Biochimica et Biophysica Acta, 1149 (1993) 93–101

BBAMEM 76016

Characterization of the chloride carrier in the plasmalemma of the alga *Valonia utricularis*: the inhibition by 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid

Ingo Spieß, Jianning Wang, Roland Benz and Ulrich Zimmermann

Lehrstuhl für Biotechnologie, Biozentrum der Universität Würzburg, Würzburg (Germany)

(Received 26 January 1993)

Key words: DIDS; SITS; Chloride carrier; Charge pulse; Turgor pressure regulation; (*V. utricularis*)

The effect of the anion transport inhibitor 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) on the Cl^--transport system located in the plasmalemma of cells of the giant marine alga *Valonia utricularis* was studied by using the charge pulse relaxation technique. Analysis of the biphasic relaxation patterns in terms of the kinetic model published previously (Wang, J., Wehner, G., Benz, R. and Zimmermann, U. (1991) Biophys. J. 59, 235–248) demonstrated that extracellular DIDS dramatically reduced the translocation rate, K_{AS}, of the Cl^--carrier complex (maximal inhibition 79%). The translocation rate of the free carrier molecules, K_S, as well as the total surface concentration of the carrier, N_o, were not affected. A Hill-plot of DIDS inhibition on K_{AS} yielded an half-maximal inhibition concentration (IC_{50}) of $3.9 \cdot 10^{-5}$ M and a Hill-coefficient of 1.61, suggesting a co-operative binding of the inhibitors to the Cl^--carrier. The maximal inhibition of DIDS was dependent on the extracellular Cl^--concentration. This inhibition was not competitive to chloride, since it increased and did not decrease with increasing chloride concentration. The DIDS effect decreased with increasing pH-value (investigated pH range between 6.5 and 10). Intravacuolar DIDS or SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid) had no effect on the biphasic voltage relaxation pattern. These results showed that the binding sites of DIDS must be located on the outer surface of the plasmalemma of *V. utricularis* and, in turn, supported previous conclusions that the Cl^--carrier (which is assumed to be part of the turgor-pressure-sensing mechanism) is only located in the outer membrane.

Introduction

Maintenance of turgor pressure is an important prerequisite of algal and plant cells for growth and survival. For this reason, many plant cells which grow in environments of fluctuating osmolarity have developed mechanisms to regulate the activity of their membrane ion-transport systems as a function of turgor pressure [1–4]. In the giant algal cells of the species *Valonia*, the K^+ influx and efflux are turgor-pressure-dependent [5]. There are also some indications that the Cl^- influx is also directly affected by changes in turgor pressure (data not shown). The finding that turgor pressure can directly interfere with solute flow has led to an intensive search for the turgor pressure sensor and for the mechanisms involved in the transformation of turgor pressure signals into transport processes during the last decade.

Charge-pulse experiments have given very good evidence for an electrogenic Cl^- transport system located within the plasmalemma of *Valonia utricularis* [6]. In contrast to electrogenic transport systems in membranes of animal cells [7,8] the voltage-independent step of the Cl^--carrier (i.e., the translocation of the free carrier) and/or the binding reaction in *V. utricularis* was rate-limiting (as in other plant cells, see Refs. 9–13). This has the consequence that the charge of the mobile Cl^--carrier complex increased the apparent capacitance of the membrane.

The Cl^--transport system could be a candidate for turgor pressure sensing because the transport properties of the system can be changed by mechanical compression of the membrane [14]. Recent studies have also shown (data not shown) that the Cl^--carrier seems to be linked to the K^+-transport system.

Correspondence to: U. Zimmermann, Lehrstuhl für Biotechnologie, Biozentrum der Universität Würzburg, Am Hubland, D-8700 Würzburg, Germany.

Abbreviations: ASW, artificial sea water; CAPSO, 3-[cyclohexylamino]-2-hydroxy-1-propanesulfonic acid; DADS, 4,4′-diaminostilbene-2,2′-disulfonic acid; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid.

In order to proceed with the evaluation of the turgor-pressure sensor, further characterization of the Cl^- transport system is therefore needed. Chemical probes have proven useful for the exploration of the molecular properties of membrane transport systems, including the identification of functional components, their arrangement in the membrane matrix, the chemical nature of transport sites, and the details of the underlying molecular mechanisms [16–19]. Inhibitors which bind covalently are also potential tools for isolation of the structural proteins of the transport system.

DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid) is a potent and very specific inhibitor for anion transport in general and inhibits anion transport in bacterial and red blood cells, as well as in a number of other animal and plant cells and organs [16–34]. Therefore, it was of great interest to explore possible interactions of this stilbene derivative (and related inhibitors, such as SITS (4-acetoamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid)) with the Cl^--transport system of *V. utricularis*.

In this article we present data from charge pulse experiments which show that DIDS (and SITS) bind highly specifically, but reversibly, to the charged Cl^--carrier complex. The translocation rate of the charged carrier molecules was markedly reduced, whereas the translocation rate of the free carrier and the total surface concentration of the carrier molecules were not affected by the presence of the inhibitors. It is also shown that DIDS did not bind competitively to the Cl^--carrier complex, but co-operatively.

Materials and Methods

Algae

Cells of *V. utricularis* were collected in the Gulf of Naples, Italy, and kept in Mediterranean Sea water under a 12 h light/dark regime at 17°C. Elliptical cells (surface area between 20 and 100 mm^2) were clamped in a small perspex™ chamber and 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 adjusted to 8.1 by addition of 10 mM Hepes and appropriate amounts of NaOH. Experiments were performed at 20°C in the dark because of the light-sensitivity of DIDS.

In the experiments in which the pH-dependence of the inhibitory potency of DIDS on the Cl^--carrier complex was investigated 10 mM Pipes was used to adjust the pH to 6.5 or 10 mM CAPSO to raise the pH to 9 or 10 (Hepes was not used). Lower Cl^- concentrations in ASW were obtained by replacing Cl^- with the appropriate amount of Mes^- [6].

Reagents

DIDS and SITS were obtained from Fluka (Neu-Ulm, Germany); CAPSO, Hepes, Mes, and Pipes from Sigma (Deisenhofen, Germany). The other chemicals (analytical grade) were from Merck (Darmstadt, Germany). DIDS (and SITS) were added to the perfusion medium from a 10 mM aqueous stock solution until the desired final concentration was established. At high concentrations DIDS and SITS were directly dissolved in ASW.

Set-up

The charge-pulse technique has been described in detail elsewhere [6,14,15]. 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 perspex chamber in which a pressure transducer (for recording the turgor-pressure, see Refs. 1,3–5) was mounted. 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 storage oscilloscope, Tektronix, Beaverton, OR, USA, model 7633) across a 10 Ω resistor connected in series with the platinum wire.

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. The potential-recording electrode was connected to a fast, differential amplifier of high input impedance. The data points were stored on a Nicolet 2090 digital oscilloscope (Frankfurt, Germany), then transferred to a PC/AT computer and analysed with a multiple-exponential-fitting program.

Data analysis

The initial voltage, V_0, the time constants τ_1 and τ_2 and the relative amplitudes a_1 and a_2 were obtained from the multiple-exponential fitting of the voltage decay to two relaxations [6,14,15].

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

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

The specific membrane capacity, C_m, was calculated from:

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

where Q is the injected charge and A the surface area of the alga.

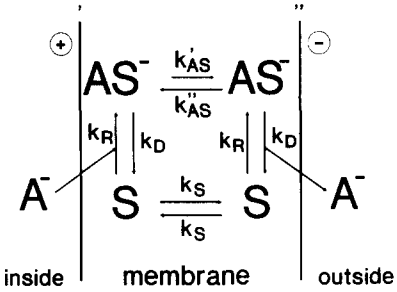

Fig. 1. Kinetic scheme for carrier-mediated Cl^--transport in the plasmalemma of *Valonia utricularis*. The rate constants k_R (association) and k_D (dissociation) refer to the heterogeneous complexation reaction between the anion (Cl^-), A, and the carrier, S. The stability constant of the Cl^--carrier complex, AS, is given by $K = k_R / k_D$. The voltage-dependent individual rate constants k'_{AS} and k''_{AS} refer to the translocation of the Cl^--carrier complex from the left to the right interface and vice versa, respectively. The rate constant k_S refers to the movement of the free carrier within the membrane.

The specific membrane resistance, R_m, and conductance, G_m, are given by Ref. 15:

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

According to the carrier model for the Cl^- transport in the plasmalemma of *V. utricularis* (Fig. 1), the information on the real rate constants 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. As shown by Wang et al. [6] only the reduced rate constants of the charged, K_{AS}, and free carrier molecules, K_S,

$$K_{AS} = k_{AS} Kc/(1 + Kc) \quad (5)$$

and

$$K_S = k_S/(1 + Kc) \quad (6)$$

respectively, as well as the total surface concentration of the carrier molecules, N_0, can be calculated from the relaxation parameters at a single chloride concentration. The kinetic parameters are given by:

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

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

$$N_0 = P_3/(2BK_{AS}) \quad (9)$$

with

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

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

TABLE I

DIDS effect on the Cl^- transport system in V. utricularis

The data give the average of four measurements for the relaxation and the kinetic parameters (calculated in terms of the kinetic model shown in Fig. 1) measured on six different cells in the presence and absence of 10^{-4} M DIDS. The turgor pressure T_P, the resting membrane potential V_m, and the conductance G_m are also shown. The standard deviations are smaller than 5% and therefore not shown. For further explanations, see text.

[DIDS] (mM)	T_p (bar)	V_m (mV)	G_m (mS/cm^2)	τ_1 (μs)	τ_2 (ms)	a_1	K_{AS} (1/s)	K_S (1/s)	N_0 (pmol/cm^2)	k_{AS} (10^3/s)	k_S (1/s)
Cell 1											
0	3.9	0	1.67	60	3.92	0.93	594	138	7.8	3.51	166
0.1	4.0	−1	1.35	141	4.28	0.92	273	128	8.1	1.61	154
0	3.8	0	1.13	63	5.51	0.92	586	98	7.0	3.46	118
Cell 2											
0	2.7	−3	4.35	51	2.03	0.94	591	263	12.5	3.49	317
0.1	2.7	−7	2.74	144	2.13	0.93	209	252	12.6	1.24	303
0	2.6	−7	4.21	55	2.08	0.94	562	256	12.5	3.32	308
Cell 3											
0	3.2	−1	2.98	62	2.14	0.90	773	260	8.0	4.57	313
0.1	3.2	−4	2.83	160	1.61	0.92	191	334	12.2	1.13	402
0	3.4	−3	3.60	55	1.70	0.90	855	326	8.0	5.05	392
Cell 4											
0	3.0	4	2.92	77	1.50	0.90	579	370	6.8	3.42	445
0.1	3.3	1	1.61	272	1.58	0.89	134	349	8.8	0.79	420
0	3.1	3	3.11	76	1.43	0.90	610	388	6.9	3.61	467
Cell 5											
0	4.3	0	1.88	90	2.01	0.88	604	282	5.1	3.57	339
0.1	4.6	−3	0.91	429	2.34	0.87	99	240	6.9	0.59	289
0	3.7	−3	1.61	95	2.46	0.87	609	232	5.0	3.60	279
Cell 6											
0	2.1	4	2.30	79	1.41	0.86	771	409	4.5	4.56	492
0.1	2.3	−3	1.47	275	1.64	0.88	148	340	7.5	0.88	409
0	2.1	4	2.39	77	1.35	0.86	781	424	4.6	4.62	510

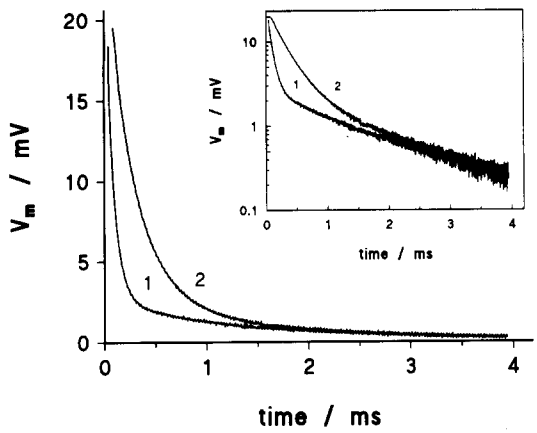

Fig. 2. Voltage relaxation patterns recorded on a cell of *V. utricularis* (surface area 72 mm^2, volume 41 mm^3, resting membrane potential +4.1 mV) in the absence and presence of $1 \cdot 10^{-4}$ M DIDS. Curve 1 is the control experiment in ASW measured 2 h after impalement of the cell with the microelectrodes. The injected charge, Q, was 12 nAs and the initial voltage, V_0, was 25 mV. Curve 2 shows the voltage relaxation pattern 30 min after addition of DIDS ($Q = 11$ nAs, $V_0 = 23$ mV). The experiments were performed in the dark because of the instability of DIDS. Inset: Semi-logarithmic plot of curves 1 and 2. Note that both in the presence and absence of DIDS the voltage relaxation pattern can be fitted by two exponentials with different time constants.

$$P_3 = a_1/\tau_1 + a_2/\tau_2 \tag{12}$$

$$B = z^2F^2/(4RTC_m) \tag{13}$$

where z ($= -1$) is the valency of the Cl^--carrier complex, K is the binding constant between the anion and the carrier, k_{AS} and k_S are the individual translocation rate constants of the charged and free carrier molecules (see Fig. 1) and c is the external Cl^- concentration; F, R and T have their usual meaning.

The analysis is based on the assumption that the interfacial reactions 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 (for details, see Ref. 6).

Results

Inhibition of the Cl^--transport system

To determine whether inhibitors of anion transport systems could affect the Cl^- transport system in the plasmalemma of *V. utricularis*, charge-pulse experiments were performed in the presence and absence of 10^{-4} M DIDS. Typical recordings are given in Fig. 2. It is clear that a 30 min incubation of the cells in DIDS-containing ASW significantly retarded the fast relaxation process compared to that of controls. In the particular experiment shown in Fig. 2 the time-constant of the fast-relaxation process τ_1 increased from 79 μs to 272 μs. Similar values were obtained for other cells (Table I).

In contrast, the time-constant of the slow relaxation process, τ_2 (1.62 ms), and the amplitude of the fast relaxation process, a_1 (0.91), were insensitive to DIDS within the limits of experimental accuracy (5%, see Fig. 2 and Table I).

A highly specific effect of DIDS on τ_1, was also observed with other cells taken from different cultures (Table I) even though the absolute magnitude of the parameters were subject to some variations. This, however, is not unusual for cells of *V. utricularis* and presumably reflects differences between developmental stages of this alga [6]. On average, τ_1 increased by a factor of 2.4 after the addition of DIDS (Table I).

It is interesting to note that in all cells investigated the turgor pressure remained constant in the absence of osmotic stress and the membrane potential remained small in the presence of DIDS (0.32 ± 0.07 MPa and -0.17 ± 3.49 mV, respectively), at least during the experimental period. The membrane conductance, however, decreased by a factor of 1.6 ± 0.4.

The time-constants and amplitudes of the biphasic relaxation pattern were extracted from the semi-logarithmic plots of the curves as shown in the inset to Fig. 2. It is obvious that the relaxation pattern of DIDS-treated cells could be fitted by two exponentials, as for control cells. A third relaxation could not be resolved after addition of DIDS. Two exponentials are expected in terms of the Cl^--carrier model (Ref. 6, see also Materials and Methods) if the heterogeneous complexation reaction between the anion and the carrier at the membrane–solution interface is always in equilibrium. The absence of a third relaxation, therefore, confirmed the adequacy of the model and justified the analysis of the voltage relaxation pattern of DIDS-treated cells according to Eqns. 7–13. As shown in Table I, DIDS affected predominantly the translocation rate of the charged Cl^--carrier complex, K_{AS}. On average, K_{AS} decreased by about 75% from 660 ± 103 s^{-1} to 176 ± 62 s^{-1}. In contrast, the translocation rate, K_S, of the free carrier and the surface concentration of the carrier, N_0, remained nearly unaltered (Table I). The real rate constants k_{AS} and k_S can be calculated by Eqns. 5 and 6 from the kinetic parameters K_{AS} and K_S, with the assumption that the binding constant $K = 0.34$ M^{-1} and the chloride concentration $c = 0.599$ M [6]. This assumption can be made because the rate constant K_S is unaltered in the presence of DIDS. The data for k_{AS} and k_S in Table I show that DIDS affects only the translocation of the charged carrier.

Removal of DIDS showed that the inhibitory effect of DIDS on τ_1 was completely reversible after about 1 h (Table I). Simultaneously, the original value of the membrane conductance (about 26.8 ± 10.3 S m^{-2}) was re-established.

SITS, another inhibitor of anion transport was less potent than the structurally-related DIDS. On average,

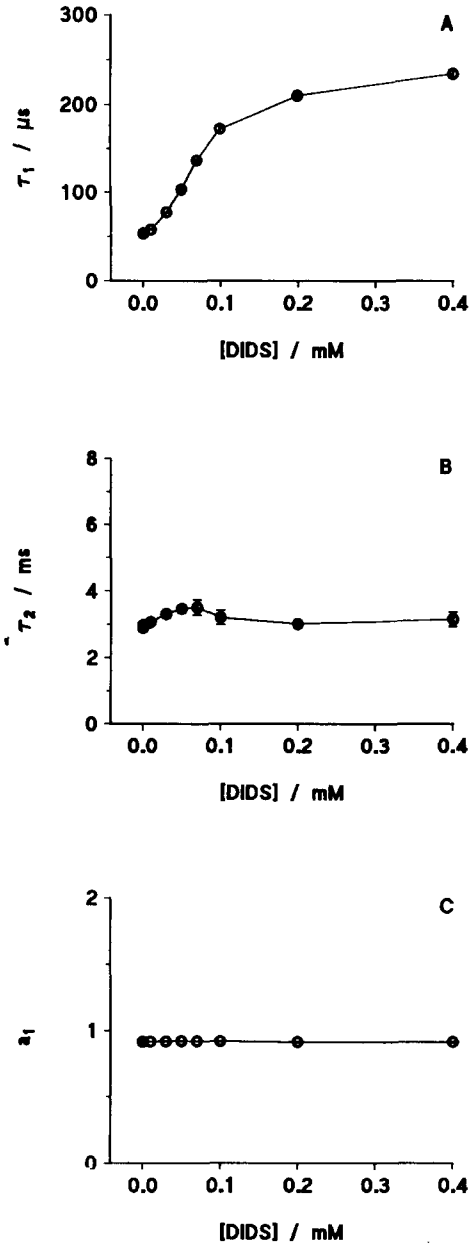

Fig. 3. Plot of the relaxation parameters τ_1 (A), τ_2 (B) and a_1 (C) as a function of the external DIDS concentration (cell no. 8). The data were obtained as follows: 2 h after insertion of the microelectrodes into the vacuole of the alga, four charge-pulse relaxation experiments were performed in DIDS-free ASW (time interval between each measurement 5 min). The ASW was then replaced by ASW containing DIDS at successively increasing concentrations. At each DIDS concentration, four charge-pulse experiments (5 min apart from each other) were performed starting 17 min after medium exchange. The values represent the mean of the four experiments. The standard deviation was extremely small.

external concentrations of $1\text{–}5 \cdot 10^{-3}$ M were required to suppress the transloction rate of the charged carrier by about 70%.

In order to identify the site of interaction of the inhibitors with the Cl^- transport, $5 \cdot 10^{-4}$ M DIDS or $2 \cdot 10^{-3}$ M SITS were injected into the vacuole via the pressure probe [1,3–5]. Intravacuolar DIDS and SITS did not affect the time constants of the two relaxation processes. This finding indicated that the reaction site of the inhibitors could be located in the plasmalemma in agreement with previous conclusions [6].

Concentration-dependence of the inhibitory effects of DIDS

The interaction of DIDS on the Cl^--transport system in the plasmalemma of *V. utricularis* was reversible. This result allowed the investigation of the saturation of the DIDS-effect on the chloride transport system in detail.

Fig. 3A shows that increasing external DIDS concentrations considerably increased the time constant τ_1. An effect of DIDS on τ_1 could be recorded at concentrations of DIDS above 10^{-5} M. The increase of τ_1 with further increase in DIDS concentration exhibited a sigmoidal saturation characteristic. τ_1 tended to plateau at a concentration of about $2 \cdot 10^{-4}$ M.

A similar trend with increasing DIDS concentrations was found for the membrane conductance. At a concentration of $2 \cdot 10^{-4}$ M the membrane conductance was about half the value measured in the absence of the stilbene derivative. Turgor pressure and membrane potential remained constant over the whole concentration range.

In contrast to the dependence of τ_1 and the membrane conductivity on the DIDS concentration, no significant effect of the inhibitor on τ_2 or a_1 could be recorded up to a concentration of $4 \cdot 10^{-4}$ M (Fig. 3B,C).

The corresponding data for N_0, K_S and K_{AS} are given in Fig. 4. As expected, K_{AS} showed a strong dependence on the concentration of DIDS, whereas K_S and N_0 remained practically unchanged. The concentration above which a plateau in the translocation rate of the charged carrier K_{AS} was reached was 10^{-4} M (Fig. 4A). The IC_{50} (i.e., the concentration of probe needed to produce 50% maximal inhibition of K_{AS}) is difficult to determine accurately from Fig. 4A because of the steep slope of the curve in the concentration range of 10^{-5} to 10^{-4} M DIDS.

A more accurate value can be obtained from a Hill plot of the average relative inhibitory potency of DIDS, $i = I/I_{max}$, against the DIDS concentration, whereby $I = (K^*_{AS} - K_{AS}([DIDS]))/K^*_{AS}$ and $I_{max} = (K^*_{AS} - K_{AS}(\infty))/K^*_{AS}$ [(1)]. The parameters I and I_{max} were normalised in order to eliminate the physiological variations of the parameter from one cell to the other. From the intercept of the linear regression curve in Fig. 5 the

[(1)] $K_{AS}([DIDS])$ is the translocation rate of the Cl^--carrier complex at a certain DIDS concentration, $K_{AS}(\infty)$ is the translocation rate at saturation and K^*_{AS} that in the control.

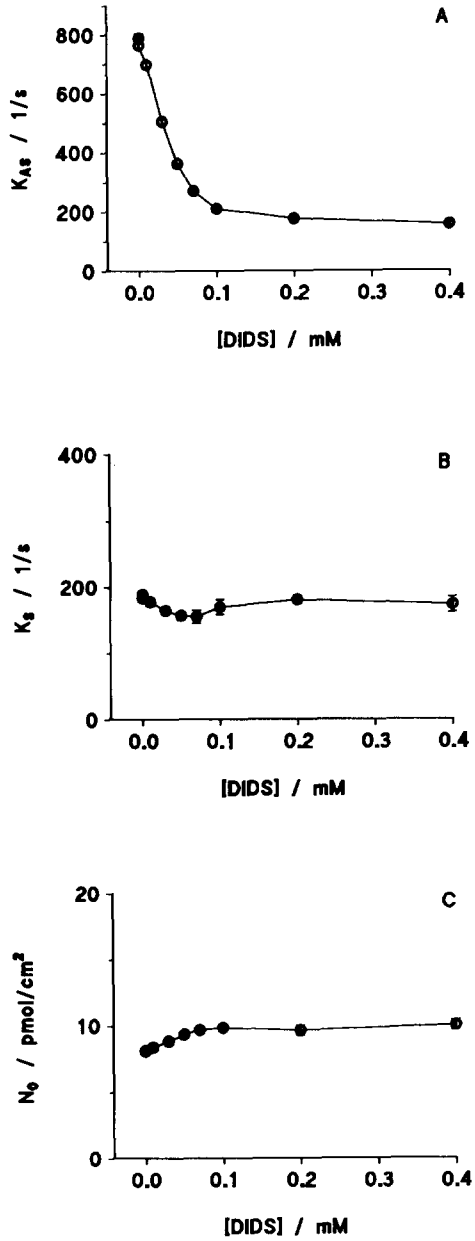

Fig. 4. Plot of the the translocation rates of the charged Cl^--carrier complex, K_{AS}, (A), and of that of the free carrier, K_S, (B), as well as of the total surface concentration of the carrier molecules, N_0, (C) as a function of the external DIDS concentration. Data were obtained by analysis of the curves in Fig. 3 (using Eqns. 7–13).

IC_{50}-value is determined to be $3.9 \cdot 10^{-5}$ M (average of 8 cells). The Hill coefficient, obtained from the slope of the linear regression curve, is 1.61. This indicates that the interaction of DIDS with the Cl^- transport system is not a first order reaction. We have to conclude that the binding of DIDS to the carrier molecules is co-operative.

Anion effects on DIDS inhibition

The inhibition of the translocation rate of the charged carrier depended on the external Cl^--concentration. Lowering of the Cl^--concentration from

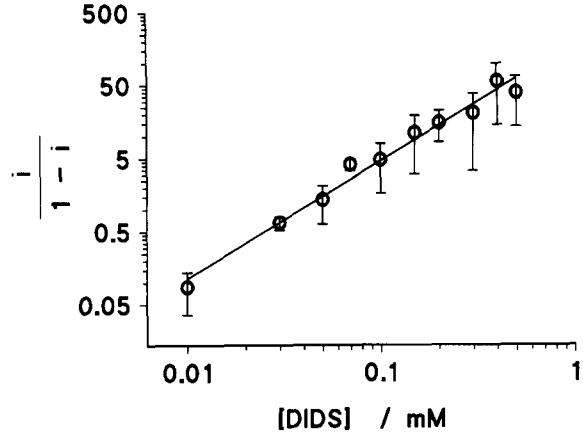

Fig. 5. Hill-Plot of the relative DIDS inhibition, $i = I/I_{max}$, of the translocation rate of the Cl^--carrier complex, K_{AS}. I and I_{max} (maximum inhibition) are normalised parameters (for definition, see text). The double-logarithmic plot of $i/(1-i)$ vs. the DIDS concentration yields a linear function ($\log(i/(1-i)) = n \log[DIDS] - n \log(IC_{50})$. From the slope of the regression curve the Hill coefficient (n) is calculated to be 1.61. From the intercept the IC_{50} value for half-maximal inhibition of the tranlsocation rate, K_{AS}, is deduced to be $3.9 \cdot 10^{-5}$ M. The data are the mean of eight experiments using different cells. The bars represent standard deviations.

599 mM to 400 mM (combined with addition of a corresponding amount of Mes^- for osmotic adjustment, see Ref. 6) gave a dependence of K_{AS} on DIDS similar to that in normal ASW. However, the IC_{50} and I_{max}-values (for definition see above) were influenced by the relatively low external Cl^--concentration (Table II). The IC_{50}-value of $5.7 \pm 1.1 \cdot 10^{-5}$ M was significantly higher than the corresponding value ($3.6 \pm 0.3 \cdot 10^{-5}$ M) in 599 mM Cl^- ($P < 0.025$) indicating that the affinity of DIDS to the charged carrier molecules decreased with decreasing Cl^--concentration. The maximum inhibition, I_{max}, in 400 mM Cl^- was 64% ± 9% and therefore smaller than in 599 mM Cl^- (79% ± 7%). The observations described above suggest that the

TABLE II

Effect of the external Cl^--concentration on the inhibitory potency of DIDS

Measurements were performed on three different cells at two Cl^--concentrations (599 mM, corresponding to the Cl^--concentration in normal ASW, and 400 mM). The values for IC_{50} and the half-maximal inhibitory potency, I_{max}, of DIDS on the translocation rate of the Cl^--carrier complex, K_{AS}, are shown. For further details, see text.

	IC_{50} (μM)		I_{max} (%)	
$[Cl^-]$ (mM):	599	400	599	400
Cell 7	34	44	84	70
Cell 8	36	60	83	68
Cell 9	39	66	71	54
Mean ± S.D.	36 ± 3	57 ± 11	79 ± 7	64 ± 9

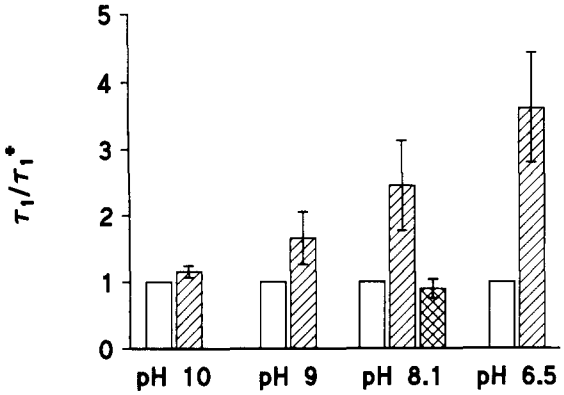

Fig. 6. Effect of $2 \cdot 10^{-4}$ M DIDS on the time constant of the fast relaxation process, τ_1, as a function of pH. The normalised values, τ_1/τ_1^*, in the presence of DIDS are given (hatched columns), i.e. the τ_1 values were divided by the (pH-dependent) value of τ_1^* at a given pH without DIDS in order to exclude the effects of H^+ on the carrier complex. The open columns which represent the respective reference-values all have the value 1. The cross-hatched column at pH 8.1 gives the value for τ_1/τ_1^* after replacement of the more acid or alkaline, DIDS-containing ASW solutions by normal ASW medium with a pH of 8.1. Note that at all pH-values investigated the effect of DIDS on the time constant was completely reversible.

binding of DIDS to the carrier molecules is not competitive to chloride.

pH effects on DIDS inhibition

To determine whether the inhibitor action on τ_1 and K_{AS} were pH-dependent, charge-pulse experiments were performed at pH 6.5, 9 and 10 in the presence of $2 \cdot 10^{-4}$ M DIDS. As already shown previously [14,15] lowering of the pH of the ASW resulted in an increase of τ_1, particularly below pH 6. In order to distinguish between the DIDS- and H^+-effects on the transport system at a given pH, the relaxation- and the kinetic-parameters measured in the presence of DIDS were normalised to the corresponding values measured in the absence of the inhibitor.

As shown in Figs. 6 and 7 the inhibitory potency of

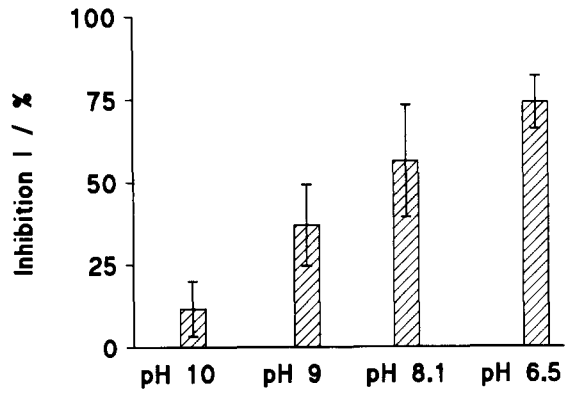

Fig. 7. Relative and normalised DIDS-inhibition, I, of the translocation rate of the Cl^--carrier complex, K_{AS}, as a function of pH. The data were calculated using Eqns. 7–13 from the relaxation parameters of the same experiment that was shown in Fig. 6. For further explanation, see text.

DIDS on the fast relaxation time, τ_1, and, in turn, on the translocation rate of the charged carrier, K_{AS}, depended significantly on the pH. After addition of DIDS at pH 6.5, τ_1 increased by a factor of 3.6 ± 0.82 ($n = 4$) compared to a factor of 2.5 ± 0.68 ($n = 7$) in normal ASW (pH 8.1). With increasing alkalinity the effect of DIDS decreased. At pH 9 τ_1 increased 1.7 ± 0.40 ($n = 3$) times and at pH 10 about 1.2 ± 0.09 ($n = 4$) times. Correspondingly, K_{AS} was inhibited in the presence of DIDS by $74 \pm 8\%$ at pH 6.5, whereas at pH 10 the decrease in K_{AS} was only $12 \pm 8\%$.

It should be mentioned that DIDS did not affect the time-constant of the slow relaxation over the investigated pH-range. In agreement with this, the kinetic parameters K_S and N_0 exhibited values which were comparable with those of the controls. The DIDS effects at all pH-values were completely reversible (see cross-hatched column in Fig. 6).

Discussion

DIDS is a relatively membrane-impermeant inhibitor which highly specifically blocks Cl^- channels and anion exchange systems operating in the membranes of many cells of various origins [17,19–21,35]. DIDS inhibition of ATPase activity in mammalian [36–38] and plant [30,34] membranes has also been reported, which suggests direct or indirect involvement of ATPase in anion transport [39]. In the cells of the giant alga *V. utricularis*, DIDS apparently interacts specifically and directly with the Cl^--carrier system in the plasmalemma. This conclusion is suggested by the charge-pulse experiments reported here.

The kinetic analysis of the voltage relaxation pattern after injection of a charge-pulse has demonstrated that anion binding to the carrier molecules and the translocation of the free carrier were obviously not inhibited by extracellular DIDS up to concentrations of 10^{-3} M. In contrast, the highly-specific effect of the inhibitor on the translocation rate of the Cl^--carrier complex was observed at concentrations where non-specific and indirect actions of DIDS can be excluded. The half-maximal inhibition value (IC_{50}) was $4 \cdot 10^{-5}$ M: this is about one order of magnitude larger than the corresponding value for the anion exchanger in human red blood cells [17,20,21], but comparable with those reported for other mammalian cell systems [17] and isolated vacuoles of sugar beet [19]. This result is interesting because the IC_{50}-values for DIDS inhibition of Cl^- transport in corn root segments [30] and in the plasmalemma of the pond water giant alga *Chara corallina* [31] were in the mM range. Since the experiments on *C. corallina* were performed at pH 6.5, the inhibitory potency of DIDS in *V. utricularis* would be even higher if the pH-dependence of DIDS inhibition of the Cl^--carrier complex is taken into account. It is conceivable [31] that interac-

tions of DIDS (which is a relatively large divalent anion, MW 453 g/mol) with the fixed negative charges of the wall during penetration require a corresponding increase in concentration. The slowness with which DIDS acted in *C. corallina* (60 min to saturate compared to about 10 min in *V. utricularis*) would support this hypothesis. If this explanation is correct we have to conclude that the walls of the marine alga *V. utricularis* bears fewer fixed negative charges than that of *C. corallina*. This is not unlikely because of the high Ca^{2+}-concentration in ASW (11 mM in contrast to 0.2 mM in pond water). It is well-known [40] that Ca^{2+}-concentrations higher than 5 mM reduce the negative Donnan potential of the cell wall. In this case, the enhancement of DIDS inhibition of the Cl^- transport both in *V. utricularis* and *C. corallina* with lowering of the pH can be explained by the assumption that the negative charge sites in the cell wall are either titrated or are electricaly shielded by the increased proton concentration. Although other explanations for the pH-dependent effect of DIDS inhibition are possible (see below) it is rather unlikely that DIDS itself is being protonated, because the pK-value of DIDS is well below 1.3 [31]. Therefore, it can be excluded that the increased inhibitory potency of DIDS towards lower pH-values was due to the presence of the undissociated form of this reactive molecule.

The sites where DIDS inhibits are assumed to be exofacial [17], since no inhibitory effects have been detected generally on the inner surface (for exceptions, see, e.g., Ref. 27). In agreement with this we found no effect on the transport system when DIDS (or SITS) was injected into the vacuole of the alga. Hedrich and colleagues [19,41] also reported that intravacuolar DIDS did not affect the Cl^--channels in the tonoplast of isolated vacuoles from sugar beet. An effect was only seen when DIDS was added to the cytoplasmic side of the vacuole [19].

DIDS blocking of cytoplasmic sites of the vacuole of *V. utricularis* under the various conditions can be excluded. As shown previously [6] the kinetic analysis of the voltage relaxation pattern in terms of the kinetic model depicted in Fig. 1 have led to the conclusion that the tonoplast resistance is rather low, despite the low permeability to salt. The time constant of the tonoplast must be less than 5 μs (the resolution of the charge-pulse technique), otherwise a third voltage relaxation would be visible. Cl^- should rapidly penetrate from the vacuole through such a highly-conductive membrane into the cytoplasm. However, this requires that the tonoplast is also highly permeable to the positively-charged counterion, K^+. If this is not the case, the occurrence of a diffusion potential will hinder the Cl^- transport out of the vacuole. Blocking of these postulated conductive Cl^--channels in the tonoplast of *V. utricularis* by DIDS acting from the cytoplasmic site would, therefore, lead to a significant increase in resistance and, in turn, to the occurrence of a third relaxation. The absence of such an effect of intravacuolar or extracellular DIDS on the Cl^- transport in *V. utricularis* is further evidence that DIDS did not penetrate the membrane barrier within the time scale of an experiment. This suggested also that the DIDS binding sites of our transport system could be located on the outer surface of the plasmalemma.

The action of DIDS on the translocation rate of the charged carrier in the plasmalemma of *V. utricularis* was completely reversible over the whole pH range. In this respect the DIDS inhibition was comparable with that found for the Cl^--influx in the plasmalemma of *C. corallina*. These results are in contrast to those in human red blood cells [21,24] and in other mammalian cells [17], as well as in the vacuoles of sugar beet [19]. However, the DIDS effect is very often partly reversible if the drug is washed off soon, although recovery is never complete [42]. The reversiblity of the inhibitory effect of DIDS also depends on temperature and the inhibitor concentration [25,43].

With prolonged exposure to the disulfonic stilbene inhibitors the block usually becomes total and irreversible, also at relatively low concentrations. In red blood cells, it is thought that the reversibility may be related to the inhibitors' ability to block the anion conductance at sites other than those where they bind covalently. Evidence is available [19] that the disulfonic stilbene derivatives bind covalently to amino groups in the membrane (ϵ-amino groups of two lysine residues on the transport protein).

The data presented here suggest that DIDS binds to the Cl^- transport system in the plasmalemma of *V. utricularis* in a different way to that in most mammalian cells. The concentration-dependence of the DIDS inhibition demonstrated that the inhibitor binds co-operatively to the Cl^--carrier complex. The experiments performed at lower external Cl^--concentration exclude the possibility of a binding of DIDS to the carrier molecules in competition with the Cl^- ions. The pH-dependence of the DIDS action on the translocation rate of the charged carrier rules out covalent binding of the inhibitor (if cell-wall effects do not play a role, see above). At pH 8.1, saturation is reached at 10^{-4} M DIDS. It is, therefore, very unlikely that the further enhancement of DIDS inhibition of the translocation rate of the charged carrier at pH 6.5 resulted from an improved binding of the inhibitor to the Cl^--carrier complex. Since a reaction between the isothiocyanate groups and amino groups can take place only with the deprotonated form [25], the opposite effect of pH on the DIDS inhibition potency is expected. Therefore, it is more likely that at lower pH the translocation of the charged carrier is blocked more effectively than at higher pH.

The reversibility of the DIDS effect on Cl^- transport in the plasmalemma of *V. utricularis* excludes the use of this inhibitor for isolation of transport proteins which are thought to be part of the turgor pressure sensor. However, recent experiments have shown that an (intermediate) degradation product of DIDS obtained after alkaline treatment highly-specific inhibits the translocation rate of the free carrier. This compound (which could not yet been identified despite much effort) reacts at concentrations comparable to those of DIDS in an irreversible manner with the free carrier. DADS, a precursor in the synthesis of DIDS, could be excluded as a possible candidate (Spieß, I., Wang, J. and Zimmermann, U., unpublished results).

Identification of this inhibitory compound and further elucidation of the action of DIDS and other related probes on the Cl^- transport will certainly pave the way for isolation of the transport proteins which are present in high concentrations in the plasmalemma of *V. utricularis*.

Acknowledgements

The authors would like to thank Dr. W. Michael Arnold for critical reading of the manuscript. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 176, projects B4 and B7) and the Fond der Chemischen Industrie to U.Z. and R.B.

References

1 Zimmermann, U. (1978) Annu. Rev. Plant Physiol. 29, 121–148.
2 Gutknecht, J., Hastings, D.F. and Bisson, M.A. (1978) in Membrane Transport in Biology, Vol. 3 (Giebisch, G., Tosteson, D.C. and Ussing, H.H., eds.), pp. 125–174.
3 Tomos, A.D. (1988) in Water Science Reviews 3 (Franks, F., ed.) pp. 186–277, Cambridge University Press, Cambridge.
4 Zimmermann, U. (1989) Methods Enzymol. 174, 338–366.
5 Zimmermann, U. and Steudle, E. (1978) Adv. Bot. Res. 6, 45–119.
6 Wang, J., Wehner, G., Benz, R. and Zimmermann, U. (1991) Biophys. J. 59, 235–248.
7 Almers, W. (1978) Rev. Physiol. Biochem. Pharmacol. 82, 96–110.
8 Armstrong, C.M. and Bezanilla, F. (1973) Nature 242, 459–461.
9 Felle, H. (1980) Biochim. Biophys. Acta 602, 181–195.
10 Felle, H. and Bentrup, F.-W. (1977) Biochim. Biophys. Acta 464, 179–187.
11 Gradmann, D. (1975) J. Membr. Biol. 44, 1–24.
12 Gradmann, D. (1989) Methods Enzymol. 174, 490–504.
13 Tittor, J., Hansen, U.-P. and Gradmann, D. (1983) J. Membr. Biol. 75, 129–139.
14 Zimmermann, U., Büchner, K.-H. and Benz, R. (1982) J. Membr. Biol. 67, 183–197.
15 Benz, R. and Zimmermann, U. (1983) Biophys. J. 43, 13–26.
16 Cabantchik, Z.I., Knauf, P.A. and Rothstein, A. (1978) Biochim. Biophys. Acta 515, 239–302.
17 Cabantchick, Z.I. and Greger, R. (1992) Am. J. Physiol. 262, C803–C827.
18 Deuticke, B. (1977) Rev. Physiol. Biochem. Pharmacol. 78, 1–97.
19 Hedrich, R. and Kurkdjian, A. (1988) EMBO J. 7, 3661–3666.
20 Cabantchik, Z.I. and Rothstein, A. (1972) J. Membr. Biol. 10, 311–330.
21 Cabantchik, Z.I. and Rothstein, A. (1974) J. Membr. Biol. 15, 207–226.
22 Knauf, P.A. (1979) Curr. Top. Membr. Trans. 12, 249–363.
24 Lepke, S., Fasold, H., Pring, M. and Passow, H. (1976) J. Membr. Biol. 29, 147–177.
25 Passow, H., Fasold, H., Jennings, M.L. and Lepke, S. (eds.) (1982) in Chloride Transport in biological Membranes, pp. 1–31, Academic Press, London.
26 Passow, H., Raida, M., Wendel, J., Legrum, B., Bartel, D. Lepke, S. and Furuto-Kato, S. (1989) Biochem. Soc. Trans. 17, 812–815.
27 Russel, J.M. and Brodwick, M.S. (1979) J. Gen. Physiol. 73, 343–368.
28 Elgavish, A., Wille, J.J., Rahemtulla, F. and Debro, L. (1991) Am. J. Physiol. 261, C916–C926.
29 Garcia-Romeu, F., Cossins, A.R. and Motais, R. (1991) J. Physiol. 440, 547–567.
30 Lin, W. (1981) Plant Physiol. 68, 435–438.
31 Keifer, D.W., Franceschi, V.R. and Lucas, W.J. (1982) Plant Physiol. 70, 1327–1334.
32 Bennet, A.B. and Spanswick, R.M. (1983) J. Membr. Biol. 71, 95–107.
33 Hille, B. (1984) Ionic channels of excitable membranes, Sinauer, Sunderland.
34 Gimmler, H., Schneider, L. and Kaaden, R. (1989) Z. Naturforsch. 44c, 128–138.
35 Pahapill, P.A. and Schlichter, L.C. (1992) J. Membr. Biol. 125, 171–183.
36 Ehrenspeck, G. and Brodsky, W.A. (1976) Biochim. Biophys. Acta 419, 555–558.
37 Faelli, A., Tosco, M., Orsenigo, M.N. and Esposito, G. (1984) Pharmacol. Res. Commun. 16, 339–350.
38 Vega, F.V., Cabero, J.I. and Mardh, S. (1988) Acta Physiol. Scand. 134, 543–547.
39 Churchill, K.A. and Sze, H. (1981) Plant Physiol. 67, S-162.
40 Rygol, J. and Zimmermann, U. (1990) Plant Cell Environ. 13, 15–26.
41 Hedrich, R. and Schröder, J.I. (1989) Annu. Rev. Plant Physiol. 40, 539–69.
42 Gray, P.T. and Ritchie, J.M. (1986) Proc. R. Soc. Lond. Biol. 228, 267–288.
43 Kokubun, S., Saigusa, A. and Tamura, T. (1991) Pflügers Arch. 418, 204–213.