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Kinetics of DIDS Inhibition of Swelling-Activated K-Cl Cotransport in Low K Sheep Erythrocytes

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Summary. The inhibitory effect of various stilbene disulfonates was examined on the swelling-activated Cl-dependent K transport (K-Cl cotransport) in low K sheep erythrocytes. Both diisothiocyanatostilbenes H_2DIDS and DIDS were found to be potent inhibitors. The DIDS concentration yielding 50% inhibition (IC_{50}) of KCl cotransport was 60 μM in the absence of external K and 3 μM at physiological K concentration. Other stilbene derivatives, such as SITS (4-acetamido-4′ isothiocyanatostilbene-2,2′-disulfonic acid), were only effective in the presence of external K, whereas DNDS (4,4′-dinitrostilbene-2,2′-disulfonic acid) and ISA (4-sulfophenyl isothiocyanate) had only slight effects at a concentration of 1 mM. The augmenting effect of external K is due to a second K site, distinguishable from the K transport site by its much higher affinity. No inhibition occurred in the absence of external Cl, whether or not external Rb(K) was present. Additionally, DIDS inhibited K-Cl cotransport activated by thiol alkylation with N-ethylmaleimide (NEM) as well as by Mg depletion in the presence of A23187 and a chelator. We conclude that allosteric sites affect the stilbene binding. When these sites are saturated, changes in external K or Cl concentration do not affect the affinity for DIDS (noncompetitive inhibition).

Key Words K-Cl cotransport · stilbenes · inhibition · sheep erythrocytes · volume-activation · N-ethylmaleimide · A23187

Introduction

Stilbene disulfonates have been used extensively to approach kinetic aspects of red cell anion transport mediated by capnophorin or band III protein (Jennings, 1989; Knauf, 1989). The binding of the stilbene derivative DIDS to the anion transport protein occurs in two distinct steps: The first involves reversible binding via the sulfonate groups whereas the second step consists of the covalent reaction of the isothiocyanate residue with the protein (Shami et al., 1978).

A variety of effects of stilbenes derivatives have been reported on Cl-dependent K fluxes. First, in swollen toadfish erythrocytes 0.1 mM DIDS prevented regulatory volume decrease mediated by a Cl-dependent K movement (Lauf, 1982). In contrast, 0.1 mM DIDS failed to significantly reduce the swelling induced Cl-dependent K influx (Kaji, 1986) as well as the NEM-induced K efflux (Garay et al., 1988) of human red cells. Also 0.15 mM DIDS had no effect in erythrocytes from patients with homozygous hemoglobin C disease, where the Cl-dependent K transport is activated (Brugnara et al., 1985). In rat red cells, 0.1 mM DIDS inhibited only partially the Cl-dependent K efflux induced by dapsone-hydroxylamine (Haas & Harrison, 1989). In sheep red cells, DIDS inhibited or activated pH-stimulated K fluxes, depending upon the experimental conditions (Zade-Oppen & Lauf, 1990). Furthermore, 0.2 mM SITS inhibited volume-sensitive K transport in LK sheep erythrocytes (Ellory & Dunham, 1980), whereas 0.1 mM SITS failed to affect the NEM-stimulated K transport (Lauf & Theg, 1980). Finally, in duck erythrocytes, both SITS and DIDS reversibly inhibited the Cl-dependent K fluxes with an IC_{50} of 30 μM and dependent on the external K concentration whereas DNDS remained without any significant effect (Lytle & McManus, 1987). Hence, DIDS inhibition of K-Cl cotransport in duck red cells shares similarities with the furosemide inhibition previously reported in sheep red blood cells (Lauf, 1984).

Experiments preliminary to our kinetic analysis of swelling-activated K-Cl cotransport in sheep red cells (Delpire & Lauf, 1991*a*) revealed that DIDS was significantly more potent than furosemide and also slightly more effective than the more recently studied dihydroindenyloxyalkanoic acid (DIOA, Garay et al., 1988). The present study examines the potency of different derivatives of the stilbene family and focuses on the substrate requirement for DIDS inhibition. The following observations were made: (1) In the absence of external K, only DIDS and H_2DIDS significantly inhibited K-Cl cotransport

with IC_{50} values of 60 and 40 μM, respectively. (2) In the presence of external K, SITS became an acceptable inhibitor while the IC_{50} of DIDS decreased to about 3 μM. (3) The apparent affinity for external K(Rb) estimated for augmentation of the DIDS inhibition was considerably higher than that found for activation of K-Cl cotransport, suggesting involvement of allosteric sites. (4) In the absence of external Cl, DIDS failed to affect the transport rate. (5) In the presence of both external Cl and Rb(K), i.e. when the presumed allosteric sites were saturated, DIDS acted as a noncompetitive inhibitor.

Materials and Methods

Chemicals

DIDS: 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (Sigma, St. Louis, MO); H_2DIDS: 4,4′-diisothiocyanatodihydrostilbene-2,2′-disulfonic acid (ICN Biomedicals, Plainview, NY); DNDS: 4,4′-dinitrostilbene-2,2′-disulfonic acid (Molecular Probes, Eugene, OR); ISA: 4-sulfophenyl isothiocyanate (Aldrich, Milwaukee, WI); A23187: (Calbiochem, San Diego, CA): NEM: N-ethylmaleimide (Sigma, St. Louis, MO); EDTA: ethylenediaminetetraacetic acid (Sigma, St. Louis, MO); DMSO: dimethylsulfoxide (Sigma, St. Louis, MO).

Blood

Blood from homozygous (LL) LK sheep was drawn by jugular venipuncture into heparinized (10 IU/ml blood) syringes just prior to the experiments. The hemoglobin concentration of the original cells was first measured by determining the ratio between the optical density at 527 nm (OD^{527}) of an appropriate dilution (D) and the hematocrit (hct): $OD^{527}_{pc} = OD^{527} xD/hct$. Then, the blood was centrifuged for 1 min at 4°C to remove plasma and buffy coat. Next, the cells were washed three times in a 295 mOsM solution containing 155 mM NaCl and 5 mM PO_4 (Na_2HPO_4/NaH_2PO_4), pH 7.4, 4°C.

Cell Swelling

The cells were washed twice in an ice-cold hyposmotic solution (240 mOsM) of 125 mM NaCl and 5 mM PO_4, pH 7.4. Relative cell volume (RCV) was determined by referring the hemoglobin concentration (hgb/hct) of the sample to that of the original cells. At 240 mOsM, the cells undergo a volume increase of 15% compared to isosmotic conditions (295 mOsM).

NEM Activation

The cells were incubated at 5% hct with 1 mM N-ethylmaleimide (from a 1 M stock solution in DMSO) in the isosmotic solution described above, for a period of 15 min, at 37°C. The cells were then washed twice before the flux experiments.

Mg Depletion

The cells were incubated in an isosmotic solution containing 1 mM EDTA, and 10 μM ionophore A23187 (made from a 191 μM stock solution in ethanol) for 20 min, at 37°C. Then the cells were washed five times with an ice-cold isosmotic solution containing 155 mM NaCl, 5mM PO_4, 1 mM EDTA and 50 mg/ml bovine serum albumin (from a stock solution 2g/100 ml, dialyzed against 1000 times its volume). The level of Mg depletion was determined by atomic absorption spectrophotometry (Perkin Elmer model 5000, Norwalk, CT).

Inhibitors

All inhibitors were tested using a 5% hct cell suspension. The reversibility of DIDS was assessed by preincubating the cells at 5% hct in the presence or absence of the inhibitor (concentration ranged between 10 μM and 1 mM), for 45 min at 37°C. The cells were then washed three times in the cold before being resuspended at 5% hct in the flux solutions.

Anion Replacement

In order to manipulate the external Cl concentration, the cells were preincubated at 5% hct during 45 min at 37°C, in the presence of 10 μM DIDS to inhibit irreversibly the anion exchanger. The cells were then washed three times, in the absence of DIDS, in a solution with the desired Cl concentration, using SO_4 as Cl substitute. Previous experiments have shown that it was easier to keep the internal Cl concentration constant with SO_4 than with NO_3 or SCN (less than 10% loss over 1 hr incubation at 37°C).

K Efflux

Zero-K-*trans* K efflux was determined by incubating the cells in K free solutions containing 155 mM NaCl (295 mOsM) or 125 mM NaCl (240 mOsM), both including 5 mM PO_4 and 0.1 mM ouabain. At time = 0, an aliquot was removed and hemolyzed in order to determine the equilibrium K concentration $[K]_{t=\infty}$. Five aliquots were removed over a period of 50 min for determination by atomic absorption spectrophotometry of the K concentration in the supernatant, $[K]_{t=x}$. The rate constant for K efflux was calculated from a linear regression analysis of $-\ln(1 - [K]_{t=x}/[K]_{t=\infty})$ as a function of the five time points. K efflux ($^{o}M_K^{QR}$) was obtained by multiplying the rate constant (h^{-1}) with the K concentration (mmol/liter original cell [LOC]). The flux was therefore expressed in mmol LOC^{-1} h^{-1}.

Rb Influx

Rb influx was determined by incubating the cells in solutions containing 125 mM (NaCl + RbCl), 5 mM PO_4 and 0.1 mM ouabain, pH 7.4, at 37°C. The uptake of Rb was followed over a period of 50 min. Five aliquots were removed at 10 min intervals, spun, and washed three times with an ice-cold $MgCl_2$/Tris-Cl (295 mOsM) solution before being lysed in a solution containing 15 mM NH_4OH, 0.031% noncationic detergent (Acationox, Baxter Healthcare, McGaw Park, IL), and 4 mM CsCl. Cellular Rb was measured using an atomic absorption spectrophotometer, while

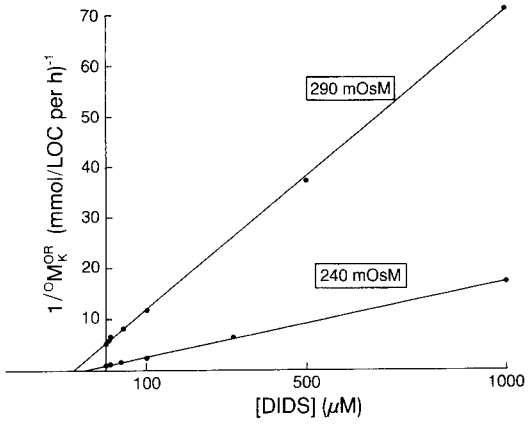

Fig. 1. Determination of IC_{50} for DIDS inhibition. The cells were preequilibrated in the cold with 290 mOsM (isosmotic) or 240 mOsM (hyposmotic) solutions. The Cl-dependent zero-K-*trans* K efflux was measured in the presence of different concentrations of DIDS. Reciprocal of flux (mmol/LOC per hr)$^{-1}$ is plotted against DIDS concentration (μM).

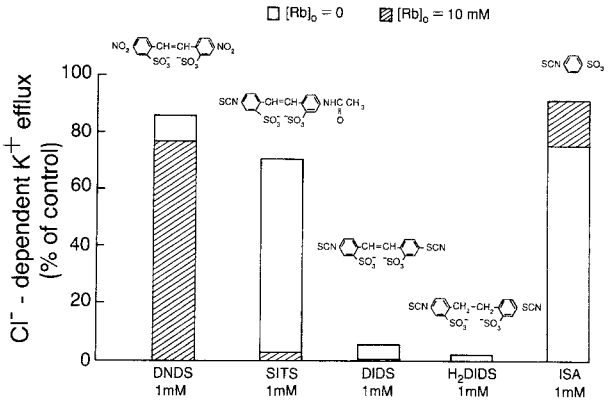

Fig. 2. Comparison between inhibitory effects of various stilbene derivatives, in the absence (open bars) or presence (dashed bars) of 10 mM external Rb. The cells were pre-equilibrated at 240 mOsM by three washes in the cold and resuspended at 5% hct in flux solutions prewarmed at 37°C in the presence of 1 mM drug concentration. All flux media were kept in the dark. Each bar represents the Cl-dependent K efflux relative to control, calculated by multiplying the rate constant of K efflux (h^{-1}) obtained from five time points with the K content expressed in mmol/LOC. Note that in the presence of external Rb, DIDS and H_2DIDS abolished the fluxes.

the hemoglobin content was determined at 527 nm with a model 300-N spectrophotometer (Gilford Instrument Laboratories, Oberlin, OH). The ouabain-resistant (OR) Rb influx, $^{i}M_{Rb}^{OR}$, was expressed in mmol LOC^{-1} h^{-1}.

Dixon Plots

The lines in the different Dixon plots are based on linear regression analysis.

Results

DIDS Inhibition of Zero-K-*trans* K Efflux

To assess the potency of DIDS as inhibitor of K-Cl cotransport in LK sheep erythrocytes, we examined the dose response of DIDS on the Cl-dependent zero-K-*trans* K efflux under isosmotic (resting state) and hyposmotic (swelling-activated state) conditions. DIDS had no effect on the Cl-independent K efflux (measured in NO_3, *not shown*). As indicated in Fig. 1, the reciprocal of the Cl-dependent efflux *versus* DIDS concentration (Dixon plot) gives a linear relationship for a large range of concentrations (0–1000 μM) and for both osmolarities. The IC_{50} values (intercepts with X axis) were 82 and 59 μM for iso- and hyposmotic conditions, respectively. Because the flux is expressed per liter original cells, the IC_{50} of 59 μM in hyposmotically swollen cells is underestimated by about 10%.

Characteristics of Inhibition

In order to compare the DIDS inhibition of K-Cl cotransport with the well-characterized DIDS inhibition of anion exchange (Barzilay & Cabantchik, 1979; Knauf, 1989), we tested different compounds of the stilbene family. It has been previously shown that external Rb(K) significantly enhanced the inhibition of K-Cl cotransport by furosemide (Lauf, 1984). Figure 2 compares the efficiency of five compounds at the concentratiion of 1 mM, in the presence or absence of external Rb(K). The results show that at least one of the thiocyanate residues is necessary for the inhibition: DIDS and H_2DIDS, which contain two of these residues, greatly inhibited Cl-dependent K efflux and completely annihilated it in the presence of external Rb, while SITS, with just one SCN residue, only inhibited significantly in the presence of external Rb. On the other hand, DNDS containing nitrate instead of thiocyanate groups and ISA, which constitutes half the molecule of DIDS, only slightly reduced K efflux (about 30% inhibition at 1 mM).

To determine the reversibility of the DIDS inhibition on K transport some cells were exposed to 100 μM DIDS (5% hct) at 37°C for 45 min while others were incubated in the absence of DIDS as control cells. After three washes in the absence of DIDS, the two samples were resuspended in flux solution to assess the transport activity. As indicated

Table 1. Reversibility of DIDS treatment

External conditions	Pretreatment	K efflux (mmol/LOC per hr)
A. NaCl (125 mM)	None	1.30 ± 0.036
	100 μM DIDS	1.30 ± 0.089
B. NaCl (105 mM)	None	1.01 ± 0.042
+ Rb Cl (20 mM)	5 μM DIDS	1.06 ± 0.023

The cells were pretreated in 240 mOsM (NaCl + RbCl) solutions with or without DIDS, for 45 min at 37°C. They were then washed three times in the same solutions in the absence of DIDS and resuspended in flux solutions. The DIDS pretreatment did not affect the fluxes ($P \gg 0.01$, t test). Note that replacement of 20 mM NaCl with 20 mM RbCl causes the well-known K(Rb) *trans*-inhibition of K efflux (Delpire & Lauf, 1991*a*).

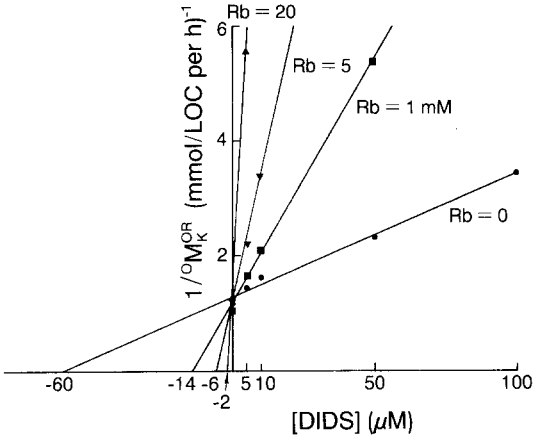

Fig. 3. Dependency of IC_{50} for DIDS on external Rb concentration. Cells were swollen in a NaCl 240 mOsM solution, resuspended at 5% hct in solutions containing different Rb and DIDS concentrations. Cl-dependent K efflux was followed over a 50-min period. Results are plotted on a Dixon plot as in Fig. 1. As Rb increased, the IC_{50} values (intercepts at X axis) decreased.

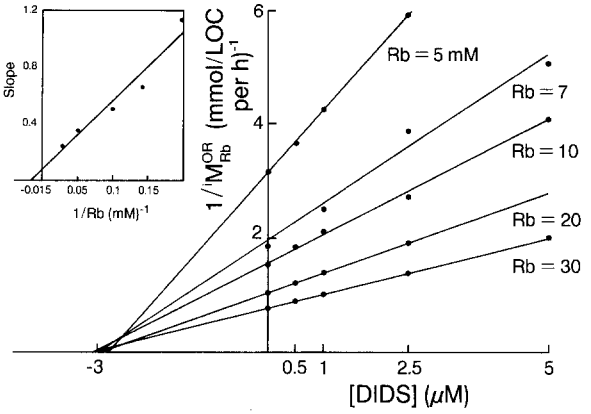

Fig. 4. Dixon plot representing the effect of DIDS on ouabain-resistant Rb influx measured at different Rb concentrations. The cells were swollen at 240 mOsM and resuspended at 37°C in solutions containing different Rb and DIDS concentrations. The Cl-dependent, ouabain-resistant Rb influx was followed over a period of 50 min. Each point represent the reciprocal of the flux calculated over five time points. *Inset*: a replot of the slope *versus* the reciprocal of the Rb concentration. The intercept on the X axis gives the negative reciprocal of the apparent affinity for Rb.

in Table 1, no statistically significant differences were found between the flux levels of cells pre-exposed to DIDS, and control cells. Furthermore, the presence of external Rb(K) in the preincubation solution, which enhances DIDS inhibition (Fig. 2) and therefore supposedly the binding, did not affect the reversibility. From these experiments we can conclude that DIDS is a good, reversible inhibitor of swelling-activated and Cl-dependent zero-K-*trans* K efflux (IC_{50} of 60 μM).

Dependence on External K(Rb)

Figure 3 reveals the sensitivity of DIDS inhibition toward external K(Rb). The IC_{50} decreases dramatically as the external Rb increases. At an external Rb concentration equivalent to the normal physiological K concentration the IC_{50} for DIDS is reduced to about 5 μM. Considering the high potency of DIDS at 5 and 20 mM Rb, only a few points were suitable for fitting. Hence, the IC_{50} of 6 μM measured at 5 mM external Rb may not be significantly different from that of 2 μM determined at 20 mM Rb. Because of *trans*-inhibition of K efflux by external Rb (*cf.* Table 1 and Delpire & Lauf, 1991*a*), the intersection of the four lines are located just left of the Y axis. In the Dixon plot of Fig. 3 (1/efflux *versus* [DIDS]) the slopes increase with higher external Rb, which is opposite to substrate effects in a classical Dixon plot. Hence, we examined the effect of external Rb on Cl-dependent Rb influx (Fig. 4). The concentration of external Rb was varied between 5 and 30 mM. It is of interest to note that the lines intercept the X axis at around −3 μM DIDS, which in the plot defines the true K_i value for a noncompetitive inhibitor. The replot of the slopes *versus* the reciprocal of the Rb concentrations (inset) gives an intercept at −0.015, corresponding to an apparent Rb affinity of 67 mM.

Dependence on External Cl

To further characterize the DIDS inhibition, we examined both the requirement and the effects of external Cl. These experiments were aided by the irrevesible nature of DIDS binding to the anion exchanger

Table 2. Absence of DIDS inhibition in the absence of external Cl (SO_4 replacement)

Internal anion	External Rb (mM)	DIDS (mM)	K efflux (mmol/LOC per hr)
NO_3	—	—	0.23 ± 0.02
Cl	—	—	1.72 ± 0.18
Cl	—	1	1.81 ± 0.08
Cl	20	—	1.80 ± 0.14
Cl	20	1	1.79 ± 0.04

The cells were pretreated at 37°C for 45 min with 10 μM DIDS (hct 5%) in NaCl (240 mOsM) or $NaNO_3$ (225 mOsM). They were then washed three times in a Na_2SO_4 (235 mOsM) and resuspended at 5% hct at 37°C with or without external Rb, in the presence or absence of 1 mM DIDS. The K efflux values are expressed in mmol LOC^{-1} hr^{-1} and represent the mean ± SD over five experimental points.

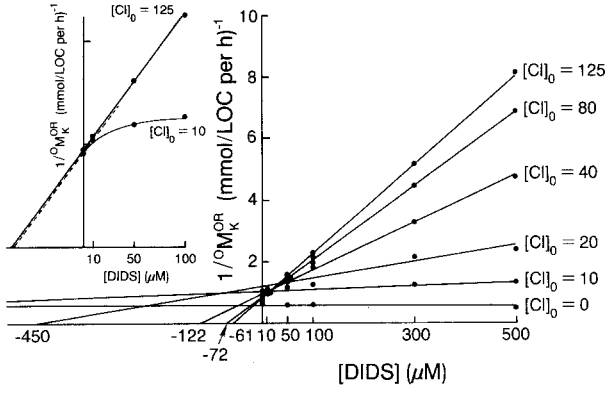

Fig. 5. Effect of external Cl concentration on DIDS inhibition of Cl-dependent K efflux. The cells were first preincubated at 5% hct, 240 mOsM, 37°C, in the presence of 10 μM DIDS, for 45 min. After three washes, the cells were resuspended in flux solutions containing different Cl (SO_4 replacement) and DIDS concentrations. Reciprocal of the flux is plotted against the DIDS concentration. The intercepts on the *X* axis give the IC_{50} values for DIDS. *Inset*: details of inhibitory effect at low DIDS concentrations showing the nonlinearity at low Cl concentrations.

and the reversible binding of DIDS to the K-Cl cotransporter. In the experiment of Table 2, we pretreated the cells with 10 μM DIDS at 5% hct in order to eliminate anion exchange through band III. After removing the excess of DIDS and restoring the activity of K transport by washings, the cells were resuspended in a solution where Cl was replaced by sulfate, in the presence and absence of 20 mM external Rb and 1 mM DIDS. K efflux was followed over a period of 50 min. The first condition represents the Cl-independent K efflux (internal Cl replaced by NO_3). In the four next conditions 1 mM DIDS failed to inhibit in the absence of external Cl, whether or not external Rb was present. Figure 5 presents the reciprocal of the efflux *versus* the DIDS concentration at different external Cl concentrations. Because the graph represents a collection of different experiments, the intersection of all lines is relatively poor. However, as in the case of Fig. 3, the common intersection must be located just left of the *Y* axis because of the *trans*-inhibition of K efflux by external Cl (*see* Delpire & Lauf, 1991*a*). Clearly, the IC_{50} for DIDS increases as external Cl decreases (from 60 μM at high Cl concentration to infinity in the absence of Cl).

It is important to note that the experimental conditions were quite different from those of Figs. 1 and 3. The cells were pretreated first with DIDS to saturate the band III protein, washed, and again exposed to DIDS. The similarity between the data shown here for 125 mM Cl and the previous data (Figs. 1 and 3) indicates that it does not matter whether or not the band III sites were DIDS-saturated prior to or during the determination of the DIDS effect on K transport. Furthermore, at low Cl, the linear relationship between 1/efflux and DIDS concentration gradually was lost. This is revealed in the inset of Fig. 5 where we chose as two extremes the data for Cl = 125 and 10 mM, respectively. In contrast, when considering the linear portion at low DIDS concentrations (*see* main part of Fig. 5), the lines intercepted the *X* axis at around 60 μM DIDS. Figure 6 examines the effect of DIDS on Cl-dependent Rb influx at three different external Cl concentrations ranging from 30 to 120 mM. An external concentration of 30 mM Rb was used to assay the influx. The experiment was therefore performed at a Rb concentration higher than the saturation level for the modulator site for external Rb. As predicted from Fig. 4, the K_i was about 5 μM. Thus, as measured by Rb influx, the affinity for DIDS was not affected when the external Cl concentration was reduced to 30 mM.

Effect on Different Activation Mechanisms

In order to ascertain that DIDS suppressed K-Cl cotransport independent of the mechanism of activation chosen, we also examined the inhibition of the NEM- as well as of the A23187-stimulated K transport. Table 3 compares the IC_{50} values of DIDS for basal, swelling-, NEM- and A23187-activated Cl-dependent K effluxes (flux in Cl minus flux in NO_3). All fluxes, measured in the absence of external Rb(K), were DIDS inhibited with IC_{50} values ranging between 40 and 80 μM and being slightly lower as the fluxes levels were greater.

Table 3. DIDS inhibition of Cl-dependent K movement activated by different manipulations

Treatment	Cl-dependent K efflux (mmol/LOC per hr)	Activation (relative)	IC_{50} (μM)
Control (isosmotic)	0.18 ± 0.012 (5)	1.0	82
Swelling (hyposmotic)	0.97 ± 0.029 (15)	5.4	59, 60, 62
A23187 (Mg depletion)	1.67 ± 0.086 (5)	9.2	59
NEM (thiol alkylation)	3.34 ± 0.015 (10)	18.4	36, 42

For control and swelling, the cells were pre-equilibrated by three washes in isosmotic (295 mOsM) and hyposmotic (240 mOsM) solutions, respectively. In order to deplete the cells of Mg, the cells were treated in isosmotic solution with 10 μM A23187 and 1 mM EDTA for 20 min at 37°C and washed five times in the presence of BSA (50 mg/100 ml) before being used. The Mg content of control cells was measured at 1141 ± 23 (n = 3) μmol/LOC in agreement with values previously reported (Delpire & Lauf, 1991*b*). After depletion, the Mg content was reduced to 3 ± 5 (n = 3) μmol/LOC. For alkylation of the thiol groups, the cells were pretreated for 15 min in the presence of 1 mM NEM, washed twice in the cold and resuspended in the flux solutions in the absence of NEM. The IC_{50} values were calculated from Dixon plots, with six to eight DIDS concentrations. For each concentration, the Cl-dependent flux was measured as the difference between the flux in Cl and the flux in NO_3, each flux being averaged over five time points. As pointed out for Fig. 1 the IC_{50} values for swollen cells are slightly underestimated.

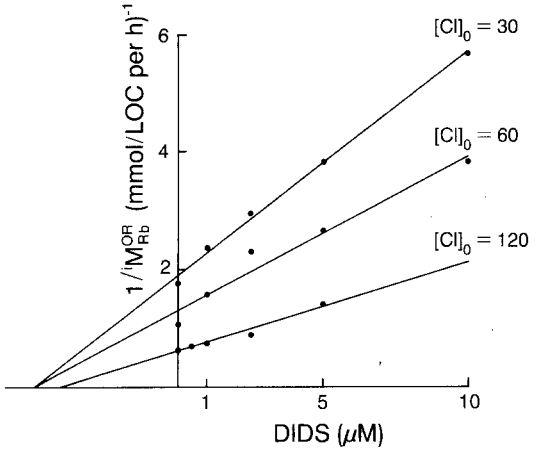

Fig. 6. Effect of external Cl on DIDS inhibition of ouabain-resistant Rb influx. The cells were swollen at 240 mOsM and pretreated with DIDS (10 μM, 37°C, 45 min). After three washes, they were resuspended in 240 mOsM solution containing 30 mM RbCl, 90 mM (NaCl × Na_2SO_4), 100 μM ouabain. Each point represents the reciprocal of the flux calculated from five time points. Between 30 and 120 mM $[Cl]_o$, the K_i was measured at 4–5 μM.

Discussion

Our study demonstrates unequivocally that the disulfonic stilbene derivatives DIDS and H_2DIDS are very potent inhibitors of Cl-dependent K fluxes (K-Cl cotransport) in sheep red cells. The potency of DIDS (IC_{50} = 60 μM) is about 30 times greater than the potency of furosemide (IC_{50} = 2 mM; Lauf, 1984). The stilbenes have, however, a very poor specificity, since they inhibit other Cl transporting molecules such as the anion exchanger (Knauf & Rothstein, 1971) and Cl channels (Bridges et al., 1989; Samman et al., 1991) as well as cation transporters such as the Na^+/K^+ pump (Teisinger, Zemkova & Vyskocyl, 1984) and the Ca^{2+} pump (Niggli, Sigel & Carafoli, 1982).

Interestingly, these data on sheep red cell K-Cl cotransport are in contrast with the absence of an effect in human red cells which has been reported independently by three groups (Brugnara et al., 1985; Kaji, 1986; Garay et al., 1988). This may suggest important differences between the two transport molecules as already suggested by kinetic analyses (Delpire & Lauf, 1991*a,c*; Kaji, 1989).

Comparing stilbene inhibition of K-Cl cotransport and anion exchange the following basic distinctions can be made. (i) Whereas the sulfonate group seems sufficient for reversible anion exchange inhibition (Barzilay & Cabantchik, 1979), the SCN group is essential for inhibition of the cotransporter (DNDS inhibits anion exchange but not K-Cl cotransport). (ii) The SCN group generates covalent (irreversible) binding to band III, but not to the K-Cl cotransporter (reversible interaction, Table 1). The very different nature of DIDS interaction with the two transporters permits studying Cl-dependent K movements in the absence of anion exchange and therefore excludes any direct participation of Band III in Cl-related K movement. SITS, which contains only one SCN group, had a very poor inhibitory effect in the absence of external Rb(K), which is consistent with an early report showing absence of inhibition on NEM-stimulated K efflux (Lauf & Theg, 1980). However, in the presence of external Rb(K), 1 mM SITS suppressed almost completely

the Cl-dependent K efflux. More surprising was the absence of a significant inhibitory effect of ISA (half the molecule of DIDS), which suggests requirement of the two aromatic rings for K-Cl cotransport inhibition. This is in contrast to the covalent binding of the benzene derivative to band III protein (Barzilay & Cabantchik, 1979).

The increase of DIDS inhibition by external K (Fig. 2) was similar to the effect on furosemide binding previously reported for the same system (Lauf, 1984) and also for bumetanide binding to the Na-K-2Cl cotransporter (Palfrey, Feit & Greengard, 1980; Haas & McManus, 1983). The fact that the effect occurs mainly at low Rb(K) concentrations (significant shift at 1 mM Rb, Fig. 3) distinguishes this Rb(K) site from the K transport site, which has a much lower affinity (Delpire & Lauf, 1991*a*). The high affinity K site is also suggested in Fig. 4, where Rb influx was measured with Rb concentrations ranging from 5 to 30 mM. Already at 5 mM the site was saturated (as demonstrated by the 'noncompetitive' intercept on the X axis indicating K_i value for DIDS binding of 3 μM). Thus a replot of the slope *versus* the reciprocal of the Rb concentration (Segel, 1975) reveals the transport site: the affinity is low and comparable to values previously reported (Delpire & Lauf, 1991*a*).

The existence of two sites also distinguishes K-Cl cotransport from Na-K-Cl cotransport since the K concentration half maximally effecting bumetanide inhibition matches that for cotransport stimulation (Haas & McManus, 1982). It is of interest to note that the estimated affinity of the 'allosteric' K site of K-Cl cotransport is in the same range as the K affinity of the transport site for Na-K-Cl cotransport. Therefore, while similarities exist between the two transport systems in regard to K requirement for inhibitor binding, the two transporters diverge in transport site affinities.

The distinction between a single site (Na-K-2Cl) and two distinct sites (transport and modulator sites for K-Cl) is important in light of the possibility to use inhibitors to assess the binding order. In a recent kinetic study, we determined by two independent methods the binding order of K and Cl at the outside configuration of the cotransporter (Delpire & Lauf, 1991*a*). It was shown that Cl binds first, followed by K. Because K modifies the binding of bumetanide to the Na-K-2Cl transporter through the transport site (Haas & McManus, 1982) and augments the furosemide inhibition of K-Cl cotransport (Lauf, 1984), we anticipated confirming the binding order using the inhibitors. However, the existence of two distinct sites (modifier and transport sites) prevents us from using these inhibitors to confirm the binding order at the transport sites. Nevertheless, it is of interest to note that DIDS inhibition of K-Cl cotransport does not occur in the absence of external Cl (Table 2 and Fig. 5), whether or not external Rb(K) is present. These results suggest that Cl must bind prior to DIDS. Again, a Cl 'allosteric' site has to be distinguished from the Cl transport site. Indeed, the data of Fig. 6 demonstrate absence of competition between DIDS and Cl. Taken together, the results of Figs. 4 and 6 indicate pure noncompetitive inhibition, i.e. DIDS binds equally well to the free transporter, its Cl loaded complex and the tertiary complex with KCl. Therefore, these results have to be reconciled with the requirement of Cl for DIDS inhibition which must occur via a site independent of the Cl transport site. Interestingly, the existence of two separate Cl sites at the Na-K-2Cl cotransporter has been shown by Kinne's group (Kinne et al., 1976). The nonlinearity of the low Cl concentration curve (Fig. 5) may represent a degree of competition between the anion groups of the inhibitor and Cl.

A question often raised concerns the identity of K movements activated by different interventions: Are the K fluxes activated by cell swelling supported by the same mechanism as those stimulated by thiol oxydation/alkylation (Lauf & Theg, 1980) or Mg depletion (Lauf, 1985; Delpire & Lauf, 1991*b*)? This study demonstrates that DIDS inhibits basal K-Cl cotransport as well as K-Cl flux activated by swelling, NEM and A23187 consistent with the hypothesis that the same macromolecular complex facilitates Cl-dependent K movements.

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References

Barzilay, M., Cabantchik, Z.I. 1979. Anion transport in red blood cells. II. Kinetics of reversible inhibition by nitroaromatic sulfonic acids. *Membr. Biochem.* **2**:255–281

Bridges, R.J., Worrell, R.R., Frizzell, R.A., Benos, D.J. 1989. Stilbene disulfonate blockade of colonic secretory Cl^- channels in planar lipid bilayers. *Am. J. Physiol.* **256**:C902–912

Brugnara, C., Kopin, A.S., Bunn, H.F., Tosteson, D.C. 1985. Regulation of cation content and cell volume in hemoglobin erythrocytes from patients with homozygous hemoglobin C disease. *J. Clin. Invest.* **75**:1608–1617

Delpire, E., Lauf, P.K. 1991*a*. Kinetics of Cl-dependent K fluxes in hyposmotically swollen low K sheep erythrocytes. *J. Gen. Physiol.* **97**:173–193

Delpire, E., Lauf, P.K. 1991*b*. Magnesium and ATP dependence of K-Cl cotransport in low K^+ sheep red blood cells. *J. Physiol.* **441**:219–231

Delpire, E., Lauf, P.K. 1991*c*. *Trans*-effects of cellular K and Cl on ouabain-resistant Rb(K) influx in low K sheep red blood cells: Further evidence for asymmetry of K-Cl cotransport. *Pfluegers Arch.* **419**:540–542

Ellory, J.C., Dunham, P.B. 1980. Volume-dependent passive potassium transport in LK sheep red cells. *In*: Membrane Transport in Erythrocytes. U.V. Lassen, H.H. Ussing, and J.O. Wieth, editors. p. 409. Alfred Benzon Symposium 14. Munksgaard, Copenhagen

Garay, R.P., Nazaret, C., Hannaert, P.A., Cragoe, E.J. 1988. Demonstration of a [K^+,Cl^-]-cotransport system in human red cells by its sensitivity to [(dihydroindenyl)oxy]alkanoic acids: Regulation of cell swelling and distinction from the bumetanide-sensitive [Na^+,K^+,Cl^-]-cotransport system. *Mol. Pharmacol.* **33:**696–701

Haas, M., Harrison, J.D. 1989. Stimulation of K-Cl cotransport in rat red cells by a hemolytic anemia-producing metabolite of dapsone. *Am. J. Physiol.* **256:**C265–C272

Haas, M., McManus, T.J. 1982. Bumetanide inhibition of (Na + K + 2Cl) cotransport and K/Rb exchange at a chloride site in duck red cells: Modulation by external cations. *Biophys. J.* **37:**214*a*

Haas, M., McManus, T.J. 1983. Bumetanide inhibits (Na + K + 2Cl) co-transport at a chloride site. *Am. J. Physiol.* **245:**C235–C240

Jennings, M.L. 1989. The anion transport: Protein structure and function. *In*: The Red Cell Membrane: A Model for Solute Transport. B.U. Raess and Godfrey Tunnicliff, editors. pp. 143–170. Humana, Clinton (NJ)

Kaji, D. 1986. Volume-sensitive K transport in human erythrocytes. *J. Gen. Physiol.* **88:**719–738

Kaji, D. 1989. Kinetics of volume-sensitive K transport in human erythrocytes: Evidence for asymmetry. *Am. J. Physiol.* **256:**C1214–C1223

Kinne, R., Kinne-Saffran, E., Schrölermann, B., Schültz, H. 1976. The anion specificity of the sodium-potassium-chloride cotransporter in rabbit kidney out medulla: Studies on medullary plasma membranes. *Pfluegers Arch.* **407:**S168–S173

Knauf, P.A. 1989. Kinetics of anion transport. *In*: The Red Cell Membrane: A Model for Solute Transport. B.U. Raess and Godfrey Tunnicliff, editors. pp. 171–200. Humana, Clinton (NJ)

Knauf, P.A., Rothstein, A. 1971. Chemical modification of membranes. I. Effect of sulfhydryl and amino reactive reagents on anion and cation permeability of the human red blood cell. *J. Gen. Physiol.* **58:**190–210

Lauf, P.K. 1982. Evidence for chloride dependent potassium and water transport induced by hyposmotic stress in erythrocytes of the marine teleost *Opsanus tau*. *J. Comp. Physiol.* **146:**9–16

Lauf, P.K. 1984. Thiol-dependent passive K/Cl transport in sheep red cells: IV. Furosemide inhibition as a function of external Rb^+, Na^+, and Cl^-. *J. Membrane Biol.* **77:**57–62

Lauf, P.K. 1985. $K^+ : Cl^-$ cotransport: Sulfhydryls, divalent cations, and the mechanism of volume activation in a red cell. *J. Membrane Biol.* **88:**1–13

Lauf, P.K., Theg, B.E. 1980. A chloride dependent K flux induced by N-ethylmaleimide in genetically low K sheep and goat erythrocytes. *Biochem. Biophys. Res. Commun.* **92:**1422–1428

Lytle, C., McManus, T.J. 1987. Effect of loop diuretics and stilbene derivatives on swelling-induced K-Cl cotransport. *J. Gen. Physiol.* **90:**28*a*

Niggli, V., Sigel, E., Carafoli, E. 1982. Inhibition of the purified and reconstituted calcium pump of erythrocytes by μM levels of DIDS and NAP-taurine. *FEBS Lett.* **13:**164–166

Palfrey, H.C., Feit, P.W., Greengard, P. 1980. cAMP-stimulated cation cotransport in avian erythrocytes: Inhibition by "loop" diuretics. *Am. J. Physiol.* **238:**C139–C148.

Samman, G., Ohtsuyama, M., Sato, F., Sato, K. 1991. K and Cl channel activation during regulatory volume decrease (RVD) in Rhesus eccrine clear cells is dissimilar to that during methacholine stimulation. *FASEB J.* **5:**A669

Segel, I.H. 1975. Enzymes Kinetics. pp. 134–135. John Wiley & Sons, New York

Shami, Y., Rothstein, A., Knauf, P.A. 1978. Identification of the Cl^- transport site of human red blood cells by a kinetic analysis of the inhibitory effects of a chemical probe. *Biochim. Biophys. Acta* **508:**357–363

Teisinger, J., Zemkova, H., Vyskocyl, F. 1984. The effect of anion channel blockers on enzymatic activity of Na^+/K^+-ATPase and the electrogenic $Na^+ : K^+$ pump. *FEBS Lett.* **175:**275–278

Zade-Oppen, A.M.M., Lauf, P.K. 1990. Thiol-dependent passive K : Cl transport in sheep red blood cells: IX. Modulation by pH in the presence and absence of DIDS and the Effect of NEM. *J. Membrane Biol.* **118:**143–151

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