Biochimica et Biophysica Acta, 643 (1981) 339—345
Elsevier/North-Holland Biomedical Press

BBA 79228

POTASSIUM CHLORIDE COTRANSPORT IN STEADY-STATE ASCITES TUMOR CELLS

DOES BUMETANIDE INHIBIT?

FELICE AULL

Department of Physiology and Biophysics, New York University School of Medicine, 550 First Avenue, New York, NY 10016 (U.S.A.)

(Received November 3rd, 1980)

Key words: Bumetanide; Cotransport; K^+; Cl^-; (Ascites tumor cell)

Summary

Bumetanide is a potent diuretic drug which has some structural features in common with furosemide. The steady-state exchange of K^+ and Cl^- was investigated in Ehrlich ascites tumor cells treated with bumetanide. This agent did not alter the cellular content of K^+ or Cl^- but the self-exchange of both ions was depressed. K^+ self-exchange was inhibited by 55% at bumetanide concentrations as low as 10^{-6} M. Cl^- self-exchange was less sensitive to this drug but at low concentrations (between 10^{-6} and 10^{-3} M) bumetanide was a more effective inhibitor of Cl^- transfer than furosemide. The steady-state K^+ flux of cells equilibrated in NO_3^- media was compared with the K^+ flux in cells treated with 10^{-4} or 10^{-3} M bumetanide; the Cl^--sensitive K^+ exchange was equivalent to the bumetanide-sensitive K^+ exchange. Since the results suggested that a bumetanide-sensitive (Cl^-, K^+) cotransport could be operative in steady-state cells, the stoichiometry of the bumetanide-sensitive fluxes was determined by measuring Cl^- and K^+ fluxes simultaneously in the same cell suspension. At $5 \cdot 10^{-4}$ and 10^{-3} M bumetanide concentrations, the ratio of these fluxes was 0.98 ± 0.07 (S.E.) and 1.04 ± 0.06, respectively, consistent with the postulated cotransport mechanism. At 10^{-4} and 10^{-5} M, however, the ratio of the bumetanide-sensitive Cl^-/K^+ flux was significantly less than 1.0. Since the magnitude of the bumetanide-sensitive K^+ flux at 10^{-4} M was close to that of the Cl^--sensitive flux, a ratio of less than 1.0 at this drug level indicates that Cl^-

---

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid.

sensitivity and drug sensitivity may not reflect inhibition of the same process under all circumstances.

Introduction

A (K^+, Cl^-) cotransport mechanism has been postulated for Ehrlich ascites tumor cells, based on observations of cells activated to accumulate K^+ [1,2]. This cotransport appears to be inhibited by furosemide. Other work suggests that such a mechanism may also operate in steady-state cells. Thus, Bakker-Grunwald et al. [3,4] have demonstrated that K^+ self-exchange was reduced to the same extent by replacement of most of the environment Cl^- and by treatment with furosemide. Based on these studies we estimate that cotransport with K^+ could account for about 60% of steady-state Cl^- exchange. Since an investigation of the stoichiometry of Cl^-- or furosemide-sensitive K^+ and Cl^- transfer was not undertaken, however, the possibility remains that Cl^- activates K^+ transport without being linked to it in an obligatory transport process.

In this study we investigated the sensitivity of K^+ and Cl^- self-exchange to bumetanide, a drug which is structurally related to furosemide [5] but more potent in its action [5,6]. The bumetanide-sensitive K^+ exchange was compared to the Cl^--sensitive K^+ exchange and to the bumetanide-sensitive Cl^- exchange. The results are consistent with a bumetanide-sensitive coupled transport of K^+ and Cl^- in the steady state, but only at drug concentrations of $5 \cdot 10^{-4}$ M or greater.

Methods

Cell suspensions and solutions

Ascites cells were grown in mice, harvested, and washed as described previously [7]. Washed cells at a concentration of 90—100 mg wet wt./ml (14—15 mg dry wt./ml) were equilibrated for 30—60 min to reach a steady state with regard to K^+ and Cl^- content. Flux studies were intiated by diluting the cells 10-fold with radioactive solutions in the presence or absence of drug, in an air atmosphere, at a temperature of 21—24°C. Depending on the study, one or both of the solutions below was used for washing and experimentation (concentration in mM): Cl^- Ringer, 154 NaCl/6 KCl/1 or 2 $CaCl_2$/10 Hepes or Mops; NO_3^- Ringer, 154 $NaNO_3$/6 KNO_3/1 $CaCl_2$/10 Mops. Solutions were titrated to pH 7.23—7.35 with NaOH.

Cl^- and K^+ self-exchange

Cl^- and K^+ fluxes were determined in the steady state by obtaining the efflux rate coefficient for each ion and then multiplying it by the cellular content of Cl^- or K^+. The rate coefficients were measured from the rate of cellular uptake of ^{36}Cl or ^{42}K, using the kinetic equations of a two-compartment closed system [8]. ^{36}Cl and ^{42}K were present at 0.15 and 0.40 μCi/ml cell suspension, respectively. Packed cells were washed with ice-cold $NaNO_3$, extracted with 7% $HClO_4$, and analyzed for radioactivity, Cl^- and K^+, as described previously [7]. When the stoichiometry of bumetanide-sensitive Cl^- and K^+ fluxes was determined, ^{36}Cl and ^{42}K were present together in the same cell suspension. To separate sample ^{42}K from ^{36}Cl, ^{42}K was counted by its Cerenkov radiation in a Searle liquid scintillation counter and then allowed to decay for 1 week, after which the small contribution of ^{36}Cl to the Cerenkov radiation was subtracted; ^{36}Cl was then monitored after the addition of scintillation fluid.

Materials

Bumetanide powder was a gift from Hoffman-LaRoche, Inc., Nutley, NJ, and from Dr. P.W. Feit, Leo Pharmaceutical Products, Ballerup, Denmark. Furosemide was a gift from Hoechst-Roussel Pharmaceuticals, Inc., Somerville, NJ. Both drugs were dissolved in 1 M NaOH and titrated to pH 7.2—7.4 with HCl or HNO_3. Isotopes were obtained from New England Nuclear; the scintillation fluid was Hydrofluor (National Diagnostics).

Results

The results of an investigation of steady-state Cl^- transfer in the presence of bumetanide are summarized in Fig. 1. For comparison, our published data of furosemide action [7] are also shown. Bumetanide appears to be the more effective inhibitor of Cl^- transport when the drug concentrations are 10^{-3} M or lower (recent unpublished work with furosemide yielded results similar to those in the earlier studies). Similar to observations on amphibian cornea [9], the curves are not parallel.

The action of bumetanide on K^+ and Cl^- self-exchange was investigated simultaneously in a number of experiments. A representative example is shown in Fig. 2, for a drug concentration of 10^{-3} M. In this example, the K^+ flux was inhibited by 56% and the Cl^- flux by 64%. As was generally the case, the cellular contents of K^+ and Cl^- were unchanged in the presence of bumetanide: cell K^+ was 450 and 441 μmol/g dry wt. in control and bumetanide-treated cells,

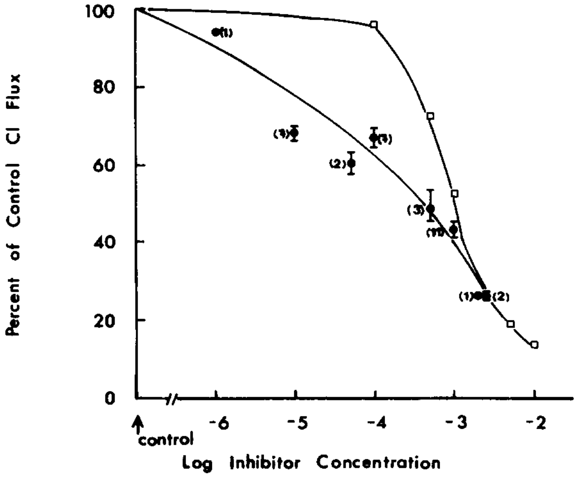

Fig. 1. Dose-response curve for action of bumetanide (●) on Cl^- self-exchange. Standard errors indicated by bars; number of experiments in parentheses. For comparison, the data with furosemide from Table I (Ref. 7) are also shown (□).

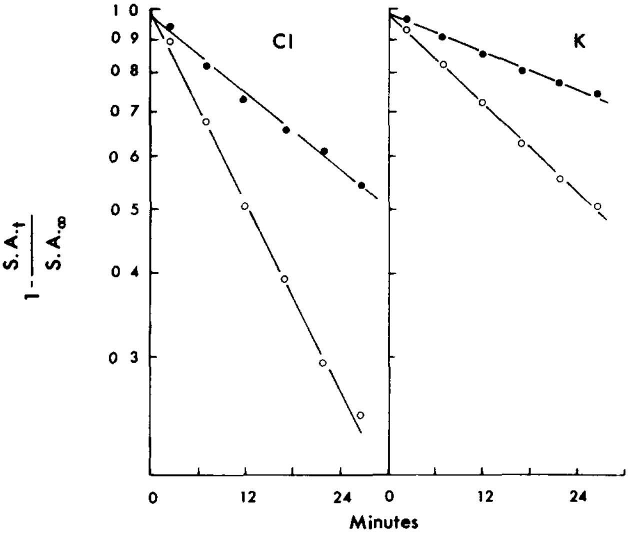

Fig. 2. Effect of bumetanide on steady-state Cl^- and K^+ exchange, determined simultaneously. Control (○), 10^{-3} M bumetanide (●). Respective rate coefficients in control and bumetanide-treated cells were 3.08 and 1.11 h^{-1} for Cl^- exchange, and 1.21 and 0.54 h^{-1} for K^+ exchange.

respectively, and Cl^- content was 186 and 187 μmol/g dry wt. Since both K^+ and Cl^- self-exchange were altered by bumetanide, it seemed possible that this agent could be acting on a (K^+, Cl^-) cotransport process. Therefore, we compared the effects on K^+ self-exchange of bumetanide and replacement of extracellular Cl^-.

The Cl^--sensitive K^+ flux was calculated as the difference between the flux in a control environment containing 162 mM Cl^- and the flux in an NO_3^- environment containing only 2 mM Cl^-. The bumetanide-sensitive K^+ flux was calculated as the difference between the flux in a bumetanide-free medium and the flux in the presence of bumetanide, either in 162 mM Cl^- or in 2 mM Cl^-

TABLE I

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

Values of flux are given in μmol/g dry wt. per h.

Bumetanide concentration (M)	Cl^--sensitive K^+ flux	Bumetanide-sensitive K^+ flux	
		Cl^- ringer *	NO_3^- ringer **
0	421	—	—
10^{-3}	—	369	30
10^{-4}	—	368	30
	(Control K^+ flux = 582)		
0	513	—	—
10^{-3}	—	540	54
10^{-4}	—	511	58
	(Control K^+ flux = 741)		

* 162 mM Cl^-.
** 2 mM Cl^-.

TABLE II

STOICHIOMETRY OF BUMETANIDE-SENSITIVE Cl^- AND K^+ FLUX

Standard errors and number of experiments (in parentheses) are indicated. Percent inhibition of K^+ flux is (1 —% of control), column 2.

Bumetanide concentration	K^+ flux (% of control)	Bumetanide-sensitive fluxes Cl^-/K^+
$1 \cdot 10^{-6}$	46 ± 1 (2)	0.14 (1)
$1 \cdot 10^{-5}$	43 ± 3 (6)	0.67 ± 0.05 (5) *
$5 \cdot 10^{-5}$	52 ± 1 (2)	0.84 ± 0.07 (2)
$1 \cdot 10^{-4}$	41 ± 2 (6)	0.61 ± 0.10 (5) *
$5 \cdot 10^{-4}$	44 ± 3 (4)	0.98 ± 0.07 (3)
$1 \cdot 10^{-3}$	36 ± 3 (8)	1.04 ± 0.06 (7)
$2 \cdot 10^{-3}$	28 (1)	1.35 (1)
$2.5 \cdot 10^{-3}$	38 ± 1 (2)	1.22 ± 0.04 (2)

* Bumetanide-sensitive Cl^- and K^+ fluxes significantly different, analyzed by paired *t*-test.

solutions. These results are shown in Table I. It can be seen that the magnitudes of the Cl^--sensitive and bumetanide-sensitive K^+ fluxes were within 5—14% of each other, and that when most of the external Cl^- had been replaced by NO_3^-, bumetanide had only a small additional inhibitory action on K^+ transfer. It is noteworthy that 10^{-4} and 10^{-3} M concentrations of bumetanide were equally effective in depressing K^+ flux.

If, as these results suggest, there is a steady-state (Cl^-, K^+) cotransport mechanism which is inhibited by bumetanide, this should be reflected by the stoichiometry of the bumetanide-sensitive Cl^- and K^+ fluxes. Accordingly, K^+ and Cl^- self-exchange were studied together, in the presence of a range of drug concentrations. The data are summarized in Table II. A coupled transport of Cl^- with K^+ would yield a ratio of 1 when the bumetanide-sensitive Cl^- flux is divided by the bumetanide-sensitive K^+ flux. As shown in column 3, the ratio was close to 1 when bumetanide was present at 10^{-3} or $5 \cdot 10^{-4}$ M and the bumetanide-sensitive Cl^- and K^+ exchanges also appeared to be equivalent at higher drug concentrations. However, at 10^{-4} M, the bumetanide-sensitive fluxes were significantly different from each other, even though there was little if any difference in the effectiveness of bumetanide in depressing K^+ self-exchange at these concentrations (Table II, column 2); furthermore, as shown in Table I, the Cl^--sensitive K^+ flux was equivalent to the bumetanide-sensitive K^+ flux at either 10^{-3} or 10^{-4} M. It should be pointed out that the difference in the flux ratios determined by compiling data from all experiments (summarized in Table II) was also true for the two experiments shown in Table I. Specifically, at 10^{-3} M, the bumetanide-sensitive Cl^-/K^+ flux ratio was 1.05 and 0.80 at 10^{-3} M but only 0.61 and 0.34 at 10^{-4} M.

In one experiment with furosemide a similar discrepancy was observed. Inhibition of the steady-state K^+ flux was 46, 46 and 39% at 10^{-4}, $5 \cdot 10^{-4}$, 10^{-3} M furosemide, respectively, but the corresponding furosemide-sensitive Cl^-/K^+ flux ratios were 0.45, 0.70, and 1.26.

Discussion

In the present study, we consider whether a (Cl^-, K^+) cotransport system can be demonstrated in steady-state ascites cells, using bumetanide as an inhibitory agent. Bumetanide inhibited both K^+ and Cl^- self-exchange; the cellular content of these ions was not altered. K^+ exchange was very sensitive to this agent; inhibition was virtually constant at about 55% over a concentration range of 10^{-6}—$5 \cdot 10^{-4}$ M (Table II). Bumetanide appeared to depress steady-state Cl^- transport at lower concentrations than has previously been demonstrated for furosemide (Fig. 1).

The results above indicated that a bumetanide-sensitive (Cl^-, K^+) cotransport system could be operative in the steady state. To test this, Cl^- and K^+ exchange fluxes were assessed simultaneously and the stoichiometry of the bumetanide-sensitive fluxes was determined. A stoichiometry of 1 Cl^- : 1 K^+ would be predicted for this mechanism; if the cotransport mechanism included Na^+ and occurred as 2 Cl^- : 1 K^+ : 1 Na^+, as postulated from net flux measurements in non-steady-state cells [2], the expected stoichiometry would be 2 Cl^- : 1 K^+.

The data are consistent with a 1 : 1 Cl^- : K^+ cotransport which is sensitive to bumetanide at drug concentrations of $5 \cdot 10^{-4}$ M and higher (Table II). Ratios approaching 2 Cl^- : 1 K^+ were not observed. At 10^{-4} M, however, as well as at 10^{-5} M drug levels, the Cl^-/K^+ ratio was significantly less than 1.0. A similar finding resulted when cells were treated with furosemide. Since the magnitude of the bumetanide-sensitive K^+ flux at 10^{-4} M was close to that of the Cl^--sensitive K^+ flux, a stoichiometry of less than 1 : 1 is difficult to account for if bumetanide sensitivity and Cl^- sensitivity both result from inhibition of (Cl^-, K^+) cotransport. Thus, Cl^- sensitivity and drug sensitivity may not reflect inhibition of the same process under all circumstances. In this regard, Bakker-Grunwald et al. [4] noted that "... the possibility of complicating drug interactions in the presence of furosemide cannot a priori be excluded". In addition, it is possible that the sensitivity of K^+ exchange to Cl^- reflects factors in addition to an obligatory coupling of Cl^- and K^+ transport. Further work evaluating the stoichiometry of steady-state Cl^- and K^+ exchange under a variety of conditions is necessary to clarify these points. This work is underway in our laboratory.

Acknowledgements

I thank Ms. Mildred Decker and Mrs. Irene St. Denny for their assistance. This investigation was supported by Grant Number CA-10625, awarded by the National Cancer Institute, DHEW.

References

1 Geck, P., Heinz, E., Pietrzyk, C. and Pfeiffer, B. (1978) In Cell Membrane Receptors for Drugs and Hormones (Straub, R.W. and Bolis, L., eds.), pp. 301—307, Raven Press, New York

2 Geck, P., Pietrzyk, C., Burckhardt, B.C., Pfeiffer, B. and Heinz, E. (1980) Biochim. Biophys. Acta 600, 432—447

3 Bakker-Grunwald, T. (1978) Biochim. Biophys. Acta 513, 292—295

4 Bakker-Grunwald, T., Andrew, J.S. and Neville, M.C. (1980) J. Membrane Biol. 52, 141—146

5 Feit, P.W. (1971) J. Med. Chem. 14, 432—439
6 Asbury, M.J., Gatenby, P.B., O'Sullivan, S. and Bourke, E. (1972) Br. Med. J. 1, 211—213
7 Aull, F., Nachbar, M.S. and Oppenheim, J.D. (1977) Biochim. Biophys. Acta 471, 341—347
8 Aull, F. (1972) J. Physiol. 221, 755—771
9 McGahan, M.C., Yorio, T. and Bentley, P.J. (1977) J. Pharmacol. Exp. Ther, 203, 97—102