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THE HUMAN ERYTHROCYTE Cl-DEPENDENT Na-K COTRANSPORT SYSTEM AS A POSSIBLE MODEL FOR STUDYING THE ACTION OF LOOP DIURETICS

J.C. ELLORY & G.W. STEWART[1]

The Physiological Laboratory, University of Cambridge, Downing Street, Cambridge CB2 3EG

1 The recent demonstration of the chloride-dependence of the red cell Na-K cotransport system suggests an analogy between this process and the active Cl^- absorption in the ascending loop of Henle, which is the target transport system for loop diuretics.

2 Using red cell K influx, four known loop diuretics, six experimental frusemide analogues, two thiazides, two K-retaining diuretics and one organomercurial were compared for inhibitory potency on the red cell Na-K cotransport system.

3 Except for mersalyl, whose exact mode of action in the kidney is still in doubt, the inhibition of the red cell system by various loop diuretics was consistent with both published whole body diuretic data and isolated perfused tubule studies, while the system did not respond to the thiazides or the K-retaining diuretics.

4 It is concluded that the human red cell Na-K cotransport system is a possible valid model process on which to study the activity of loop diuretics.

Introduction

The principal action of loop diuretics is believed to be the inhibition of active Cl transport in the thick ascending limb of the loop of Henle (e.g. see Burg, Stoner, Cardinal & Green, 1973; Burg & Stoner, 1976). To study the mechanism of these drugs it would be convenient to have a simple model system with properties reflecting the transport characteristics of the isolated tubule preparation. In this context, both the human red cell anion transport system (Band 3, Brooks & Lant, 1978; Gunn, 1979) and the avian erythrocyte cyclic adenosine 3',5'-monophosphate (cyclic AMP)-stimulated cation cotransport system (Palfrey, Feit & Greengard, 1980) have been proposed as possible candidates, based on pharmacological studies.

An alternative which may present an appropriate pharmacological model is the human red cell Na-K cotransport system, which is Cl-dependent and is inhibited by loop diuretics (Dunham, Stewart & Ellory, 1980).

Similar linked Na-Cl and/or K-Cl transporting systems have been characterized in other tissues, e.g. epithelia (Frizzell, Field & Schultz, 1979; Ramos & Ellory, 1980), nerve (Russell, 1979), smooth muscle (Brading, 1979) and Ehrlich ascites tumour cells (Geck, Heinz, Pietrzyck & Pfeiffer, 1978). When examined, these cation-Cl linked systems have been found to be loop diuretic-sensitive (Geck *et al.*, 1978; Frizzell *et al.*, 1979; Ramos & Ellory, 1980). Thus the red cell Na-K cotransport system may represent an example of a generally-distributed Cl-dependent cation-linked transporter, which may, in epithelia, give secondary active transport (i.e. using the energy from K or Na gradients) as an essential component of salt transport.

In the present paper we have measured the potency of four known loop diuretics (frusemide, bumetanide, piretanide and ethacrynic acid (EA)) as inhibitors of the human Na-K cotransport system. The results are compared with previously published data from both *in vitro* work on isolated perfused tubule (Burg & Stoner, 1976) and whole body diuretic studies (Roberts, Homeida, Roberts & Bogie, 1978). We have also tested six structural analogues of frusemide and have examined two thiazides (metolazone and chlorothiazide), two K-sparing diuretics (triamterene and amiloride), and one mercurial, mersalyl, as inhibitors of this system. In the case of mercurials, there is some controversy over their exact site of action (e.g. see Carfuny, 1968). The available data points to an effect on the thick ascending limb and in fact Burg & Green (1973a) have demonstrated inhibition of active Cl transport by mersalyl in the isolated tubule preparation. However, mercurials are also known to cause non-specific increases in membrane-cation permeability (Weed, Eber & Rothstein, 1962).

[1]Present address: The Medical Unit, St Mary's Hospital Medical School, University of London, Paddington, London W2 1PG.

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Our results indicate that the human red cell Na-K cotransport system may represent a valid simple model system for studying loop diuretics, giving good agreement between *in vitro* and *in vivo* pharmacological activities.

Methods

Fresh heparinized human red cells from normal volunteers were used throughout.

Tracer flux measurements

K influx was measured using ^{86}Rb as a tracer by methods previously described (Beaugé & Lew, 1977; Dunham & Ellory, 1980) in a medium containing (mM): K 7.5, Na 142.5, Cl 150, morpholinopropane sulphonic acid buffer (MOPS) 15 (pH 7.4), glucose 5 plus ouabain 10^{-4} M and the diuretic under test. The results were expressed in terms of mmol of K taken up by unit volume of cells in unit time (mmol l cells^{-1} h^{-1}). If external K was varied over the range 5–50 mM, NaCl was used to maintain isotonicity. Previously described control experiments with choline (Dunham *et al.*, 1980) showed that the consequent variation in Na concentration did not affect the results. Anion replacement with methyl sulphate was carried out as previously described (Dunham *et al.*, 1980). Diuretics were dissolved in either isotonic saline or dimethyl sulphoxide (DMSO) and diluted by at least ×100 before use, usually at the final concentrations of 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} M. Control experiments indicated that the resulting 1% v/v concentration of DMSO did not affect the fluxes. The total cotransport K influx was defined as that fraction of the ouabain-insensitive K influx which was dependent on the presence of Cl (methyl sulphate replacement): operationally this was identical with the flux inhibited by bumetanide 10^{-4} M (e.g. Figure 1, see also Dunham *et al.*, 1980).

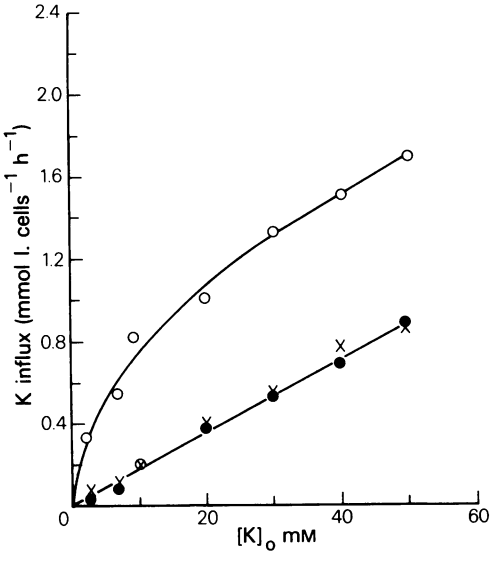


Figure 1 Ouabain-insensitive K influx as a function of external K concentration ([K]$_o$) in erythrocytes: (○) chloride medium, ouabain 10^{-4} M; (●) chloride medium, ouabain 10^{-4} M plus bumetanide 10^{-4} M; (×) ouabain only, chloride replaced by methyl sulphate. The saturable component of ouabain-insensitive K influx is inhibited by either the addition of bumetanide 10^{-4} M or by the substitution of chloride with methyl sulphate.

It has been reported (Burg & Green, 1973b) that ethacrynic acid (EA) is a more potent inhibitor of isolated tubule Cl^- transport if cysteine is also present. EA was therefore used both alone and in the presence of equimolar concentrations of cysteine (EA-cys). Cysteine alone did not inhibit K influx.

The reversibility of loop diuretic inhibition of the red cell cotransport system was tested by first incubating the cells with maximally inhibitory concentrations of bumetanide (10^{-4} M), frusemide (10^{-3} M) and EA-cys (10^{-4} M each) at 37°C for 15 min before three rapid washes by centrifugation (10000 *g*, 1 min) and subsequent K influx measurement over 30 min.

Materials

Frusemide, piretanide and compounds A–F were gifts from Hoechst Pharmaceuticals, Hounslow, Middlesex; bumetanide was a gift from Leo Laboratories, Princes Risborough, Bucks; ethacrynic acid, chlorothiazide and amiloride were gifts from Merck, Sharpe & Dohme, Hoddesdon, Herts; triamterene was supplied by Smith, Klein & French, Welwyn Garden City, Herts and mersalyl was obtained from Sigma Ltd, Dorset.

Results

Figure 1 shows the dependence of the ouabain-insensitive K influx on external K concentration in human red cells. Influx was measured in a medium containing 150 mM Cl with and without bumetanide 10^{-4} M and in the absence of Cl, using substitution with 150 mM methyl sulphate. The Na-K cotransport system, defined as the saturable component of this influx, is equally inhibited by either the addition of bumetanide 10^{-4} M or by the replacement of Cl by methyl sulphate.

Figure 2 shows log inhibitor-concentration curves for the three commonly used loop diuretics frusemide, piretanide and bumetanide, tested on K

influx. All three compounds gave similarly shaped curves, although there was a 60 fold difference in potency. From such curves, estimates of the concentration of inhibitor giving half-maximal inhibition (K_i^{app}) were made for these three diuretics, EA, EA-cys and six analogues of frusemide (labelled A–F). These data, together with structural formulae are given in Table 1. Compounds are listed in decreasing order of potency. All of the known loop diuretics inhibited the cotransport system with a high affinity. The relative inhibitor effectiveness of the series bumetanide, piretanide, frusemide was 56:8:1 which is in quantitative agreement with the results described by Roberts *et al.* (1978, Table 2) for the relative natriuretic activities of the same series in human studies.

Of all the compounds tested, bumetanide was the most potent. The series bumetanide, piretanide, F shows the importance of substitutions at the R_3 group, since compound F, the succinamide derivative, was without effect, while bumetanide, which differs from piretanide only in having the pyrollidine group opened to a chain, was 10 × as effective as piretanide. In contrast, variations at the R_4 group, compounds A–D and piretanide, gave relatively little variation in potency.

Ethacrynic acid by itself was a poor inhibitor (30% inhibition at 10^{-4} M) but in the presence of equimolar concentrations of cysteine, the EA-cys adduct was effective at micromolar concentrations, consistent with the observations of Burg & Green (1973b) on the isolated perfused tubule.

The effects of both frusemide and EA-cys on the isolated perfused tubule are reversible (Burg *et al.*, 1973; Burg & Green, 1973b), consistent with their short duration of action as diuretics in man (Roberts *et al.*, 1978). The reversibility of bumetanide, frusemide and EA-cys inhibition of the red cell Na-K cotransport system was determined by measuring K influx following incubation with inhibitor and washing (see Methods for details). When cells were washed immediately after exposure to the three diuretics, the levels of inhibition were 50, 40 and 20% of the maximal response respectively, indicating significant reversal for all three compounds. In fact, after a further 30 min incubation in the absence of inhibitor, bumetanide reversal was complete.

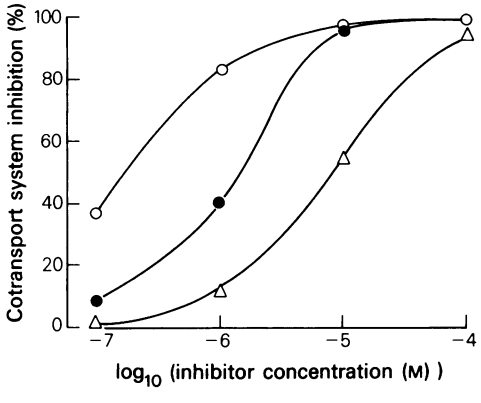


Figure 2 Log inhibitor concentration vs cotransport K influx inhibition in erythrocytes for bumetanide (○), piretanide (●) and frusemide (△).

Results for the effect of mersalyl on ouabain-insensitive K influx are shown in Table 2. At 10^{-4} M mersalyl, a large increase in the ouabain- and bumetanide-resistant flux was found, without a significant inhibition of the cotransport system. At 10^{-5} M mersalyl, there was no significant effect on the fluxes. In the present experiments on red cells we therefore conclude that mersalyl is not an inhibitor of the cotransport system at concentrations $< 10^{-5}$ M, above which concentrations, its effects on membrane cation permeability make it impossible to assess its action on the cotransport system.

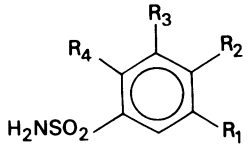
In contrast to the loop diuretics, the two thiazides, metolazone and chlorothiazide had no detectable inhibitory effect on the cotransport K influx at 10^{-4} M. Similarly the K-sparing drugs, triamterene and amiloride, were without effect at this concentration.

Discussion

In this paper, we have presented data comparing the effects of various diuretics and six analogues of frusemide on the human red cell chloride-dependent Na-K cotransport system. The K influx via this system was inhibited by all of the established loop diuretics (frusemide, bumetanide, piretanide and ethacrynic acid with cysteine) and the relative potency of frusemide, bumetanide and piretanide was quantitatively consistent with published whole body pharmacological studies (Roberts *et al.*, 1978). The greatly increased potency of the cysteine adduct of ethacrynic acid compared with the drug alone is consistent with previous reports on the behaviour of this drug at the isolated perfused tubule (Burg & Green, 1973b). One anomaly in comparing the present results with those of Burg & Green (1973a) on the tubule preparation is the apparent ineffectiveness of mersalyl on the red cell transport system at 10^{-4} M. This may merely reflect non-specific mercurial binding effects to the red cell membrane.

Although the human red cell anion exchange transporter (Brooks & Lant, 1978; Gunn, 1979) has previously been proposed as a model for epithelial Cl transport, the present results for the red cell Na-K cotransport system suggest that it is more appropriate

Table 1 Concentrations giving half maximal inhibition (K_i^{app}) of the human red cell cotransport system for frusemide, piretanide, 6 analogues of these diuretics (A–F), ethacrynic acid and ethacrynic acid-cysteine, with structural formulae for the frusemide derivatives



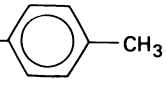
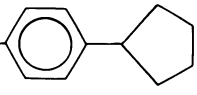
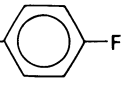
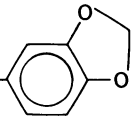
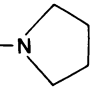
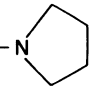
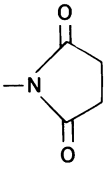
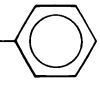
Compound	R_1	R_2	R_3	R_4	K_i^{app} (M)
Bumetanide	—COOH	—H	—NH_2 $(CH_2)_3CH_3$	—O— 	1.6×10^{-7}
Ethacrynic acid-cys					4.0×10^{-7}
A	—COOH	—H	—N 	—S—  —CH_3	4.5×10^{-7}
B	—COOH	—H	—N 	—O— 	6.0×10^{-7}
C	—COOH	—H	—N 	—O—  —F	9.5×10^{-7}
D	—COOH	—H	—N 	—O— 	1.2×10^{-6}
Piretanide	—COOH	—H	—N 	—O— 	1.2×10^{-6}
Frusemide	—COOH	—$NH.CH_2$ 	—H	—Cl	9.0×10^{-6}
E	—C(=O)—NH_2	—H	—N 	—O— 	2.8×10^{-5}
Ethacrynic acid					10^{-4}
F	—COOH	—H	—N 	—O— 	Ineffective

Table 2 Effect of mersalyl on ouabain-insensitive K influx

Inhibitor concentration (M)		*Total K influx*	*Bumetanide-sensitive*
Mersalyl	*Bumetanide*	(mmol l cells^{-1} h^{-1})	
—	—	1.29 ± 0.01	1.18 ± 0.01
—	10^{-4}	0.11 ± 0.01	
10^{-4}	—	1.61 ± 0.20	0.87 ± 0.20
10^{-4}	10^{-4}	0.74 ± 0.04	
10^{-5}	—	1.28 ± 0.02	1.16 ± 0.02
10^{-5}	10^{-4}	0.12 ± 0.01	

Values are ± s.e.mean, $n = 3$.
K influx was measured as described in the text with ouabain 10^{-4} M present in all solutions.

as a possible model for studying the action of loop diuretics. In this context it is not only more sensitive (K_i^{app} for frusemide inhibition of anion transporter $= 2 \times 10^{-4}$ M (Brazy & Gunn, 1976), compared with 9×10^{-6} M (present data)), but also more specific since, unlike the anion exchanger, it is not inhibited by thiazides. The avian erythrocyte cyclic AMP-stimulated cation cotransport system has also been used as a model system for studying diuretic action, with relative affinities of diuretic inhibition in close agreement with the present results (Palfrey *et al.*, 1980). However, the human system has the advantage of convenience and the lack of species differences. In this regard it is interesting to note that the order of potency for diuretic activity in the rat (Merkel, Bormann, Mania, Muschawek & Hropot, 1976) is piretanide > frusemide > bumetanide, unlike man, showing that species differences can be important. Of course, such results may reflect differences in absorption, metabolism and secretion as well as inhibitor binding at the transport site. However, model systems like the present one may play a useful role in eliminating ineffective compounds on the basis of direct action on the transport system.

The present limited studies on structure-activity relationships between the frusemide analogues on the human red cell cotransport system demonstrate that inhibitory potency is more sensitive to R_3 group variations than R_4. There is at present no human (or animal) data available with which to compare these data with diuretic potency for the compounds A–F.

The reversibility of bumetanide and ethacrynic acid-cysteine inhibition of the red cell cotransport system is consistent with both *in vitro* studies on the isolated perfused tubule (Burg *et al.*, 1973; Burg & Green, 1973b) and the known short action of loop diuretics in human studies.

The data presented here demonstrate similar pharmacological responses between the red cell and thick ascending limb transport systems. However, it should be noted that Cl transport via the red cell system has not as yet been demonstrated, due to the presence in the red cell membrane of the high capacity anion exchange system (Band 3), although the Cl-dependence of the red cell cotransport system is not in doubt. However, we feel that the human red cell Na-K cotransport system offers a simple model system in which to study the cellular action of loop diuretics.

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