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INHIBITION OF EHRLICH ASCITES CELL ANION TRANSPORT BY 1-ISOTHIOCYANATE-4-BENZENESULFONIC ACID

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Summary

The effects of 1-isothiocyanate-4-benzene sulfonic acid on steady state Cl^- and SO_4^{2-} transport in Ehrlich mouse ascites tumor cells were investigated. At 10 mM, 1-isothiocyanate-4-benzenesulfonic acid reduced SO_4^{2-} exchange by 94% but Cl^- exchange was reduced by only 37%; Cl^- exchange was not further inhibited by as much as 60 min of preincubation with 1-isothiocyanate-4-benzenesulfonic acid. Inhibition of Cl^- exchange was completely reversible following 30—45 min of contact with 1-isothiocyanate-4-benzenesulfonic acid whereas under the same conditions, inhibition of SO_4^{2-} exchange was irreversible. The effect of 1-isothiocyanate-4-benzenesulfonic acid on SO_4^{2-} transport could be reversed, however, when exposure to 1-isothiocyanate-4-benzenesulfonic acid lasted for only 2 min. In these respects the action of 1-isothiocyanate-4-benzenesulfonic acid resembles that of 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid and 4,4′-diisothiocyano-1,2-diphenylethane-2,2′-disulfonic acid; the results are compatible with separate membrane sites for Cl^- and SO_4^{2-} transport. The K_i for reversible inhibition of SO_4^{2-} transport, determined from a Dixon plot, was 4.8 mM and the inhibition appeared to be noncompetitive.

Introduction

There is considerable evidence for mediated exchange transport of Cl^- [1—4] and SO_4^{2-} [5] in the Ehrlich ascites tumor cell. The disulfonic stilbenes,

Abbreviations: H_2Dids, 4,4′-diisothiocyano-1,2-diphenylethane-2,2′-disulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Ibs, 1-isothiocyanate-4-benzenesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; Sits, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid.

which have been so useful in characterizing the erythrocyte anion transport system [6,7] have been employed to study ascites cell anion transport as well. Interestingly, disulfonic stilbenes do not affect ascites cell Cl^- transport in the same manner as they influence SO_4^{2-} transport. Whereas SO_4^{2-} transport is virtually abolished by both Sits and H_2Dids [8,9], Cl^- exchange is inhibited by no more than 37% [9,10]. Furthermore, both Sits and H_2Dids can reduce SO_4^{2-} exchange irreversibly [8,9,11] while the effect of these inhibitors on Cl^- exchange is completely reversible [9,10].

It has been shown that H_2Dids reacts covalently with ascites cell membrane proteins and that it might be feasible to identify a SO_4^{2-} transport protein with it [11,12] but disulfonic stilbenes may not be useful for identifying a Cl^- transport site. A compound which has not heretofore been utilized in ascites cell anion transport studies and which has been helpful in characterizing the red cell anion transport system is Ibs. This substance is capable of irreversibly inhibiting red cell phosphate [13] and SO_4^{2-} [14,15] transport; it binds covalently to band-3 protein [13], which is considered to participate directly in anion exchange. It was of interest to determine whether Ibs could irreversibly inhibit a significant fraction of ascites cell Cl^- exchange and to compare the effects on SO_4^{2-} exchange.

Methods

Cell suspensions and solutions

Ehrlich ascites cells were grown in mice, harvested, and washed as described previously [10]. Washed cells were brought to a concentration of $7.5 \cdot 10^7$—$8.5 \cdot 10^7$ cells/ml (equiv. to 90—120 mg wet weight or to 11—18 mg dry weight/ml, determined as in Ref. 10). All studies were performed in an air atmosphere at 21—24°C. Depending on the type of study, one of the solutions below was used for washing and experimentation (concentration in mM). Solution I: 154 NaCl/6 KCl/2 $CaCl_2$/10 Hepes or 10 Mops; Solution II: 134 NaCl/10 Na_2SO_4/6 KCl/2 $CaCl_2$/10 Hepes or 10 Mops; Solution III: 154 NaCl/0.5 Na_2SO_4/6 KCl/2 $CaCl_2$/10 Mops; Solution IV: 149 NaCl/2.5 Na_2SO_4/6 KCl/2 $CaCl_2$/10 Mops. All solutions were titrated to pH 7.2 with NaOH; osmolarity was 297—316 mosM/l.

Chloride and sulfate efflux coefficient

Cl^- and SO_4^{2-} exchanges were determined in the steady-state by obtaining the efflux rate coefficient for each ion. The efflux coefficient represents the fractional exchange rate or turnover of the particular ion. The Cl^- efflux coefficient was measured from the rate of $^{36}Cl^-$ uptake and cell Cl^- content as previously described [10,16]. Studies were initiated when cells were diluted 1/10 with Solution I or II containing $^{36}Cl^-$ at 0.15 μCi/ml suspension, in the presence or absence of Ibs.

SO_4^{2-} turnover was measured by washout of $^{35}SO_4^{2-}$ from preloaded cells, according to the procedure of Levinson [17], in which the rate of loss of cellular radioactivity is assessed. Cells suspended in Solution II were loaded with $^{35}SO_4^{2-}$ (1.5 μCi/ml suspension) for 90—120 min which was long enough to reach isotopic equilibrium; cell SO_4^{2-} content was then determined [5]. Cells

were washed twice with ice-cold Solution II to remove extracellular $^{35}SO_4^{2-}$ (there were approx. 1 ml packed cells/45 ml wash solution per wash). Exchange studies were initiated when resuspended cells were diluted 1/10 with Solution II in the presence or absence of Ibs. In most cases, $^{36}Cl^-$ was also present so that $^{36}Cl^-$-uptake could be measured simultaneously.

When the purpose of the experiment was to assess whether the effect of Ibs on anion exchange was reversible, cells were incubated during the last 30—45 min with Ibs and then washed twice with cold medium containing 0.5% albumin and once more with cold albumin-free medium. $^{36}Cl^-$-uptake and/or $^{35}SO_4^{2-}$ loss were then determined as described above.

Initial sulfate influx

In two types of studies the initial 2 min influx of SO_4^{2-} was measured. This procedure was used when the reversibility of brief exposure to Ibs was assessed and when competition between SO_4^{2-} and Ibs was studied. Cells were incubated for 90—120 min in Solution III or IV to achieve a steady-state; in the reversibility experiments cells were then exposed to Ibs for 2 min, washed twice with cold medium and resuspended in Ibs-free medium before further use. Initial SO_4^{2-}-influx was determined by mixing, in a centrifuge tube, 1 ml cell suspension with 3 ml Solution III or IV containing $^{35}SO_4^{2-}$ at a final concentration of 1 μCi/ml, in the presence or absence of Ibs. After 2 min, ice cold wash solution (see below) was added, the cells centrifuged and treated as described under 'Cell analysis'. The influx procedure was carried out in quadruplicate. Initial SO_4^{2-} influx was calculated from the amount of $^{35}SO_4^{2-}$ taken up by the cells in 2 min and the environment specific activity [8].

Cell analysis

Cell samples were washed twice with ice-cold $NaNO_3$ wash solution (171 mM $NaNO_3$, buffered to pH 7.2 with 0.003 M Na-Hepes or Na-Mops, osmolarity 301—311). The packed cell pellets were extracted with 7% perchloric acid for at least 30 min. The extracts were analyzed for Cl^- by electrometric titration and for K^+ by flame photometry. Radioactivity was monitored by liquid scintillation counting; when $^{36}Cl^-$ and $^{35}SO_4^{2-}$ were present together one channel was set to count only $^{36}Cl^-$ and spillover of $^{36}Cl^-$ into the $^{35}SO_4^{2-}$ channel was corrected for. Cell water and dry weight were determined as described previously [10].

Materials

Ibs was purchased from Pierce Chemical Co. Isotopes were from New England Nuclear.

Results

Chloride and sulfate flux in the presence of Ibs

When the efflux coefficients for Cl^- and SO_4^{2-} were determined in the presence of Ibs, without prior exposure to the inhibitor, an effect on both anions was detected within 1 to 2 min following contact with Ibs. The results of a representative experiment are given in Table I. The inhibitor reduced SO_4^{2-}

TABLE I

EFFECT OF INCREASING Ibs CONCENTRATION ON CHLORIDE AND SULFATE SELF EXCHANGE

Efflux coefficients for Cl^- and SO_4^{2-} were measured simultaneously (see Methods). Exchange with radioactive isotope was initiated at the same time that cells were mixed with Ibs. Results with Ibs-treated cells are expressed as a percent of the control efflux coefficient, which for Cl^- was 2.7 h^{-1} and for SO_4^{2-} was 1.8 h^{-1}.

Ibs concn. (mM)	Percent of control	
	Cl^-	SO_4^{2-}
2	88	72
4	80	41
6	70	33
8	62	24
10	59	7

turnover much more effectively than Cl^- turnover. At 10 mM, Ibs inhibited SO_4^{2-} exchange by 94% (n = 3) but Cl^- exchange at the same concentration of Ibs was inhibited by only 37% (n = 13).

Preincubation with Ibs for 60 min prior to the addition of $^{36}Cl^-$ did not result in further reduction of Cl^- exchange. In one experiment, for example, the efflux coefficients for Ibs-treated cells after 1 min and 60 min were 2.0 h^{-1} and 2.3 h^{-1}, respectively, while the comparable control values were 3.1 and 3.4.

Reversibility of inhibition by Ibs

To test whether or not inhibition of anion exchange by Ibs was reversible, cells were treated with the inhibitor for 30—45 min, washed and the anion efflux coefficients were then determined. The effects of 10 mM Ibs on Cl^- exchange were completely reversible. One of six such experiments is shown in Fig. 1. The treated, washed cells had a Cl^- turnover rate of 4.2 h^{-1} while the untreated, washed cells had a turnover of 3.6 h^{-1}. Typical inhibition by Ibs was seen, however, when untreated, washed cells were then exposed to 10 mM Ibs (turnover rate = 2.3 h^{-1}). Note also that when Ibs was present there was no effect on cell Cl^- content, indicating that a one for one exchange process was affected.

In contrast, SO_4^{2-} turnover was irreversibly reduced following 30—45 min of pre-treatment with Ibs. A summary of the results is shown in Fig. 2. The efflux rate coefficients measured in the presence of Ibs are not distinguishable from those of cells which were pretreated with Ibs and then washed free of the inhibitor.

It was possible to reverse the inhibitory effect of Ibs on SO_4^{2-} flux when the duration of exposure to Ibs was 2 min or less. In one experiment after 2 min of contact with Ibs followed by washing, the initial SO_4^{2-} influx (see Methods) was 2.80 μmol/g dry weight per h while the control flux was 2.86. Another such experiment gave very similar results.

Dixon plot of reversible inhibition of SO_4^{2-} flux by Ibs

During the first 2 min of contact with Ibs, SO_4^{2-} flux is reversibly inhibited

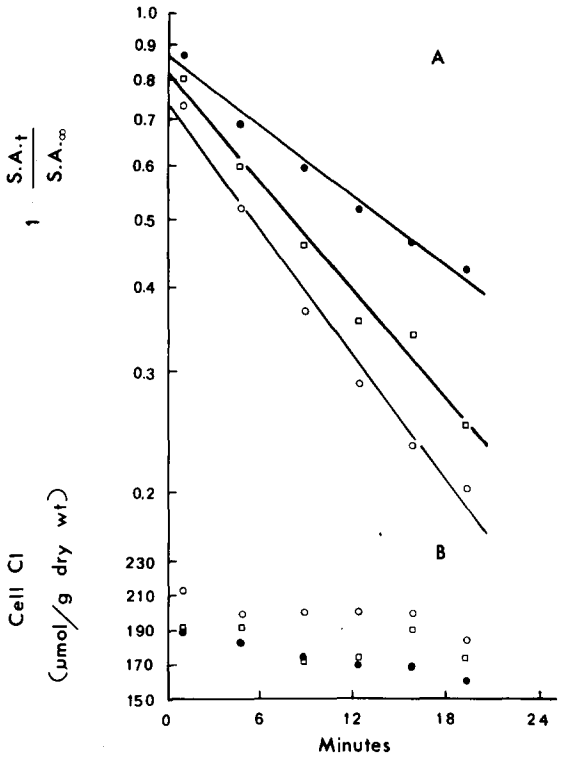

Fig. 1A. Reversibility of Ibs inhibition of Cl^- exchange. Cl^- efflux coefficient is obtained as described in Ref. 16, from slope of the linear regression equation. $S.A._t$ = specific activity of cells in cpm/μmol; $S.A._\infty$ is obtained from the environment and is constant. ○, cells treated with 10 mM Ibs for 38 min, washed and at time zero mixed with $^{36}Cl^-$ in the absence of Ibs; □, untreated cells incubated, washed and mixed with $^{36}Cl^-$; ●, untreated cells incubated, washed and mixed with $^{36}Cl^-$ in the presence of 10 mM Ibs. The respective rate coefficients were: 4.2, 3.6, and 2.3 h^{-1}. B. Cell Cl^- content in the same experiment during the $^{36}Cl^-$ uptake period.

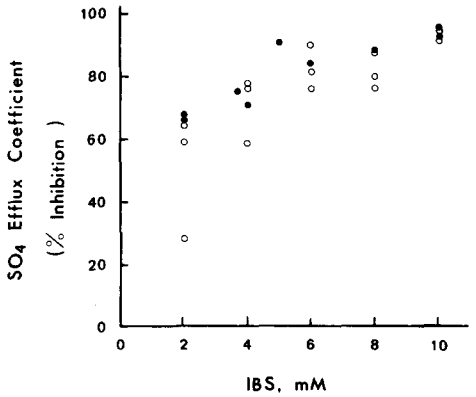

Fig. 2. Irreversibility of Ibs effect on SO_4^{2-} exchange at different external Ibs concentrations. Efflux coefficients determined from $^{35}SO_4^{2-}$ loss, as described in Ref. 17. Individual data from five different experiments. ○, cells were exposed to Ibs during $^{35}SO_4^{2-}$ washout; ●, cells were exposed to Ibs for 30—45 min, washed, and $^{35}SO_4^{2-}$ washout was followed in the absence of Ibs. Percent inhibition is 1- (efflux coefficient in Ibs/control efflux coefficient) × 100. Mean control efflux coefficient was 1.9 h^{-1} ± 0.18 (S.E.), n = 5; mean control cell SO_4^{2-} content was 7.93 μmol/g dry weight ± 0.26 (S.E.), n = 5.

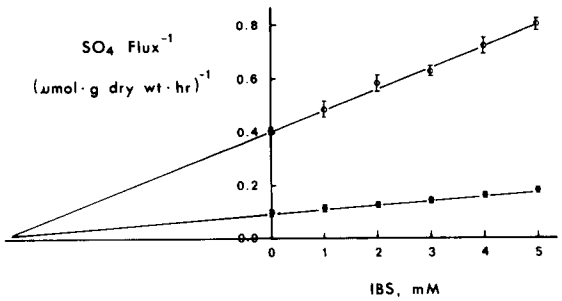

Fig. 3. Dixon plot: the reciprocal of the initial SO_4^{2-} influx (see Methods) as a function of extracellular Ibs concentration, at two external SO_4^{2-} concentrations; ○, 0.5 mM SO_4^{2-} ●, 2.5 mM SO_4^{2-}. Mean values ± S.E. from three experiments.

so that it should be possible to determine the K_i for inhibition and whether or not inhibition is competitive from the initial SO_4^{2-} influx during this brief exposure to Ibs. A Dixon plot [18] from the results of three studies is shown in Fig. 3. The K_i was 4.8 mM; at this point of intersection of the two lines the value of the ordinate does not differ significantly from zero ($0.2 < P < 0.3$). Inhibition of SO_4^{2-} flux by Ibs, therefore, does not appear to be competitive.

Discussion

The results of this study show that Cl^- and SO_4^{2-} transport in the ascites tumor cell are affected differently by the benzene sulfonic acid, Ibs. The differences are the same as those found previously for the action of the disulfonic stilbenes, Sits and H_2Dids: Cl^- exchange is inhibited by only 37% when inhibition of SO_4^{2-} exchange is greater than 90%; further, inhibition of Cl^- exchange is completely reversible while SO_4^{2-} exchange can be inhibited irreversibly. This pattern thus differs from the response of human erythrocytes, for which there is no evidence of such different effects on Cl^- vs. SO_4^{2-} transport [6,7,14,15].

The results are compatible with, but do not prove, the existence of separate membrane transport systems or sites for Cl^- and SO_4^{2-} in the ascites cell membrane. Although Levinson [9,17] has given evidence for interaction between SO_4^{2-} and Cl^- in ascites cell anion exchange, direct kinetic studies have not demonstrated competition between these two anions (unpublished results). The existence of separate transport pathways for Cl^- and SO_4^{2-} remains a possibility which requires further investigation.

It is interesting that inhibition of Cl^- turnover by 10 mM Ibs was 37% since that was the maximum effect observed with Sits [10]. Although we did not study anion exchange at higher concentrations of Ibs the results suggest that Sits and Ibs (and probably H_2Dids) act on the same moiety of Cl^- transport. This interpretation is supported by an experiment in which we assessed the combined effect of 10 mM Ibs plus a maximally effective concentration of Sits (0.6 mM) and found that the inhibition was also 37%. It should be pointed out however, that Cl^- transport can be reduced by as much as 80—90% with furosemide [10] and phloretin [9].

A concentration of Ibs which was at least 10 times greater than that of Sits and

H_2Dids was required for comparable depression of Cl^- exchange and 100 times the concentration was needed for inhibition of SO_4^{2-} transport [9,10]. These results are consistent with studies of anion transport in human erythrocytes in which 10 mM Ibs was needed to inhibit inorganic phosphate [13] and SO_4^{2-} [15] transport effectively, whereas Sits [19] and H_2Dids [20] were 100 and 400 times more potent.

In the ascites cell, 10 mM Ibs did not appear to harm the cells. Cell Cl^- content was unchanged (Fig. 1B). There was no detectable effect on K^+ transport since cells maintained normal intracellular K^+ levels and were even able to accumulate K^+, both against a steep concentration gradient. In the experiment shown in Table I, for example, cells maintained the intracellular K^+ at 131 and 127 mequiv./kg cell water in control and Ibs treated cells, respectively; in another experiment, during 38 min of contact with 10 mM Ibs, cells accumulated K^+ from 123 to 134 mequiv./kg cell water while in control cells K^+ went from 122 to 139 mequiv./kg cell water.

Villereal and Levinson [8] concluded that Sits competitively inhibited SO_4^{2-} transport in the ascites cell. The Dixon plot of Ibs action on SO_4^{2-} transport failed to demonstrate competition. Further experiments would be needed to determine whether or not these agents act on SO_4^{2-} transport by different mechanisms.

The present results emphasize once again that in the ascites cell Cl^- transport is relatively insensitive to specific anion transport inhibitors and that these agents bind reversibly to the Cl^- transport site, in contrast to their interaction with a SO_4^{2-} transport site. A means of labeling a Cl^- binding site in the ascites cell membrane remains to be developed.

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