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SPECIFIC DRUG SENSITIVE TRANSPORT PATHWAYS FOR CHLORIDE AND POTASSIUM IONS IN STEADY-STATE EHRLICH MOUSE ASCITES TUMOR CELLS

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A major aim of this investigation was to determine whether, in steady-state ascites cells, Cl^- transport can be partitioned into a furosemide-sensitive cotransport with K^+ and a separate 4,4′-isothiocyanostilbene-2,2′-disulfonic acid (DIDS) sensitive self-exchange. Both Cl^- and K^+ fluxes were studied. The furosemide- and Cl^- sensitive K^+ fluxes were equivalent, both in normal ionic media and when the external K^+ concentration, $[K^+]_o$, was varied from 4 to 30 mM. The stoichiometry of the furosemide-sensitive Cl^- and K^+ fluxes was 2 Cl^- : 1 K^+ at 0.1 and 0.5 mM drug levels but increased to 3 Cl^- : 1 K^+ at 1.0 mM furosemide. DIDS at 0.1 mM had no effect on the K^+ exchange rate but inhibited Cl^- exchange by 39% ± 2 (S.E.). The effects of DIDS and 0.5 mM furosemide on Cl^- transport were additive but 1.0 mM furosemide and DIDS had overlapping inhibitory actions. Thus furosemide acts on components of K^+ and Cl^- transport which are linked to each other, but the drug also inhibits an additional DIDS-sensitive Cl^- pathway, when present at higher concentrations. The dependence of the furosemide-sensitive K^+ and Cl^- transport on $[K^+]_o$ was also studied; both fluxes fell as the $[K^+]_o$ increased. The latter results recall those in an earlier study by Hempling (Hempling, H.G. (1962) J. Cell. Comp. Physiol. 60, 181–198).

Introduction

Chloride-linked cation movements in Ehrlich ascites tumor cells have been described by a number of investigators [1–4]. Geck and coworkers [3] postulated that there was a cotransport system of Cl^- with Na^+ and/or K^+ which functioned to regulate cell volume and was sensitive to the drug, furosemide. Recent data from Hoffmann's laboratory also implicate a furosemide-sensitive cotransport process in cell volume regulation [5].

Steady-state cells, in which there are no net ion or water movements, exhibit Cl^--dependent cation transfer that is sensitive to furosemide [1,2] and to bumetanide [4]. We showed that Cl^- transport is also inhibited by these agents [4,6] and that the stoichiometry of the bumetanide-sensitive Cl^- and K^+ flux was 1 : 1 at 0.5 mM and 1.0 mM drug concentrations [4]. Based on this data, 50–60% of steady-state Cl^- transfer could represent cotransport with cation.

The disulfonic stilbenes, on the other hand, reduce Cl^- self exchange by about 40% [6,7]. If they act on an independent Cl^- transport process their effects should be additive with furosemide or bumetanide; acting together these agents could depress steady-state Cl^- transfer almost completely. An aim of the present study, therefore, was to determine whether, in steady-state cells, furosemide specifically inhibits cotransport of Cl^-

Abbreviations: DIDS, 4,4′-isothiocyanostilbene-2,2′-disulfonic acid; H_2DIDS, 4,4′-isothiocyano-1,2-diphenylethane-2,2′-disulfonic acid; IBS, 1-isothiocyanate-4-benzenesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; SITS, 4-acetamido-4-isothiocyanostilbene-2,2′-disulfonic acid.

with K^+ and whether the disulfonic stilbene, DIDS acts on a separate Cl^- transport pathway. In addition, the steady-state cotransport of KCl was characterized further by studying K^+ and Cl^- transfer as a function of external K^+ concentration $[K^+]_o$.

Methods

Cell preparation and solutions

Ascites cells were grown in mice, harvested, and washed as described previously [6]. Cells were harvested with control Ringer which had the following composition (concentration in mM): 154 NaCl/6 KCl/1 $CaCl_2$/10 Mops, pH 7.35. Cells were washed three times and then incubated for 30–120 min until a steady state was reached. The preparative washing solution depended on the experiment: cells were washed with the Ringer in which they were subsequently incubated and sampled. Low Cl^-, normal K^+, normal Na^+ medium (NO_3^- Ringer in Table I) was (in mM); 154 $NaNO_3$/6 KNO_3/1 $CaCl_2$/10 Mops. Extracellular K^+ was varied either by replacing some Na^+ with K^+ (Table II) or by holding Na^+ constant and maintaining the osmolarity with choline (Fig. 1). When K^+ was increased at the expense of Na^+ (Table II) the two stock solutions from which mixtures were made were (in mM): 130 NaCl or $NaNO_3$/30 KCl or KNO_3/1 $CaCl_2$/10 Mops and 160 NaCl or $NaNO_3$/1 $CaCl_2$/10 Mops. When K^+ and choline were varied (Fig. 1) the two stock solutions from which mixtures were made were 130 NaCl/30 choline chloride/1 $CaCl_2$/10 Mops and 130 NaCl/30 KCl/1 $CaCl_2$/10 Mops. All studies were carried out in an air atmosphere at 21–24°C. The final cell concentration was 17–20 mg dry wt./ml (cytocrit 11%).

Steady-state fluxes

An experiment was initiated by adding radioactivity and, when appropriate, furosemide and/or DIDS to a cell suspension. The rate of cell uptake of $^{36}Cl^-$ or $^{42}K^+$ was measured and the efflux rate coefficient was obtained from the ratio of cell to environment specific activity using the kinetic equations of a two-compartment closed system [8]; the steady-state flux was then calculated from the rate coefficient and cell ion content [4,8]. Modification of procedures previously published [4,6] was as follows. Aliquots of 0.5 ml of cell suspension were pipetted into centrifuge tubes containing 10 ml of ice-cold $NaNO_3$ wash solution [6], centrifuged, and then washed once again with fresh cold $NaNO_3$ solution. Zero-time samples were obtained by pipetting a 0.5 ml aliquot into cold wash solution containing radioactivity; the zero-time radioactivity in the cell pellet was subtracted from subsequent samples [7,8]. In most studies 6–8 aliquots were removed over a 20–30 min period following addition of radioactivity to a cell suspension; in the experiments in which $[K^+]_o$ was varied (Table II and Fig. 1) 12 or 18 groups were handled and therefore only four samples were removed, over a span of 10 min. In the latter experiments six groups were sampled simultaneously, followed immediately by another set of six. Each of these experiments was repeated at another occasion, using a different sequence for the sets of six.

Analytical procedures

To obtain cell specific activity of Cl^- and/or K^+ [4,6,8] the packed washed cells were extracted with 7% $HClO_4$ and analyzed for radioactivity, Cl^-, and K^+ [6]. In the experiments summarized in Table III and Fig. 1, $^{36}Cl^-$ and $^{42}K^+$ were present together in the same cell suspension and radioactivity was analyzed as reported [4].

The washing procedure described in the previous section yields very accurate data from which to determine the efflux rate coefficients; control group linear regression equations had correlation coefficients of 0.99 ± 0.002 (S.E., $n = 19$) and 1.00 ± 0.001 (S.E., $n = 13$) for Cl^- and K^+, respectively; the intercept values were 0.98 ± 0.02 for Cl^- and 1.01 ± 0.01 for K^+. At least 20% of the cells, however, are lost during the washing procedure, as determined from dry weight measurements. This would lead to inaccurate cell ion content if no correction were made for the lost cells. To assess the cell K^+ content accurately we used either one of two procedures which agreed with each other within 5%: analysis of an unwashed cell pellet, using [^{3}H]inulin or [^{3}H]dextran to measure trapped extracellular space and correct for trapped extracellular K^+; in the second procedure an aliquot of cell suspension (cells plus environment)

was analyzed for its K^+ content as was the supernatant environment of a comparable aliquot, and the cell K^+ content was calculated as the difference in K^+ content between the two samples. For cell Cl^- content, the most reliable and reproducible results were obtained by multiplying the Cl^- content of washed cells by the ratio, (cell K^+ content in unwashed cells)/(cell K^+ content in washed cells). Wet and dry weights were measured as described [6]. The mean control cell concentrations of Cl^- and K^+ were 58 ± 1 mM and 183 ± 3 mM, respectively ($n = 11$). Furosemide and DIDS had no effect on cell ion content or concentration.

Inhibitors

Furosemide as the dry powder was a gift from Hoechst Pharmaceuticals, Inc. It was dissolved in 1 M NaOH, diluted with the appropriate Ringer, and titrated to pH 7.35 with HCl or HNO_3. DIDS was purchased from Polysciences, Inc. Both drugs were prepared fresh for each experiment and stored in the dark until used.

Results

Furosemide- and Cl^--sensitive K^+ flux

Bakker-Grunwald [1] showed that Rb^+ uptake was depressed to the same extent by 1.2 mM furosemide as by lowering the environment Cl^- to 2 mM. The data in this section demonstrate that in steady-state cells, the furosemide-sensitive K^+ flux is equal to the Cl^--sensitive K^+ flux at several drug concentrations and even when the $[K^+]_o$ is varied over a 7-fold range.

Table I shows that the Cl^-- and furosemide-sensitive fluxes are equivalent (Cl^--sensitive K^+ flux is the K^+ flux in cells equilibrated in Cl^- medium minus the flux in cells equilibrated in NO_3 medium; furosemide-sensitive is the flux in control cells minus the flux in furosemide-treated cells). The Cl^--dependence of the furosemide-sensitive K^+ flux is emphasized further by the experiments of Table II, in which $[K^+]_o$ was varied at a constant furosemide concentration of 0.5 mM. The ratio of the furosemide- and Cl^--sensitive K^+ flux was close to 1.0 in all instances.

In Table I, inhibition of K^+ flux is the same at 0.1 and 1.0 mM furosemide. In other experiments where more than one furosemide concentration was assessed inhibition changed little at drug levels of 0.1, 0.5 and 1.0 mM. When all data were pooled, inhibition was $43\% \pm 3$ (S.E., $n = 5$), $58\% \pm 8$ ($n = 9$) and $46\% \pm 2$ ($n = 5$) at 0.1 mM, 0.5 mM, and 1.0 mM, respectively.

TABLE I

COMPARISON OF FUROSEMIDE-SENSITIVE AND Cl^--SENSITIVE STEADY-STATE K^+ FLUX

Flux in mol/g dry wt. per h.

Furosemide concn. (mM)	Cl^--sensitive K^+ flux	Furosemide-sensitive K^+ flux	
		Cl^- Ringer [a]	NO_3^- Ringer [b]
0	138	–	–
0.1	–	140	2
1.0	–	144	7
(Control K^+ flux, 0.5 $h^{-1} \times 600$ mol/g dry wt.)			

[a] 162 mM Cl^-.
[b] 2 mM Cl^-, 160 mM NO_3^-.

Stoichiometry of furosemide-sensitive Cl^- and K^+ flux

From the results above and the fact that Cl^- turnover is also inhibited by furosemide, it is likely that the fraction of steady-state K^+ transfer sensitive to the drug represents a coupled transport

TABLE II

COMPARISON OF FUROSEMIDE-SENSITIVE AND Cl^--SENSITIVE STEADY-STATE K^+ FLUX IN VARYING ENVIRONMENT K^+ CONCENTRATION, $[K^+]_o$

$[K^+]_o$ was increased at the expense of Na^+, in the presence or absence of 0.5 mM furosemide, and in 162 mM Cl^- or 2 mM Cl^-, 160 mM NO_3^-.

$[K^+]_o$ (mM)	Furosemide-sensitive K^+ flux	
	Cl^--sensitive K^+ flux	
	Expt. 1	Expt. 2
4.5	1.03	1.09
7.0	0.99	1.05
9.0	1.00	0.99
18.0	0.97	1.04
23.0	0.99	0.98
29.0	0.91	0.96

TABLE III

THE COUPLING RATIO OF FUROSEMIDE-SENSITIVE Cl^- AND K^+ FLUX IN STEADY-STATE CELLS

Cl^- and K^+ fluxes were measured simultaneously in the same cell suspension, in control Ringer. Mean±S.E. shown; n = number of experiments.

Furosemide concn. (mM)	n	Furosemide-sensitive Cl^- flux / Furosemide-sensitive K^+ flux
0.1	5	2.0±0.5
0.5	6	2.1±0.3
1.0	5	3.2±0.4

with Cl^-. We next investigated whether the converse is also true, namely, whether the furosemide-sensitive steady-state Cl^- flux represents cotransport with K^+ and/or Na^+. One would then predict that the furosemide-sensitive Cl^- and K^+ fluxes in steady-state cells must be equal so that their ratio must be at least 1.0. If Na^+ were also coupled to Cl^- transport, as proposed by Geck and colleagues [3], the ratio of the inhibited fluxes would be 2 Cl^- : 1 K^+. If furosemide is specific for that fraction of Cl^- transport which is linked to cation movement, then the predicted ratios should not change over the range of 0.1 to 1.0 mM furosemide, based on observations reported in the previous section. The results of experiments in which the steady-state transfer of Cl^- and K^+ were assessed simultaneously in the same cell suspension are given in Table III. A stoichiometry of 2 Cl^- : 1 K was found at 0.1 and 0.5 mM drug levels but at 1.0 mM the stoichiometry appeared to increase to 3.

Effects of furosemide and DIDS on Cl^- flux

The results thus far suggest that at 0.1 mM and 0.5 mM, furosemide is specific for a fraction of Cl^- movement which is coupled to the transfer of cation. At 0.5 mM, the mean inhibition of Cl^- flux was 43% ± 5, $n = 6$, leaving 57% of steady-state Cl^- transfer unaffected, and presumably uninvolved in cotransport. The furosemide-insensitive Cl^- transport could represent an independent self exchange which was specifically sensitive to disulfonic stilbenes. To test this hypothesis we investigated the effects of DIDS, and the combined effects of DIDS and furosemide on steady-state Cl^- flux.

DIDS was present at a concentration of 0.1 mM, which was maximally effective under the usual experimental conditions (control Ringer, room temperature, no pre-incubation with drug). The mean inhibition of Cl^- exchange was 39% ± 2 in six experiments. K^+ flux was not altered by DIDS when measured simultaneously with Cl^- fluxes in two of the experiments. Control vs. experimental K^+ fluxes were, respectively, 264 vs. 229 mol/g dry wt. per h and 245 vs. 245.

If DIDS and furosemide are specific for functionally distinct fractions of Cl^- transport, then their effects should be additive at 0.1 mM and 0.5 mM furosemide. When the combined effects of DIDS and furosemide were assessed the results were consistent with this view. The inhibitory action of 1 mM furosemide, however, overlapped with that of DIDS.

The predicted and actual outcome in two representative experiments is given in Table IV; there was additive inhibition of Cl^- self exchange when furosemide was present at 0.5 mM, less than additive actions at 1.0 mM furosemide and variable results at 0.1 mM furosemide. In four out of five cases, 0.5 mM furosemide and DIDS inhibited

TABLE IV

COMBINED EFFECTS OF DIDS AND FUROSEMIDE ON STEADY-STATE Cl^- FLUXES

DIDS was present at 0.1 mM. Percent inhibition of Cl^- flux is (experimental flux/control flux)×100.

Inhibitor concentration	Inhibition of Cl^- flux (%)	
	Predicted [a]	Actual
DIDS+0.5 mM furosemide	69	69
DIDS+0.1 mM furosemide	55	58
(Control Cl^- flux, 4.0 h^{-1} × 164 mol/g dry wt.)		
DIDS+1.0 mM furosemide	87	68
DIDS+0.5 mM furosemide	66	61
DIDS+0.1 mM furosemide	37	52
(Control Cl^- flux, 3.0 H^{-1} × 180 mol/g dry wt.)		

[a] Calculated as the sum of the separate effects of DIDS and furosemide, measured in the same experiment.

Cl^- transport additively (within 5% of the expected value), as predicted from our other data. In all of five experiments with 1.0 mM furosemide, actual inhibition was less than predicted from the sum of the separate effects of DIDS and furosemide. Thus at the higher furosemide concentration, the agent is acting partly on DIDS sensitive Cl^- exchange. Consistent with this result is the higher ratio of furosemide-sensitive $Cl^-:K^+$ flux at the 1.0 mM level (Table III). At 1 mM, furosemide no longer appears to be specific for that Cl^- transport which is linked to K^+ (and Na^+) transport. In the presence of DIDS plus 0.1 mM furosemide inhibition was variable (Table IV). While independent, additive actions were observed (first experiment), the two agents may also potentiate each other's effects when present together (second experiment). Potentiation was seen in two of six instances, indicating that DIDS and furosemide may interact when they alter Cl^- transport.

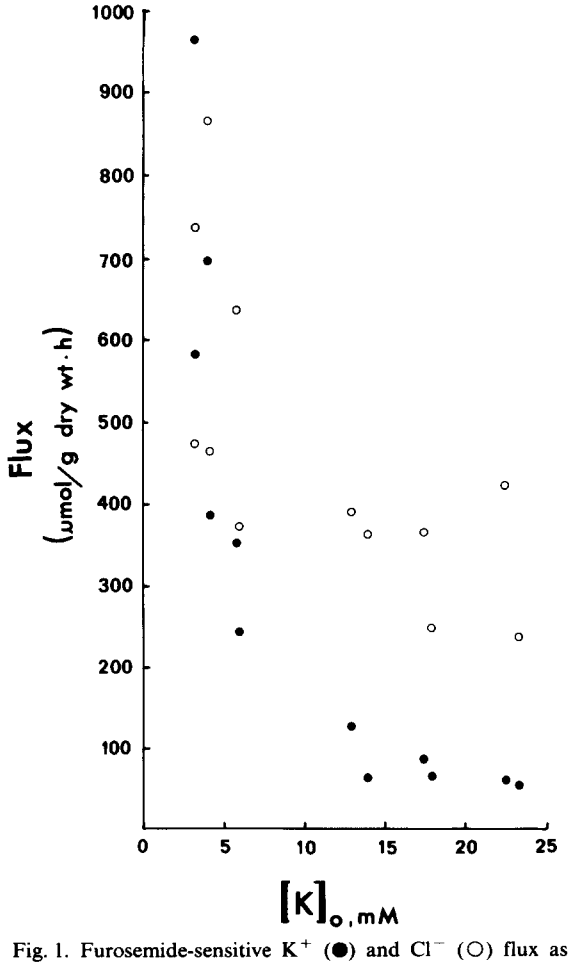

Fig. 1. Furosemide-sensitive K^+ (●) and Cl^- (○) flux as a function of external K^+ concentration. Data from two experiments measuring Cl^- and K^+ fluxes simultaneously in the same cell suspension. Furosemide concentration was 0.5 mM. K^+ was varied by replacement with choline, keeping Na^+ constant.

Furosemide-sensitive K^+ and Cl^- fluxes and $[K^+]_o$

The data reported here appear to demonstrate that the furosemide-sensitive K^+ flux in steady-state ascites cells represents a specific transport process. One might expect that when furosemide-sensitive K^+ flux was determined as a function of $[K^+]_o$, saturation behavior would be observed. As shown in Fig. 1, however, the K^+ flux fell in a concave relationship to $[K^+]_o$. Furosemide-sensitive Cl^- flux, measured in the same experiments, followed a similar pattern in relation to $[K^+]_o$, although the Cl^- flux was 4–7-times greater than the comparable K^+ flux at high $[K^+]_o$. The Cl^--sensitive K^+ flux, studied in the experiments of Table II, also fell as $[K^+]_o$ was raised. In experiment 1 of Table II, for example, the Cl^--sensitive K^+ flux was 852 mol/g dry wt. per h at 4.5 mM K_o^+, 615 at 18 mM K_o^+, and 523 at 29 mM K_o^+.

Discussion

Bakker-Grunwald and colleagues studied Rb^+ uptake in steady-state ascites cells and suggested that a component of K^+ transport was linked to the transport of Cl^- and was sensitive to furosemide [1,2]. The present study of K^+ and Cl^- transport confirms the presence of furosemide-sensitive cotransport in steady-state cells and shows also that an additional fraction of the steady-state Cl^- transport is not linked to cation and is specifically sensitive to DIDS. The stoichiometry of the furosemide-sensitive fluxes was 2 Cl^-:1 K^+ at furosemide concentrations (0.1 mM and 0.5 mM) which inhibited K^+ exchange to the same extent as removal of external Cl^-, and at which there were additive effects of furosemide and DIDS on Cl^- exchange. DIDS did not alter K^+ exchange. While furosemide appears to be specific for that component of K^+ transport which is linked to Cl^- movement, when present at 1.0 mM the drug

inhibited Cl^- self exchange in addition to Cl^--K^+ cotransport.

These results in steady state cells are similar to those which were found for cells undergoing net ion and water movements [3] in that a furosemide-sensitive cotransport of 2 Cl^- : 1 K^+ was observed. This would be consistent with the cotransport mechanism postulated by Geck and colleagues [3] in which 1 Na^+, 1 K^+ and 2 Cl^- are transferred electroneutrally. In an earlier study we found a bumetanide-sensitive K^+ and Cl^- transport for which the stoichiometry was 1 Cl^- : 1 K^+ [4]. Reasons for the different stoichiometry are not clear but different experimental conditions could play a role. In the earlier work the cells were incubated at one tenth the concentration. In a recent experiment where dilute and concentrated cells were studied simultaneously when treated with 0.5 mM furosemide, the ratio of furosemide-sensitive Cl to furosemide-sensitive K^+ fluxes was 2.3 in the concentrated suspension and only 1.1 in the diluted cells.

There is also variability in the coupling ratio as a function of $[K^+]_o$. This can be seen from Fig. 1, but is shown more explicitly in Table V, which lists the ratio of the furosemide-sensitive fluxes at each $[K^+]_o$ for both experiments in the figure. As shown earlier, however (Table III), the stoichiometry is reasonably close to 2 Cl^- : 1 K^+ at the normal $[K^+]_o$ of 5.8 or 5.6 mM. There was a tendency for the cells to swell with increasing $[K^+]_o$; in the experiments of Fig. 1, at $[K^+]_o$ of 22–23 mM cell water was greater by a factor of 1.16 ± 0.03 (S.E., $n = 4$) than it was at $[K^+]_o = 3$ mM. It is possible that cell volume is important in determining the stoichiometry of cotransport. There was not, however, a reproducible difference between the cell volumes of furosemide-treated cells and the untreated cells.

TABLE V

THE COUPLING RATIO OF FUROSEMIDE-SENSITIVE CL^- AND K^+ FLUX AS A FUNCTION OF ENVIRONMENT K^+ CONCENTRATION

Individual data from the two experiments illustrated in Fig. 1.

$[K^+]_o$ (mM)	Furosemide-sensitive Cl^- flux / Furosemide-sensitive K^+ flux
3.0	0.8
4.0	1.2
5.8	1.5
13.8	6.0
17.7	3.7
23.2	4.5
3.0	0.8
3.8	1.2
5.6	1.8
12.8	3.1
17.3	4.1
22.3	7.0

The conclusion that there are functionally distinct elements of steady-state Cl^- transport which are sensitive to different drugs has its counterpart in non-steady state ascites cells. Thus the volume regulatory KCl^- and water uptake which followed extracellular tonicity changes was sensitive to furosemide but not to DIDS [5].

In this investigation some 39% of steady-state Cl^- transfer was sensitive to DIDS and represented self exchange. This figure agrees well with inhibition in the presence of other disulfonic stilbenes. SITS depressed Cl^- exchange by 37% but did not alter K^+ transfer [6], and H_2DIDS inhibited Cl^- flux by about 36% [7]. The benzene sulfonic acid, IBS, also reduced Cl^- exchange by 37% [9]. It is likely that all of these agents act on that moiety of Cl^- transport which is not linked to cation movement. Another 43% of the steady-state Cl^- transport appears to represent furosemide-sensitive transport with cation; this is a minimum value based on the mean depression of Cl^- exchange at 0.5 mM furosemide where the drug acts additively with DIDS and where the stoichiometry of furosemide sensitive fluxes is 2 Cl^- : 1 K^+.

The relationship between the furosemide-sensitive K^+ flux and $[K^+]_o$ (Fig. 1) is not like that found for most transport systems even though Cl^- dependence of the flux was demonstrated at every $[K^+]_o$ (Table II). In human erythrocytes, for example, the ouabain-insensitive, Cl^--dependent K^+ flux is saturable [10,11]. It is possible that furosemide and K^+ compete with each other for the cotransport pathway and that this might account for the inverse relationship between the furosemide-sensitive K^+ flux and $[K^+]_o$. This cannot

be the entire explanation, however, since the Cl^--sensitive K^+ flux also fell as $[K^+]_o$ was raised. Furthermore, the present results are strikingly like data obtained 20 years ago by Hempling, in an investigation of K^+ transport in Ehrlich mouse ascites tumor cells [12]. Hempling showed that the steady-state K^+ flux decreased with increasing $[K^+]_o$, reaching a minimum value which occurred at abour 25 mM. While that study described the total unidirectional K^+ flux and the data of Fig. 1 depict the furosemide-sensitive K^+ flux, we have found that total K^+ exchange follows a similar relationship as well (data not shown). Mills and Tupper [13] also studied K^+ transport in ascites cells and noted a complex relationship between $[K^+]_i$ and a component of steady-state K^+ efflux which was dependent on $[K^+]_o$; this efflux component was sensitive to furosemide [14]. The present results cannot be compared readily with those, however, since the $[K^+]_i$ was 15–120 mM in their experiments while in our work the $[K^+]_i$ was 140–200 mM. There is not enough information yet available to account for the characteristics of K^+ transport which have been described in these different investigations. Further, while one would expect the furosemide-sensitive Cl^- flux to decrease as the K^+ flux falls (Fig. 1), it is not clear why the ratio of the furosemide-sensitive fluxes of $Cl^- : K^+$ increases as $[K^+]_o$ is raised. These questions await further investigation.

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