fig. 7. Effects of cyanide and antimycin A on the ATP level of cells of *V. utricularis* in the light (open columns) and in the dark (hatched). The data are means ± S.D.$_{n-1}$ of 5 to 10 experiments performed on different algal cells. I: controls in ASW without the inhibitors (see Figs. 5 and 6), II and III measurements conducted after 30 min incubation with 30 µM and 3 mM KCN, respectively, and IV: experiments performed after 30 min incubation with 10 µM antimycin A. For experimental details, see text.

that 30 µM cyanide only caused a decrease of the translocation rate of the free carrier in the dark.

The effect of 10 µM antimycin A on the ATP level of cells of *V. utricularis* was comparable to that observed in the presence of 30 µM cyanide (Fig. 7, columns IV). However, on average the reduction was less (0.4 ± 0.1 mM, n = 5). This was consistent with the finding that the translocation rate of the free carrier was only reduced by 50% in the presence of antimycin A as already mentioned above.

In the presence of 3 mM cyanide the ATP level decreased by about 60% in the dark, and by about 70% in the light (Fig. 7, columns III). Similar reductions of the ATP content are reported for other algae, fungi, higher plant and animal cells, when cyanide was added in millimolar concentrations (e.g., [29,41–47]).

3.8. Effect of cyanide and antimycin A on vacuolar pH

The cytosolic pH of *Neurospora crassa* has also been shown to be dependent on the cytosolic ATP-level [48]. The depletion of ATP leads in this case to a strong decrease of the cytosolic pH, which in turn may influence the transport systems. Similarly, a lowering of the external pH has a strong influence on the chloride carrier in *V. utricularis* [16,26]. To test whether the external addition of KCN has a similar effect on the pH inside the algal cells we measured the vacuolar pH in *V. utricularis* cells, which had been preincubated in 30 µm or 3 mm cyanide. Surprisingly we did not observe any decrease of the vacuolar pH. Instead, the pH increased slightly from 6.3 ± 0.2 (n = 10) to 7.3 ± 0.3 (n = 10) at 30 µm cyanide in the dark, to 7.1 ± 0.4 (n = 10) in the light and to 7.0 ± 0.5 (n = 10) at 3 mM. The addition of 10 µm antimycin a had no effect on vacuolar pH. This means that the effect of these compounds on the kinetics of the chloride carrier were not trivial pH-effects.

4. Discussion

4.1. Validity of the Läuger-model

In a previous study we presented evidence that the electrogenic transport system in the cell membrane of *V. utricularis* is a chloride carrier [14]. For the interpretation of the experimental data we used a simple model, the Läuger-model [18]. This model assumes a symmetric carrier and voltage-dependence only for the charged form. It provides the minimum requirements to explain the results of the voltage-clamp and charge-pulse experiments [14,15], although also more complicated models may explain the experimental data. However, the use of these models would not allow a straightforward analysis of the experimental results. Furthermore, we did not find any indication that the carrier is asymmetric [14,15], nor that the carrier system is highly voltage-dependent [15]. In this investigation we studied the effect of metabolic energy on the chloride transporter. Again the simple model provided an excellent explanation of the experimental results and it was possible to give a quantitative description of the effects of cyanide and antimycin A on the carrier system in the presence and absence of light.

4.2. Experimental possibilities for demonstration of an ATP-driven Cl^--carrier system

An active electrogenic transport process is coupled to metabolic energy [31,49]. Sensitivity of transport systems to conditions that reduce or inhibit ATP synthesis (such as darkness, low temperature, or addition of uncouplers or metabolic inhibitors) are taken as an indication for the presence of active transport. However, conclusions from such experiments are only straightforward for cells showing a resting membrane potential, which is distinctly hyperpolarized beyond the diffusion potential and which changes when ATP synthesis decreases. In *Valonia* the vacuolar resting membrane potential is not more negative than the diffusion potentials of K^+ and Cl^-. On the contrary (and unlike to other marine algae, see [50]), *Valonia* cells display, on average, a small positive, vacuolar resting membrane potential of a few millivolts ($V_m = +2.4 \pm 6.4$ mV, see also [51–53]). As shown here, the membrane potential, the ATP level (Fig. 7) and the kinetic parameters of the electrogenic Cl^--carrier system (Figs. 5 and 6) do not change when illumination ceases. In contrast, the membrane potentials of *H. parvula* and *A. mediterranea* are light- and ATP-dependent [20,23,24,54–56], whereas in other algae (e.g., *Chara*, [30]) the intracellular ATP level was also not changed by darkness. Low temperature modulates the activity Cl^- pump in *H. parvula* and *A. mediterranea*. In *V. utricularis*, the translocation rate of the mobile charges of Cl^--carrier also decreases 10-fold with lowering of the temperature from 18 to 5°C [28]. However, the interpretation of this result is not clear-cut because low temperatures may not only affect metabolism, but also the physical state of the membrane lipids and thus the carrier proteins.

Uncouplers such as CCCP and DNP inhibit ATP-driven membrane transport processes by increasing the H^+-permeability of the energy-conserving membranes of mitochondria and chloroplasts, thereby inhibiting ATP synthesis. However, uncouplers – even at lower concentrations – can also increase the permeability of the plasma membrane for H^+-ions [29,57–60]. In addition, charge pulse relaxation studies on phospholipid bilayer membranes have shown that the uncoupler FCCP produced a similar voltage relaxation pattern to the Cl^--carrier in the plasmalemma of *Valonia* [61,62]. Uncouplers are, therefore, not the optimum tools to explore the ATP – dependence of the Cl^--carrier system in *Valonia*.

Cyanide is often used as a rapid metabolic blocker to differentiate between the energy-dependent and energy-independent components of the membrane potential in algae, fungi, higher plant and animal cells (e.g., [29,31,44–47,49]), and to dissect the whole-cell current-voltage relations into their active and passive components [41–43]. The major reason for using cyanide is that (1) – at least at low concentrations – its action in blocking respiration at cytochrome-*c* oxidase seems to be specific and (2) it yields simple kinetics for changes both in ATP level and in membrane voltage [29,63].

Complications may arise in the presence of an electrogenic anion transport process because CN^- may directly bind to the carrier. This must, however, not necessarily evoke difficulties in the interpretation of the data if the effects of cyanide on the ATP level and on the carrier system can be clearly separated by distinct differences in their sensitivity to the inhibitor. The results reported here demonstrate that this is apparently the case in *V. utricularis*.

4.3. ATP-dependence of the Cl^--carrier system

The biphasic dependence of the time constants and the translocation on increasing cyanide-concentration can be easily explained when we assume that in the micromolar range cyanide inhibit exclusively the oxidative phosphorylation by binding to cytochrome-*c* oxidase, but not to the carrier. The effect of cyanide on the Cl^--carrier only occurs in the dark and is paralleled by a corresponding decrease in the ATP level. Under light conditions photophosphorylation apparently maintains the ATP level constant despite inhibition of respiration. The carrier can, therefore, operate as usual without cyanide. Like other plant cells [30,45,64,65], *Valonia* can apparently keep its ATP level constant exclusively either by respiration or by photosynthesis. Support for the hypothesis that micromolar cyanide-concentrations interact with the Cl^--carrier via inhibition of the oxidative phosphorylation also arise from the finding that antimycin A exhibited a similar, light-de-

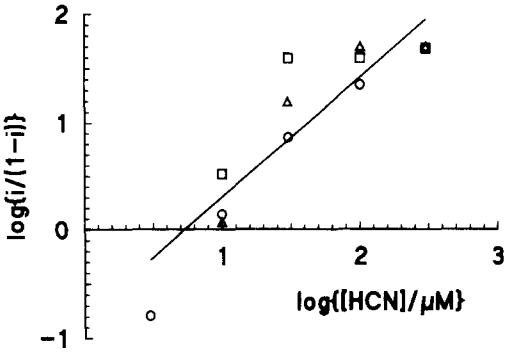

Fig. 8. Hill-Plot of the relative cyanide-inhibition, $i = I/I_{max}$, of the translocation rate of the free Cl^- carrier, K_S, whereby $I = K_S^* - K_S$ and $I_{max} = K_S^* - K_S^{**}$. K_S^* and K_S are the translocation rates in the absence and presence, respectively, of cyanide, whereas K_S^{**} is the value of the translocation rate measured in 300 μM cyanide. The double logarithmic plot of $i/(1-i)$ versus the cyanide concentration yields a linear function, $\log(i/(1-i)) = n\log([HCN]) + n\log(K)$. From the slope of the linear regression the Hill coefficient is calculated to be $n = 1.1$. From the intercept, the apparent binding constant is deduced to be $K = 1.9 \cdot 10^5$ M^{-1}. Data were calculated from experiments as shown in Figs. 5 and 6 performed on three different cells (as indicated by the symbols).

pendent effect on the Cl^--carrier and on the ATP level. Strong evidence is further added by analysis of the saturation kinetics of the high-affinity cyanide-effect in the micromolar range. Fig. 8 shows the Hill-plot of the relative cyanide-inhibition of K_S for the concentration range of 3–300 μM. From the straight line the apparent binding constant is calculated to be $1.9 \cdot 10^5$ M^{-1} and the Hill coefficient to be 1.1. These values are very similar to those determined for the reaction of cyanide with cytochrome-*c* oxidase. The binding constants of cyanide to the oxidized resting and oxidized pulsed cytochrome-*c* oxidase were $2.1 \cdot 10^5$ M^{-1} and $1.1 \cdot 10^5$ M^{-1} respectively. The Hill coefficients were close to unity [63].

Millimolar cyanide-concentrations usually inhibit both photophosphorylation and oxidative phosphorylation [66]. According to this, we found that the ATP level in *V. utricularis* was independent of light when 3 mM of cyanide was present. The ATP level decreased by about 70% indicating that under normal physiological conditions a small amount (< 30%) of ATP is presumably synthezised by the cyanide-resistant, respiratory pathway [67].

However, the pronounced, additional effect of millimolar cyanide-concentrations on the charge pulse relaxation pattern cannot be explained by additional inhibition of the photophosphorylation because these changes in the time constants and the translocation rates of the free and charged carrier molecules were also observed in the dark. Furthermore, the cyanide effect was different to that observed with azide. Therefore, we have to conclude that cyanide at millimolar concentrations inhibits the mobility of the carrier by direct binding to the transporter, as seen with various other anions (such as Br^-, NO_3^- and to a small extent Mes^-, [14,15]). Further experiments are needed to study the cyanide effect on the chloride transporter.

The response of the ATP pool in *V. utricularis* upon addition of cyanide was relatively slow (as compared to 4 to 30 s in other algal, fungal and higher plant cells, see [29,41,42,60]. However, despite rapid variation of the ATP pool, the short-circuit current in *Chara corallina* dropped also more slowly (by approximately 65% within the first 2 min [41]). This is comparable with the response of the Cl^--carrier system in *Valonia* (Fig. 3). The delayed response of the Cl^--transport system in *Valonia* is presumably due to the diffusion limitations in the 10 μm thick and highly negatively charged cell wall as well as due to the unfavourable surface to volume ratio. In other work with *Valonia*, Ba^{2+} [39], DIDS [17] and anaesthetics [68] also gave a similar slow responses, which means that the cell wall acts as a permeability barrier also in these cases.

4.4. Is the Cl^--transport in Valonia an active or passive process?

Considering the results presented here it is clear that the electrogenic Cl^--carrier system in the plasmalemma of *V. utricularis* depends on ATP. On the other hand, the current evidence is inadequate to answer the question of whether the electrogenic Cl^--transport is active or passive. For the related species of *Acetabularia* (and probably also for *Halicystis*) the postulation of an electrogenic Cl^--pump is straightforward because of the magnitude of the membrane potential and its dependence on ATP, light and temperature (see above). However, the characteristic features of the vacuolar membrane potential in *Valonia* do not exclude the possibility that the ATP-dependent Cl^--carrier is a Cl^--pump. The actual voltage that an electrogenic pump can induce under certain conditions depends upon the stoichiometry of pump action, and the general conductance or leakiness of the membrane. The electrogenic Cl^--transport in the plasmalemma is integrated into the overall complex regulation of ion transport processes both across the plasmalemma and the tonoplast, which finally counterbalance each other.

The electrical properties of the two membranes cannot be studied separately in a simple way as is generally the case for plant cells since the cytosol is inaccessible [50,52,53,69,70]. However, analysis of the charge pulse relaxation patterns performed under various environmental conditions [14–17,19,26] have shown conclusively that the tonoplast must exhibit a high electrical conductance (although only a low permeability to salt). Therefore, the tonoplast potential cannot assume very high values. Furthermore, it is likely that the postulated pump induces only a relatively small potential across the plasmalemma. Therefore, if changes in the plasmalemma potential lead to corresponding regulatory changes of the tonoplast potential, the total (vacuolar) membrane potential may be held ultimately constant.

An explanation of the results reported here in terms of a Cl^--pump must additionally account for the observation that the (turgor pressure-dependent) K^+-influx is apparently linked to the Cl^--transporter (see above and [10,71,72]). Coupling can be easily envisaged through the membrane potential at the plasmalemma or by chemically interactions of both fluxes within the plasmalemma. The latter explanation also holds, when we reject the concept of a chloride pump in *Valonia* and instead of this assume a co-transport between K^+ and Cl^-. If the coupling ratio is 1:1, the K^+/Cl^- co-transport is electroneutral and does not contribute directly to the vacuolar membrane potential. In this case, the ATP-dependent, electrogenic Cl^- transport could be a submode or a part of a K^+/Cl^- co-transport system. The possibility that a co-transporter may operate in several alternative modes has been shown for the Na^+/K^+ pump (for details, see [73] and the literature cited there). If this explanation is true, the ATP-dependent step in the reduced 4-state model in Fig. 1 should be extended to include the K^+-transport [74,75].

Regardless of the mechanisms underlying the Cl^--transport and its coupling to the K^+-transport in *Valonia* the question how far the Cl^--carrier system is the putative turgor pressure sensor also remains open. We failed to find an effect of cyanide on turgor pressure – at least within 3 h treatment. In addition, inhibitors of the Cl^--carrier system did not change the hydrostatic and osmotic pressure balance in *V. utricularis* [17]. However, these findings do not contradict the idea of turgor-sensing via changes of the translocation rates of the Cl^--carrier system due to electromechanical membrane compression [19,76,77]. The electromechanical model favours the view that only disturbances of the balance between turgor pressure and the osmotic pressure difference can be sensed and transformed into transport processes.

Further progress in the elucidation of the electrogenic Cl^--carrier system and its role in the physiology of the cell and in turgor pressure sensing can be expected if the vacuolar content can be manipulated in perfused cells under maintenance of high turgor pressures. Direct access to the inner vacuolar membrane in turgescent cells will certainly provide a more comprehensive picture to the complex ion regulation pattern in *Valonia* than is currently possible.

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