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CHLORIDE SELF EXCHANGE IN EHRLICH ASCITES CELLS

INHIBITION BY FUROSEMIDE AND 4-ACETAMIDO-4′-ISOTHIOCYANOSTILBENE-2,2′-DISULFONIC ACID

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Summary

The effects of furosemide and 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS) on steady-state Cl^- flux were studied in Ehrlich mouse ascites cells. At 10 mM, furosemide inhibited isotopically-determined Cl^- flux by 86% without changing cell Cl^- content, indicating that influx and efflux were depressed by the same amount. These results suggest that at least 86% of the steady-state Cl^- flux may occur as a one for one exchange. Half of the inhibitory effect was not reversed by vigorous washing with albumin-Ringer. A smaller portion of steady-state Cl^- flux was inhibited by SITS. The maximum effect of SITS was reached near 0.6 mM; at this concentration Cl^- flux was reduced by 37% without an alteration in cell Cl^- content. Possible competition of environment Cl^- and SITS was investigated by replacing environment Cl^- with acetate or NO_3. These anions reduced the efficacy of SITS because they depressed cell Cl^- turnover themselves, apparently acting on the same exchange process.

Introduction

It has been postulated that Cl^- transfer in Ehrlich ascites tumor cells is carrier mediated. This conclusion was based on the inhibitory effect of certain anions on Cl^- self exchange [1,2], the demonstration of saturation kinetics in some instances [1,2] and measurements which suggested that the Cl^- conductance of the cell membrane was less than predicted from the isotopic exchange

Abbreviations: SITS, 4-acetamido-4′-isothiocyano-stilbene-2,2′-disulfonic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid.

flux [2,3]. However, there is uncertainty about the relative contribution of carrier-mediated movement to the total Cl^- flux. Heinz and co-workers [3] estimated that Cl^- exchange flux was about 20 times faster than net Cl^- flux but Levinson and Villereal [1] concluded that mediated exchange represented 40% of total Cl^- flux.

In order to clarify this question and to learn more about the Cl^- exchange system we studied the effect of two agents known to inhibit ion exchange transfer in cells: furosemide and SITS. It has been known for several years that furosemide exerts its powerful diuretic action by depressing active Cl^- transport in the thick ascending limb of Henle's loop [4]. Recently this agent was shown to inhibit carrier-mediated Cl^- exchange in the human erythrocyte [5] and was postulated to inhibit a Cl^- exchange pathway in frog skin [6]. The effects of furosemide are not confined to anion transport, however. Tupper [7] demonstrated that furosemide inhibited a one for one exchange component of Na^+ and K^+ transfer in Ehrlich ascites cells and Sachs [8] found inhibition of Na^+-Na^+ exchange in the human erythrocyte. SITS, on the other hand, specifically depresses anion transport in the human red blood cell [9]. In addition, the ability of irreversibly bound SITS to inhibit the transfer of SO_4^{2-} but not of Cl^- or PO_4^{3-} was evidence obtained by Villereal and Levenson [10] in support of separate mechanisms for SO_4^{2-} and Cl^- movement in the ascites cell. In this report, based on the action of furosemide, it is suggested that at least 86% of the total Cl^- flux takes place by a one for one exchange process. A portion of Cl^- exchange is also sensitive to SITS.

Methods

Cell suspensions. Ehrlich ascites cells (hyperdiploid) were grown in HA/ICR Swiss mice by intraperitoneal transplantation. After 9—12 days of growth cells were harvested by aspiration immediately following cervical dislocation. They were washed three times by gentle centrifugation and resuspension in control Ringer (9 g NaCl, 40 ml 0.154 M KCl, 100 ml of 0.1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES) buffer, titrated to pH 7.4 with NaOH and brought to 1 l with distilled water; osmolarity 305—318). Washed cells were diluted with Ringer 1/11 such that the final cell concentration was $7 \cdot 10^6$—$8 \cdot 10^6$ cells/ml. An experiment was initiated immediately or within 30 min by addition of $^{36}Cl^-$ to the suspension (0.15 μCi/ml suspension).

The effect of low external Cl^- concentrations on the ability of SITS to inhibit Cl^- flux was studied in some experiments. After harvesting cells in control Ringer, they were washed with solutions in which sodium acetate or $NaNo_3$ replaced NaCl mol for mol. The cells were exposed to these media for a total of 2 h before mixing with $^{36}Cl^-$, in order to achieve a constant internal Cl^- content.

Sampling and analysis. All studies were performed at room temperature (20—24°C) and in an air atmosphere. Samples of cell suspensions were removed at intervals following addition of $^{36}Cl^-$ to the medium, centrifuged and the supernatant either saved for subsequent analysis or discarded. The packed cells were washed with ice-cold $NaNO_3$ (14.5 g $NaNO_3$/l, buffered to pH 7.4 with 0.0003 M Na-HEPES, final osmolarity 301—311), according to procedures pre-

viously described [11]. We found $NaNO_3$ preferable to choline dihydrogen citrate [1] wash solution because, in contrast to the latter, no loss of Cl^- or K^+ was detectable during the washing procedure.

The packed cell pellets were extracted with 7% $HClO_4$ for at least 30 min. Clear extracts of the cell pellets and the supernatant environments (see above) were analyzed for Cl^- by electrometric titration and for K^+ by flame photometry [11]. Radioactivity was monitored in a Searle liquid scintillation counter, using Hydromix™ (Yorktown Research) scintillation fluid.

Cell water and dry weight were measured by packing 1-ml samples of concentrated cells ($8 \cdot 10^7$ cells/ml) for 2 min in a Brinkman table top centrifuge. Trapped extracellular fluid was 0.23 ml/g wet weight ±0.01 (S.E.), $n = 8$, as detected with [^{3}H]dextran. Dry weights were obtained after drying the pellets for 24 h at 95—110°C. In 13 separate experiments the mean dry weight per 10^7 cells was 2.43 ± 0.13 (S.E.) mg.

Calculation of chloride exchange rate. The rate of Cl^- exchange was determined from isotopic uptake by cells in the steady state, using the kinetics of a two-compartment closed system. The equations have been given earlier [11]. An efflux rate coefficient (k_e) is obtained which represents the fractional exchange rate of cell Cl^-. When multiplied by cell Cl^- content, a steady-state flux is calculated.

Materials. Furosemide powder was a gift from Hoechst Pharmaceuticals, Inc. (Somerville, N.J.). It was dissolved in Ringer and titrated to pH 7.4 with NaOH. In a few experiments we used the commercial preparation, Lasix, diluted with Ringer and titrated to pH 7.4 with HCl. There was no difference in effects detectable between the two types of preparations. SITS was purchased from ICN Pharmaceuticals or from British Drug House (U.S. outlet Gallard-Schlesinger, Carle Pl., N.Y.). SITS from both sources gave comparable results; however, material from the latter source was more stable. Both SITS and furosemide were made up freshly for each experiment and stored in the dark until use. Isotopes were from New England Nuclear; $H^{36}Cl$ was neutralized with NaOH prior to use.

Results

Chloride flux in the presence of furosemide and SITS

Furosemide depressed steady-state Cl^- flux within 1 min after contact with cells. A typical experiment is shown in Fig. 1A. At 10 mM, furosemide reduced the Cl^- efflux coefficient (k_e) by 86% and at 1 mM the reduction was 57%. In spite of these effects cell Cl^- content remained normal and essentially constant (Fig. 1B). If furosemide inhibited only the simple diffusion of Cl^- one would expect to detect a change in cell Cl^- content under these conditions. Since no change was seen, it is likely that furosemide inhibited a one for one exchange process. The results of a number of experiments at different concentrations are summarized in Table I. Cl^- influx and efflux were apparently always inhibited equally since cell Cl^- content remained the same as control values.

Cl^- self exchange was examined following incubation with furosemide and subsequent thorough washing with albumin and albumin-free Ringer. Depression of Cl^- exchange by 10 mM furosemide was only partially reversible. From

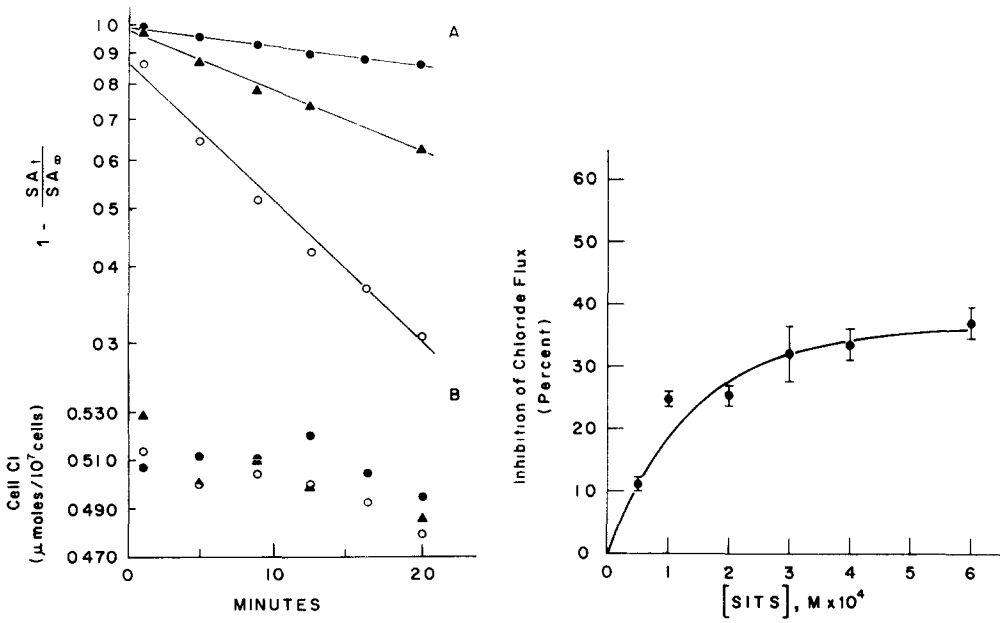

Fig. 1. (A, B) Effect of furosemide on Cl^- efflux coefficient (A) and cell Cl^- content (B). Furosemide and $^{36}Cl^-$ were added to cells at time zero. ○, control; ▲, 1 mM furosemide; ●, 10 mM furosemide. In A, $S.A._t$ = specific activity of cells in cpm/μmol; $S.A._\infty$ is obtained from the environment and is constant. Efflux coefficient is determined as described in ref. 11, from slope of the linear regression equation. The values were. control, 3.14 h^{-1}; 1 mM furosemide, 1.36 h^{-1}; 10 mM furosemide, 0.44 h^{-1}.

Fig. 2. Concentration response curve of SITS action on steady state Cl flux. In all studies ^{36}Cl and SITS were added simultaneously to a cell suspension at time zero A control was run in each experiment and results expressed in reference to it. The number of experiments at the 6 concentrations tested was, in the order of increasing concentration, 2, 4, 2, 3, 2, and 6. Vertical bars show S E. Curve was drawn by eye.

six experiments the mean inhibition following treatment with 10 mM furosemide and washing was 40.5% ± 3.9 (S.E.). In contrast to these findings the inhibitory action of 1 mM furosemide was reversed completely. Therefore close to half of the inhibitory effect of 10 mM furosemide appears to be irreversible (40.5% compared to 86.7%).

TABLE I

EFFECT OF INCREASING FUROSEMIDE CONCENTRATION ON CHLORIDE SELF EXCHANGE

Except for one experiment at 10 mM, in which cells were preincubated with furosemide for 30 min, all flux studies were initiated when cells were mixed with the drug. Cell Cl^- was in a steady state in all cases Percent inhibition is 1 — (flux in furosemide/control flux) × 100. Mean control flux was 1.27 μmol/10^7 cells per h (2.78 ± 0.16 (S.E.) h^{-1} × 0.456 ± 0 017 (S.E.) μmol/10^7 cells). To convert to flux per mg dry weight, divide by 2.43 mg/10^7 cells.

Furosemide concn. (mM)	Inhibition of Cl^- flux (% ± S.E.)
0.1 (n = 4)	14.0 ± 3.4
0.5 (n = 2)	28.0 ± 1.0
1.0 (n = 6)	46.7 ± 3.0
5.0 (n = 2)	80.5 ± 0.5
10.0 (n = 3)	86.7 ± 0.7)

Cl^- self exchange was also inhibited by SITS without alteration of cell Cl^- content. Reduction of steady-state Cl^- flux was detectable at $5 \cdot 10^{-5}$ M. The concentration vs. response curve obtained from a number of experiments is given in Fig. 2. The greatest inhibition observed was 37.0% ± 2.5 (S.E.), $n = 6$, at 0.6 mM SITS. Isotopic Cl^- exchange was not measured above this concentration because SITS at 1 mM caused significant net KCl loss from the cells. It is apparent from Fig. 2, however, that depression of Cl^- self exchange approached maximal levels around 0.6 mM.

To test whether SITS was specific for anion exchange, cell K^+ flux was also determined in one experiment. The K^+ efflux coefficient measured in steady-state control cells was 0.50 h^{-1} and was 0.48 h^{-1} in cells treated with 0.6 mM SITS. Since K^+ content remained constant and at control levels during exposure to SITS, there was no detectable action of this agent on K^+ flux.

Villereal and Levinson [10] demonstrated that Cl^- self exchange was normal when SITS-treated ascites cells were washed with albumin-Ringer prior to study. We found that the inhibition reported above was reversed completely when the cells were washed with albumin-Ringer; indeed albumin was not necessary in the Ringer since the effect was also reversed completely by Ringer alone.

Influence of low external Cl^- on SITS effect

We considered the possibility that extracellular Cl^- competed with SITS for binding sites on the cell membrane and thus reduced the inhibitory effect. Therefore Cl^- flux was measured using media in which acetate or NO_3^- replaced most of the Cl^-. SITS, at concentrations from 0.1 to 0.6 mM, was less effective in reducing steady-state cell Cl^- turnover (a higher k_e) after cells had equilibrated with solutions containing these foreign anions. For example, in 156 mM Cl^- Ringer, SITS at 0.1 mM reduced the control k_e by 24.5% ± 1.2 (S.E.), $n = 4$, but in 124 mM acetate + 32 mM Cl^- Ringer the k_e was reduced by only 3.0% ($n = 1$) and by 6.0% ($n = 1$) in 124 mM NO_3^- + 32 mM Cl^- Ringer. With 0.6 mM SITS the respective inhibition was 37.0% ± 2.5 (S.E.), $n = 6$; 26.0% ($n = 1$); 20% ($n = 1$). These results might be expected if SITS, acetate, and NO_3^- were inhibiting the same exchange process. That this was likely was seen when steady-state Cl^- turnover in acetate or NO_3^- media was evaluated in the absence of SITS.

After equilibration in acetate-chloride, the k_e was 2.12 h^{-1} ± 0.21 (S.E.), $n = 3$, while in control steady-state cells the k_e was 3.15 h^{-1} ± 0.14 (S.E.), $n = 10$ ($0.001 < P < 0.01$). The Cl^- turnover was also reduced by external NO_3^- as shown in Fig. 3. If Cl^- flux occurred by simple diffusion, k_e should not be altered by altering the external anions. Further, after equilibration in NO_3^-, cell Cl^- content was reduced but cell H_2O and K^+ content were at control levels, implying that a one for one exchange of Cl^- for NO_3^- had been the predominant form of Cl^- loss during the equilibration period. Thus in six control groups of cells, the mean wet to dry weight ratio was 4.644 ± 0.077 (S.E.), while for the same cell populations equilibrated in different NO_3^- solutions (Fig. 3), the ratio was 4.314 ± 0.029 (S.E.) ($n = 18$), demonstrating a cell H_2O loss of only 7%. Mean cell K^+ was 125 mmol/kg cell H_2O ± 6.7 (S.E.) in control cells and 126.2 ± 2.4 (S.E.) after incubation in NO_3^- Ringer. Since in the steady state the frac-

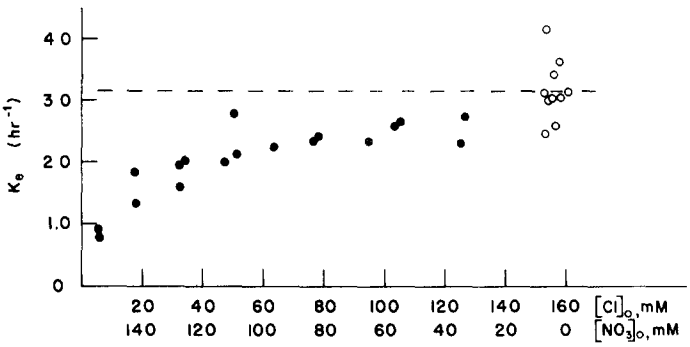

Fig. 3. Cl^- efflux coefficient as a function of external Cl^-, using NO_3^- as the replacement anion. Cells were equilibrated in NO_3^- Ringer until cell Cl^- was constant, before adding $^{36}Cl^-$. •, Data from five different NO_3^- experiments, each with several different concentrations; ○, control data from the same five experiments (range of k_e = 2.98—4.15 h^{-1}) and five additional control points obtained during the same 3 month period (range = 2.45—3.60 h^{-1}). Dotted line is mean control k_e, 3.15 h^{-1} ± 0.14 (S.E.).

tional turnover of cell Cl^- was reduced, the data taken together suggest that NO_3^- and Cl^- share a carrier which transports NO_3^- less readily than Cl^-. Inhibition of Cl^- turnover by acetate may occur in a different manner; incubation of cells in 124 mM acetate, 32 mM Cl^- resulted in a 17% increase of cell H_2O content and an 18% fall in cell K^+ concentration (data from one experiment; similar results were reported by Levinson and Villereal [1]). The data support the interpretation that SITS may act on carrier transfer of Cl^- because prior reduction of the transfer by NO_3^- or acetate diminished the efficacy of SITS.

Discussion

The major finding in this study is inhibition by furosemide of a very substantial portion of Cl^- self exchange. Since cell Cl^- content did not change, influx and efflux were apparently affected equally and it is probable that furosemide acts on a one for one exchange flux. Similar reasoning led Tupper [7] to conclude that Na^+ and K^+ exchange diffusions in ascites cells were inhibited by this drug. If this interpretation is correct, the present results imply that at least 86% of total Cl^- flux across the ascites cell occurs by such an exchange process.

Another important observation was that at high concentration about half of the inhibitory effect of furosemide was not readily reversible. Investigators who reported that furosemide was a reversibly acting inhibitor in other cells made their measurements at lower concentrations of 1 mM [12] and 0.01 mM [4]. Our data suggest it may be possible to use labeled furosemide to identify a specific Cl^- binding membrane protein. This will depend in part upon whether the Na^+ and K^+ exchange systems are reversibly sensitive to furosemide. Indeed, since this drug inhibits exchange of Cl^-, Na^+, and K^+ in the ascites cell, it may act by binding to some membrane component (protein or lipid) which produces a generalized membrane alteration.

SITS depressed a smaller portion of steady-state Cl^- transfer; its action was completely reversible and was specific in that K^+ exchange was not affected. The reasons why SITS was only half as effective as furosemide in depressing

this exchange are not apparent but may relate to: (1) the ease with which an agent reaches its site of action, (2) a competitive effect of environment Cl^- on the action of SITS.

Anion replacement experiments provided further evidence for carrier exchange of Cl^-. The data are consistent with a carrier mechanism which transports NO_3^- poorly in comparison to Cl^-. SITS appears to act on this pathway because pre-equilibration with NO_3^- diminished the effectiveness of SITS. Inhibition by acetate also reduced SITS action but was probably non-competitive. Consistent with our findings, Hoffmann and collaborators [2] noted inhibition of steady-state Cl^- transfer by NO_3^- and acetate. In the human erythrocyte as well, acetate inhibits Cl^- self exchange and in a non-competitive manner [13]. On the other hand Levinson and Villereal [1] found that Cl^- turnover in the ascites cell increased as external Cl^- was replaced by acetate, and estimated on the basis of these experiments that 40% of Cl^- exchange was carrier mediated. The reasons for the discrepant results with acetate are not clear. The present findings with furosemide suggest that exchange transport may account for a much larger fraction of Cl^- flux, consistent with conclusions of Heinz and collaborators [3].

In a recent study of SO_4^{2-} transport in the ascites cell Villereal and Levinson [14] concluded that following equilibration of cells in a SO_4^{2-} medium, SITS (0.2 mM) inhibited Cl^- uptake which occurred in exchange for SO_4^{2-} while furosemide (0.1 mM) partially blocked the net Cl^- uptake which was independent of SO_4^{2-} efflux. It remains to be determined whether the furosemide-sensitive pathway of net Cl^- uptake and the furosemide-sensitive pathway for Cl^- exchange reported here are the same.

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