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OCCURRENCE OF PASSIVE FUROSEMIDE-SENSITIVE TRANSMEMBRANE POTASSIUM TRANSPORT IN CULTURED CELLS

J.F. AITON [a,*], A.R. CHIPPERFIELD [b], J.F. LAMB [a], P. OGDEN [a] and N.L. SIMMONS [a]

[a] *Department of Physiology, University of St Andrews, St Andrews, KY16 9TS Fife and* [b] *Department of Physiology, The University, Dundee DD1 4HN (U.K.)*

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Furosemide ($1 \cdot 10^{-4}$ M) inhibits a proportion of the total passive (ouabain-insensitive) K^+ influx into primary chick heart cell cultures (85%), BC_3H1 cells (75%), MDCK cells (40%) and HeLa cells (57%). This action of furosemide upon K^+ influx is independent of ($Na^+ + K^+$)-pump inhibition since the furosemide-sensitive component of the K^+ influx is identical in the presence and absence of ouabain ($1 \cdot 10^{-3}$ M). For HeLa cells the passive, furosemide-sensitive component of K^+ influx is markedly dependent upon the external K^+, Na^+ and Cl^- content. Acetate, iodide and nitrate are ineffective as substitutes for Cl^-, whereas Br^- is partially effective. Partial Cl^- replacement by NO_3^- gave an apparent affinity of 100 mM [Cl]. Na^+ replacement by choline$^+$ abolishes the furosemide-sensitive component, whereas Li^+ replacement reduces this component by 48%. Partial Na^+ replacement by choline$^+$ gives an apparent affinity of 25 mM [Na^+]. Variation in the external K^+ content gives an affinity for the furosemide-sensitive component of approx. 1.0 mM. Furosemide inhibition of the passive K^+ influx is of high affinity, half-maximal inhibition being observed at $5 \cdot 10^{-6}$ M furosemide. Piretanide ($1 \cdot 10^{-4}$ M) and phloretin ($1 \cdot 10^{-4}$ M) inhibit the same component of passive K^+ influx as furosemide; ethacrynic acid and amiloride (both $1 \cdot 10^{-4}$ M) partially so. The stilbene, SITS ($1 \cdot 10^{-6}$ M), was ineffective as an inhibitor of the furosemide-sensitive component.

Introduction

A proportion of the passive transmembrane flux of Na^+, K^+ and Cl^- in human red cells is mediated by a $Na^+ + K^+$ cotransport system which is highly Cl^--dependent and is inhibited by loop diuretics such as furosemide and piretanide [1–4].

Passive, linked ion fluxes (e.g., $Na^+ + Cl^-$, $K^+ + Cl^-$) are a feature of cell types other than red cells; in particular, $Na^+ + Cl^-$ co-transport has been identified in thick ascending limb of the Loop of Henle [5] shark rectal glands [6], human placenta [7] smooth muscle [8] and Ehrlich ascites cells [9]. Often furosemide has been shown to markedly inhibit such coupled fluxes [5–7,9]. Recently both Chipperfield [2,3] and Geck et al. [9] proposed that a common furosemide-sensitive $Na^+ + K^+ + Cl^-$ co-transport, may be responsible for the experimentally observed co-transport (or countertransport) of ion pairs [2,3,9]. Thus in Ehrlich ascites cells, the 'paradoxical' Cl^- movement previously attributed to coupled KCl movement is now thought to be dependent upon a furosemide-sensitive $Na^+ + K^+ + Cl^-$ co-transport [9].

The present series of experiments was designed to determine whether a furosemide-sensitive component of the passive (ouabain-insensitive) ion transport is indeed a common feature of transmembrane ion

* To whom correspondence should be addressed.

exchange in cells of widely differing properties and to compare the properties of such a system with data obtained from the well-characterized human red cell [2–4,10]. We have accordingly measured the passive ouabain-insensitive K^+ influx in four cultured cell types chosen to cover a spectrum of cellular properties. The cell lines were primary chick heart cell cultures, BC_3H1 smooth muscle cells, MDCK epithelial cells and HeLa cells.

Our major finding is that a furosemide-sensitive component of K^+ influx is present in all four cell-types studied and that for HeLa cells, the properties of the system closely resemble the known features of the $Na^+ + K^+ + Cl^-$ co-transport present in human red cells. A preliminary account of some of the present data has been published [11].

Methods

(i) Cell culture

(a) HeLa cells. HeLa cells were grown in Eagle's Basal Medium supplemented with 10% new-born calf serum [12,13]. For experimental purposes, monolayers of cells were grown to confluency in 60 mm 'Sterilin' plastic petri dishes (approx. $2 \cdot 10^6$ cells/plate). The HeLa cell is an example of a de-differentiated cell, the ion transport properties of which are well characterized [12,13].

(b) Chick heart cells. Monolayer cultures of spontaneously contracting myocardial cells were derived from 11-day-old embryonic chick hearts subjected to multiple periods of tryptic digestion [14,15]. Non-muscle cell overgrowth was minimized by using the cell cultures within 3 days of plating. The myocardial cells were grown on 35 mm 'Nunclon' petri dishes in Medium 199 supplemented with 10% foetal bovine serum and 2% chick embryo extract. Myocardial cell cultures were incubated at 37°C in a humidified environment of 96% air/4% CO_2.

(c) MDCK epithelial cells. MDCK cells form monolayers possessing epithelial structure and are capable of transmonolayer ion transport [16–18]. Cultures of MDCK cells were obtained at 60 serial passages from Flow Laboratories (Irvine, Scotland) and maintained in serial culture in Roux flasks in Minimal Essential Eagle's Medium supplemented with non-essential amino acids (2 mM glutamine, 1 unit/cm^3 gentamycin antibiotic and 10% v/v foetal bovine serum (Flow)) at 37°C in a 95% air/5% CO_2 atmosphere. For experimental purposes cells were grown in 60 mM 'Sterilin' plastic petri dishes. Sub-confluent cell monolayers were used.

(d) BC_3H1 smooth muscle cells. The cloned BC_3H1 smooth muscle cell-line was a gift from Dr. J. Patrick [19,20]. BC_3H1 cells are electrically excitable and responsive to acetyl choline application [21]. The cells were grown at 37°C in Eagle's medium (Dulbecco's modification) with 10% (v/v) foetal calf serum in a humidified atmosphere of 88% air/12% CO_2. For experiments, confluent monolayers were grown on 60 mm 'Sterilin' petri dishes.

(ii) Experimental measurements

The techniques used in this study have been described in detail elsewhere [12,22,23] and are summarized below. All experiments were carried out at 37°C.

^{86}Rb was used as an isotopic tracer to indicate potassium movements (this was checked for all the cell lines used, in separate experiments). The ^{86}Rb influx into cell monolayers was measured during a 10 min (HeLa), 5 min (BC_3H1, MDCK) or 1 min (chick) incubation in Krebs' solution containing 0.2 μCi/ml ^{86}Rb. Incubation times were varied to ensure that flux measurements were made during that time corresponding to the linear portion of the uptake curve. At the end of the influx period, the monolayer was rapidly rinsed four times with ice-cold Krebs' solution to remove extracellular isotope. The washing protocol removed all of an extracellular space marker ($[^{14}C]$inulin) in separate experiments in all of the cell types used. Loss of intracellular ^{86}Rb during the wash (no more than 20s) in ice-cold Krebs' solution was less than 1%. Cell layers were then trypsinized to form a cell suspension. Aliquots of this cell suspension were used to determine the cell number and mean cell volume with a Coulter Counter (ZF) fitted with a Channelyser (C1000). An aliquot of the cell suspension was also used to determine the ^{86}Rb activity in a liquid scintillation spectrometer (Packard model 3255) by the Cerenkov method.

(iii) Solutions

Flux experiments were carried out at 37°C; for control conditions the Krebs' solution contained 137 mM NaCl, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.2 mM

TABLE I

INHIBITION BY FUROSEMIDE OF K^+ INFLUX INTO CULTURED CELLS

K^+ influx (tracer ^{86}Rb) was measured in the four cell lines as described in Methods; ouabain ($1 \cdot 10^{-3}$ M) and furosemide ($1 \cdot 10^{-4}$ M) were added as indicated. All results are the mean of three determinations ± S.E. except where indicated. Student's *t*-test of differences between the ouabain-sensitive fluxes in the presence and absence of furosemide, or between the furosemide-sensitive fluxes in the presence and absence of ouabain shows no significant differences ($P > 0.05$ for all data sets for four degrees of freedom).

K^+ influx (mmol · l^{-1} · min^{-1})					Differences			
Cell type	Control	+Ouabain	+Furosemide	+Furosemide +ouabain	Ouabain-sensitive without furosemide	Ouabain-sensitive with furosemide	Furosemide-sensitive without ouabain	Furosemide-sensitive with ouabain
Chick heart	9.44 ± 0.3	5.76 ± 0.18	5.22 ± 0.18	0.84 ± 0.06	3.68 ± 0.31	4.38 ± 0.32	4.22 ± 0.31	4.92 ± 0.19
HeLa	3.42 ± 0.06	2.28 ± 0.05	1.84 ± 0.02	0.54 ± 0.06	1.12 ± 0.07	1.30 ± 0.07	1.58 ± 0.07	1.74 ± 0.07
MDCK (n = 8)	2.20 ± 0.07	1.24 ± 0.16	1.63 ± 0.09	0.49 ± 0.04	1.19 ± 0.06	1.14 ± 0.09	0.57 ± 0.09	0.75 ± 0.16
BC_3H1	4.33 ± 0.08	2.78 ± 0.16	2.16 ± 0.09	0.70 ± 0.02	1.55 ± 0.18	1.46 ± 0.10	2.17 ± 0.08	2.08 ± 0.16

$MgSO_4 \cdot 7H_2O$, 0.3 mM NaH_2PO_4, 0.3 mM KH_2PO_4, 12 mM HCl, 14 mM Tris base and 11 mM glucose supplemented with 1.0% (v/v) new-born calf serum, pH 7.4.

The Na^+ content of the experimental media was varied by isosmotic replacement of NaCl by choline chloride and LiCl. The Cl^- content of the media was varied by isosmotic substitution of NaCl by NaBr, NaI, $NaNO_3$ and sodium acetate. In some cases Tris-maleate replaced Tris-HCl.

The K^+ content of the media was varied in one experiment from 0 to 9 mM KCl without adjustment of osmolarity.

For experiments in which Na^+, Cl^- or K^+ was varied, serum was dialysed overnight against two changes of 50 vol. distilled water.

(iv) Materials

Whenever possible, inorganic salts of Analar quality were used. ^{86}Rb was purchased from the Radiochemical Centre, Amersham (U.K.). Tissue culture supplies were obtained from Flow Laboratories (Irvine, Scotland) and Gibco-Biocult Ltd. (Paisley, Scotland). New-laid fertilized Hubbard hatching eggs were purchased from Buxted Poultry Ltd., Kinglassie Hatchery, Fife.

The stilbene, 4-acetamide-4-isothiocyano-2,2'-stilbene disulphonic acid (SITS) was obtained from B.D.H. Chemicals (Poole U.K.) and dissolved in 10 mM Tris base to make a 10^{-2} M stock solution.

4-Aminopyridine, phloretin, tetrodotoxin and ouabain were obtained from the Sigma Chemical Co., Fancy Road, Poole, U.K.; stock solutions were made in Krebs' solution, 10 mM Tris base and distilled water, respectively.

Ethacrynic acid and amiloride were gifts from Merck, Sharpe and Dohme and stock solutions were made in 10^{-2} M Tris and Krebs solution.

Furosemide and piretanide were gifts from Dr. S. Dombey, of Hoechst Pharmaceuticals, Hounslow, Middlesex, U.K. Stock solutions were also prepared in 10^{-2} M Tris.

Statistics

Variation in results is expressed as the standard error of the mean. Tests for significance of differences were made by a two tailed Student's *t*-test (unpaired means solution).

Results

(i) Furosemide sensitivity of passive K^+ influx

The results in Table I show the effects of ouabain and furosemide on K^+ influx into the four cultured cell lines tested. The total, ouabain-sensitive and ouabain-insensitive fluxes of the cell lines tested (chick heart, HeLa and MDCK) agree satisfactorily with previous results from this laboratory [11–13, 24,25], whilst the magnitude of the total K^+ influx measured in BC_3H1 cells agrees well with previously reported measurements of the transmembrane K^+ exchanges [21]. A substantial furosemide-sensitive flux (last column) is observed in all four cell types in the presence of ouabain. This component ranged from 40% of the passive influx in MDCK cells up to 85% in chick heart cells: in BC_3H1 and HeLa cells it was 75% and 57%, respectively. The furosemide-sensitive, ouabain-insensitive flux appears to be the major component of the passive flux. There appears to be, however, no fixed proportionality between the furosemide-sensitive and -insensitive passive components in the different cell types. In human red cells, where flux rates are some 100-fold less, an even larger proportion of the passive K^+ influx, namely 94%, is inhibited by furosemide [1–4].

In the absence of ouabain, there was again a furosemide-sensitive flux (Table I) which was no greater than in media containing ouabain. Moreover, the

TABLE II

LOSS OF FUROSEMIDE-SENSITIVE, OUABAIN-INSENSITIVE K^+ INFLUX INTO HeLa CELLS IN CHLORIDE-DEPLETED MEDIA

K^+ influx (tracer ^{86}Rb) in HeLa cells was measured as described in Methods; ouabain ($1 \cdot 10^{-3}$ M) was present in all media, and furosemide ($1 \cdot 10^{-4}$ M) as shown. In the control media, $[Cl^-]$ was 160 mM and in the depleted media, $[Cl^-]$ was 23 mM and the replacement anion 137 mM. All results are the mean of three determinations ± S.E.

Major anion	K^+ influx ($mmol \cdot l^{-1} \cdot min^{-1}$)		
	Control	+Furosemide	Difference
Chloride	3.27 ± 0.28	1.45 ± 0.15	1.83 ± 0.52
Bromide	2.57 ± 0.32	1.86 ± 0.04	0.71 ± 0.32
Iodide	1.62 ± 0.31	1.37 ± 0.25	0.24 ± 0.39
Acetate	1.08 ± 0.18	1.29 ± 0.14	−0.21 ± 0.22

ouabain-sensitive fluxes were the same in furosemide-containing or furosemide-free media (Table I). Furosemide does not, therefore, inhibit sodium pump-mediated K^+ influx. Whilst generally the greater the passive flux the greater the pump flux, no direct relationship between pump-mediated and furosemide-sensitive fluxes could be found.

(ii) Anion dependence of furosemide-sensitive K^+-influx

The nature of the diuretic-sensitive passive K^+ influx was examined in more detail using HeLa cells. Of the four cell lines tested, these cells have the simplest structure and are well characterized in respect of their transmembrane ion fluxes. When most of the chloride in the media was replaced by iodide, acetate, bromide or nitrate there was a clear loss of furosemide-sensitive influx (Table II and Fig. 1). With the exception of bromide, which reduced the furosemide-sensitive flux by only 60%, the other anions tested abolished the furosemide-sensitive component. Moreover, with iodide, nitrate and acetate replacements, the furosemide-insensitive flux was not significantly affected ($P > 0.2$) for each anion replacement versus Cl^- (Table III and Fig. 1). Bromide was exceptional in that it raised the furosemide-insensitive influx ($P < 0.05$ for Br^- vs. flux in chloride medium plus furosemide). The reason for this is unknown.

The chloride dependence of the furosemide-sensitive K^+ influx using nitrate as the replacement anion is shown in Fig. 1. Over the range tested there was an upward-curving, non-saturable dependence of the furosemide-sensitive K^+ influx. This is not inconsistent with the linear [2,3] or sigmoidal curves [1] observed in other systems, since the experimental discrepancy between the linear and slightly sigmoidal curves reported previously and the curve in Fig. 1 is not great. In contrast to the furosemide-sensitive influx, the furosemide-insensitive influx was largely independent of the chloride concentration from 10 to 160 mM, similar to human red cells [1–3,26].

The chloride dependence with acetate replacements (Fig. 2) contrasts with the results obtained with nitrate. There was almost no dependence of the furosemide-sensitive influx up to 120 mM chloride. The furosemide-insensitive influx was again independent of the chloride concentration over the range tested (data not shown). These results suggest that acetate

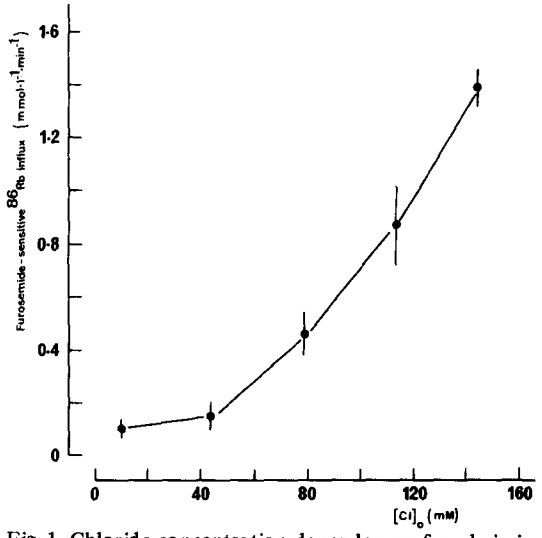


Fig. 1. Chloride concentration dependence of ouabain-insensitive K^+ influx into HeLa cells. K^+ influx (tracer ^{86}Rb) was measured as described in METHODS in this and subsequent figures: ouabain ($1 \cdot 10^{-3}$ M) was present in all media, furosemide was used at $1 \cdot 10^{-4}$ M. Chloride was replaced by nitrate as shown. All results are the mean of three determinations. Errors where shown are ±S.E. The total ouabain-insensitive K^+ flux is not shown.

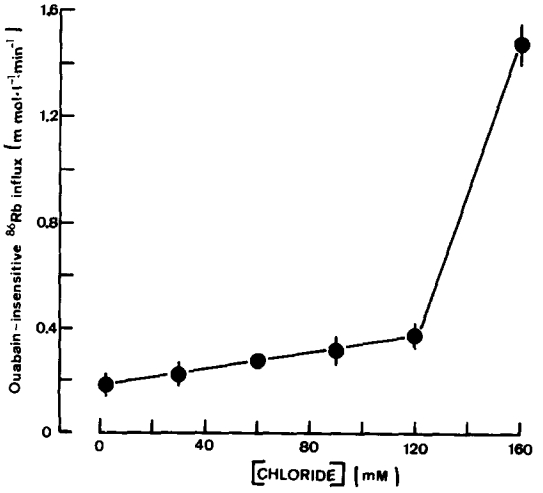


Fig. 2. Chloride concentration dependence of ouabain-insensitive K^+ influx into HeLa cells with acetate replacement. Ouabain ($1 \cdot 10^{-3}$ M) was present in all media and furosemide ($1 \cdot 10^{-4}$ M) added. All results are the mean of three determinations.

TABLE III

EFFECT OF ANIONS AT LOW CONCENTRATIONS ON OUABAIN-INSENSITIVE K^+ INFLUX INTO HeLa CELLS

K^+ influx (tracer ^{86}Rb) in HeLa cells was measured as described in Methods: ouabain ($1 \cdot 10^{-3}$ M) was present in all media as shown; in the control media, [Cl^-] was 160 mM and in the test media [Cl^-] was 140 mM with 20 mM of the anion indicated. All results are the mean of three determinations ± S.E.

Added anion	K^+ influx (mmol · l^{-1} · min^{-1})		
	Control	+Furosemide	Difference
None, Cl^- only	1.80 ± 0.25	0.73 ± 0.08	1.07 ± 0.26
Bromide	2.43 ± 0.34	0.96 ± 0.05 [a]	1.47 ± 0.12
Iodide	2.10 ± 0.16	0.53 ± 0.12	1.57 ± 0.20
Nitrate	2.12 ± 0.22	0.58 ± 0.16	1.54 ± 0.27
Acetate	0.79 ± 0.04 [b]	0.65 ± 0.14	0.14 ± 0.14 [b]

Significantly different from chloride values (Student's *t*-test):
[a] $P < 0.05$.
[b] $P < 0.01$.

inhibits the furosemide-insensitive, chloride-dependent K^+ influx. To test whether acetate and other anions may inhibit, the effects of anions at fixed low concentration (20 mM) were tested in the presence of 140 mM chloride (Table III). In this experiment, there was no significant furosemide-sensitive K^+ influx when acetate was added. In contrast, the other anions (bromide, iodide and nitrate) had no significant effect upon the furosemide-sensitive K^+ influx at low concentration, except for Br^-, where a 30% elevation was observed (see above). Thus, bromide can produce a marked increase in passive K^+ influx.

(iii) Potassium dependence of furosemide-sensitive K^+-influx

On raising potassium over the range 0 to 10 mM, the ouabain-insensitive furosemide-sensitive K^+ influx increased in a saturable fashion (Fig. 3). Half-maximal stimulation was observed at approx. 1 mM. The ouabain-insensitive, furosemide-insensitive component of K influx was approximately linearly related to K^+ concentration consistent with the notion that this component of K^+ influx represents transmembrane diffusion (Fig. 3).

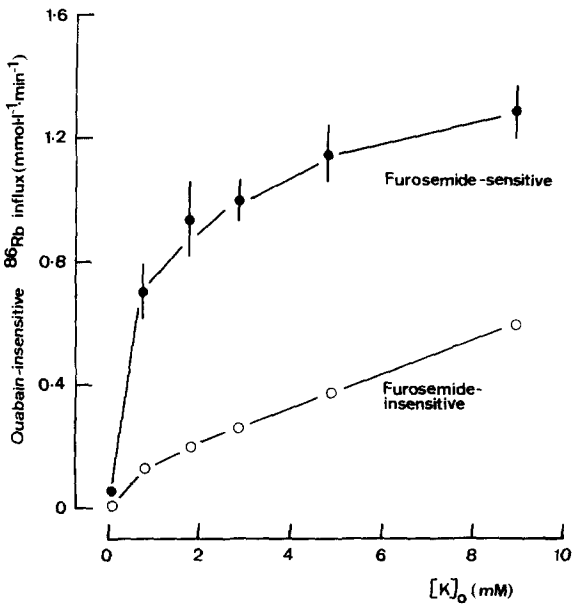


Fig. 3. Potassium concentration dependence of ouabain-insensitive K^+ influx into HeLa cells. Ouabain ($1 \cdot 10^{-3}$ M) was present in all media and furosemide ($1 \cdot 10^{-4}$ M) and potassium was added as indicated. All results are the mean of three determinations. Furosemide-sensitive, •; furosemide-insensitive, ○.

(iv) Sodium dependence of furosemide-sensitive K^+ influx

On replacing sodium with either lithium or choline, there was a reduction in the furosemide-sensitive K^+ influx (Table IV). However, the effects of the two sodium substitutes were quite different. With lithium, the furosemide-sensitive influx was reduced by only 48% and the furosemide-insensitive influx was almost unaffected. With choline the furosemide-sensitive influx fell by 89% and the furosemide-insensitive influx increased 4-fold: the total ouabain-insensitive K^+ influx was thus unchanged on replacing sodium with choline. This effect of choline on the furosemide-insensitive influx has not been seen in human red cells [4,2,7,28], but otherwise the effects of sodium replacement agree.

The sodium concentration dependence was tested over the range 0 to 140 mM using choline as the replacement cation (Fig. 4). This range was chosen because K^+ influx into human red cells is half maximally activated at about 35 mM with choline replacement. As before (Table IV, Fig. 4) the passive K^+

TABLE IV

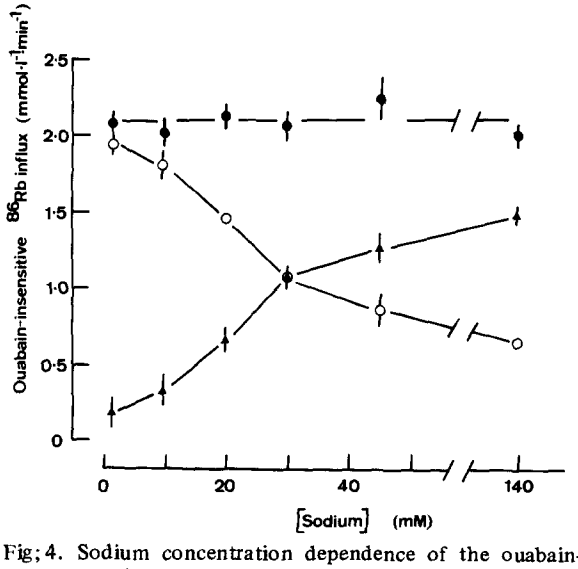
LOSS OF FUROSEMIDE-SENSITIVE, OUABAIN-INSENSITIVE K^+ INFLUX INTO HeLa CELLS IN SODIUM-DEPLETED MEDIA

K^+ influx (tracer ^{86}Rb) in HeLa cells was measured as described in Methods: ouabain ($1 \cdot 10^{-3}$ M) was present in all media and furosemide ($1 \cdot 10^{-4}$ M) as shown; in the control media Na^+ was 140 mM and it was replaced by 140 mM Li^+ or choline$^+$ as shown. All results are the mean of at least three determinations ± S.E.

Major cation	K^+ influx (mmol · l^{-1} · min^{-1})		
	Control	+Furosemide	Difference
Sodium	1.93 ± 0.10	0.54 ± 0.03	1.39 ± 0.10
Choline	2.09 ± 0.12 [a]	1.94 ± 0.01 [d]	0.15 ± 0.12 [d]
Lithium	1.12 ± 0.03 [d]	0.41 ± 0.03 [b]	0.72 ± 0.03 [c]

Significantly different from Na^+ media values:
[a] Nonsignificant.
[b] $P < 0.05$.
[c] $P < 0.002$.
[d] $P < 0.001$.

influx was unchanged on replacing sodium with choline but the furosemide-sensitive component increased as medium Na^+ was increased. There



Fig; 4. Sodium concentration dependence of the ouabain-insensitive K^+ influx into HeLa cells. All results are the mean of three observations. Total ouabain-insensitive K^+ influx, •; furosemide- and ouabain-insensitive, ○; the difference, i.e., ouabain-insensitive, furosemide-sensitive, ▲.

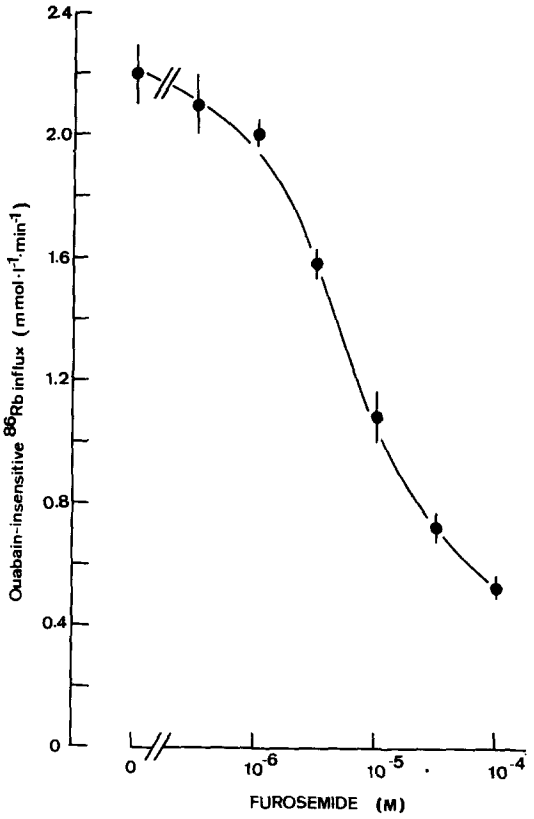


Fig. 5. Furosemide inhibition of ouabain-insensitive K^+ influx into HeLa cells.

appeared to be both saturable and non-saturable components, with the saturable components being half-maximally stimulated at about 25 mM Na^+.

(v) Inhibition by furosemide and other drugs

The concentration of furosemide routinely employed was $1 \cdot 10^{-4}$ M, since half-maximal inhibition in human red blood cells is $5 \cdot 10^{-6}$ M: above $1 \cdot 10^{-4}$ M, furosemide produces no further inhibition [2,3, 10]. The concentration dependence of furosemide inhibition in HeLa cells was very similar (Fig. 5). concentration giving half-maximal inhibition of the furosemide-sensitive component was $5 \cdot 10^{-6}$ M.

The effects of certain other drugs on passive K^+ influx were tested (Table V). The effects of piretanide and furosemide were closely similar. This is consistent with work on red cells showing that both drugs will block diuretic-sensitive passive K^+ influx at the high concentrations employed [10]. In human

TABLE V

EFFECT OF DRUGS ON OUABAIN-INSENSITIVE K^+ INFLUX INTO HeLa CELLS

K^+ influx (tracer ^{86}Rb) in HeLa cells was measured as described in Methods; ouabain ($1 \cdot 10^{-3}$ M) was present in all media with additions of drugs (all $1 \cdot 10^{-4}$ M, except SITS = $1 \cdot 10^{-5}$ M) as shown. All results are the mean of four determinations ± S.E.

Drug	K^+ influx (mmol · l^{-1} · min^{-1})	
	Influx	Difference from control
None, control [a]	1.92 ± 0.09	–
Furosemide	0.36 ± 0.05	1.56 ± 0.10 [e]
Piretanide	0.31 ± 0.02	1.61 ± 0.09 [e]
Ethacrynic acid	1.24 ± 0.03	0.68 ± 0.09 [d]
Phloretin	0.45 ± 0.02	1.47 ± 0.09 [e]
Amiloride	1.55 ± 0.02	0.37 ± 0.09 [c]
SITS	1.88 ± 0.05	0.04 ± 0.10 [b]

[a] K^+ influx without ouabain in this experiment was 4.24 ± 0.16 (S.E.) mmol · l^{-1} · min^{-1}, giving an ouabain-sensitive influx of 2.32 ± 0.18 mmol · l^{-1} · min^{-1}.

Significantly different from zero (Student's one tailed t-test):

[b] Non-significant.

[c] $P < 0.02$.

[d] $P < 0.01$.

[e] $P < 0.001$.

red cells, ethacrynic acid [10] and phloretin [2,3] are known to block the diuretic-sensitive pathway but their affinities are less than furosemide; in HeLa cells, these substances inhibited, but not completely. Again, as in human red cells [1], SITS did not inhibit K^+ influx; thus separating the Cl^--dependence of the system from the SITS-sensitive Cl^--transporter found in various tissues. Amiloride produced a significant, but small reduction in K^+ influx.

Besides the drugs given in Table V, 4-aminopyridine and tetrodotoxin were tested on the K^+ influx. At $1 \cdot 10^{-4}$ M, 4-aminopyridine produced no effect. At $1 \cdot 10^{-5}$ M, tetrodotoxin produced a slight fall in K^+ influx: however, further confirmation is required on this point.

Discussion

The present results show that in all four of the cell lines studies there is a furosemide-sensitive component of the passive K^+ influx; thus, despite marked differences in the cellular phenotype (cardiac, smooth muscle, epithelial and cancerous) the furosemide-sensitive K^+ influx is indeed a common feature, as is the ouabain-sensitive (Na^+-K^+)-pump. The present findings are comparable to earlier work on red cells [1–4,28] and on ascites cells [26,29]. The furosemide-sensitive component is greatest in magnitude where the passive fluxes are greatest and, in turn, where the active fluxes are largest. However, there appears to be no direct relationship between the magnitudes of the different components of K^+ influx. The present results, combined with the work on red cells and ascites cells mentioned above, lend no support to the idea [27] that some passive fluxes are mediated by the sodium pump. Of the four cell types studied, the furosemide-sensitive component is largest in those cells which are electrically excitable. The effect of chloride on passive K^+ fluxes found here is similar to that observed in other systems [1–4,26]. In this paper, detailed evidence on this point is presented for the HeLa cells, but similar evidence is available for MDCK cells (Table VI) [11]. A dependence of part of the passive K^+ influx on chloride may therefore be a general phenomenon.

An important feature of the present results is the clear parallel between HeLa and human red cells, suggesting that the molecular basis is similar (Na^+ +

TABLE VI

AFFINITIES OF FUROSEMIDE-SENSITIVE, OUABAIN-INSENSITIVE K^+ INFLUX IN RED BLOOD CELLS, HeLa CELLS AND MDCK CELLS

The data for the affinities are taken from Chipperfield (Refs. 4, 5 and unpublished observations) for human red blood cells (RBC), from this work for HeLa cells and for MDCK cells (unpublished data).

Substance	Concentration giving half-maximal activation or inhibition		
	Human RBC	HeLa	MDCK
Sodium (mM)	35	25	10
Potassium (mM)	5.5	1.0	1.0
Chloride (mM) [a]	70–80	100	100
Furosemide (M)	$5 \cdot 10^{-6}$	$5 \cdot 10^{-6}$	$3 \cdot 10^{-6}$

[a] Nitrate replacement: for the $K_{1/2}$ given, the maximum activity is taken as the flux observed in 150 mM Cl^- media (see Fig. 1 and text).

$K^+ + Cl^-$ cotransport). First, the quality of the chloride dependences agrees well: thus, of the anions tested, only bromide supports a substantial furosemide-sensitive K^+ influx. Second, the furosemide-sensitive K^+ influx is similarly dependent upon external Na^+ in red cells [4,28] and HeLa cells. Third, furosemide is found to be just one of several diuretics that may inhibit. Thus, the powerful inhibition by piretanide, and the weaker inhibitions by ethacrynic acid and phloretin, of K^+ influx into HeLa cells are all consistent with the effects observed in red cells [1–3,10]. Moreover, the affinity for furosemide in HeLa is high and comparable to red cells. In this connection, there is some evidence that at high concentrations, furosemide may produce rather indiscriminate inhibitory effects [30,31] but the high affinities observed here seem to rule out any non-specific effect of furosemide. In addition, chronic treatment (more than 12 h) of HeLa cells by inclusion of $1 \cdot 10^{-4}$ M furosemide in the growth medium has no detectable effect upon intracellular cation concentrations (unpublished observation).

In general, therefore, passive K^+ influx in HeLa cells shows striking similarities to passive K^+ influx in red cells. On the other hand, there are some interesting differences. Principally, these are in the ion affinities (Table VI). The affinities for both Na^+ and K^+ are significantly greater in HeLa than red cells. In contrast, the affinity for chloride (insofar as the linear or nearly-linear chloride concentration dependence allows it to be assessed) is lower than red cells. The same features occur in both HeLa and MDCK cells (Table VI). This may mean that human red cells are, in this respect, unusual. However, it is difficult to rule out the possibility that, just as the magnitude and proportion of the furosemide-sensitive fluxes differ from from one cell type to another, the affinities may differ as well.

A further difference between HeLa and red cells lies in the effects elicited by choline and bromide upon the furosemide-insensitive fluxes: in HeLa, they can increase the furosemide-insensitive K^+ influx, whereas in red cells they do not. These results bring to mind the work of Funder and Weith on red cells [32] where there were increases in K^+ efflux with nitrate and iodide: again, Wiley and Cooper [4] found that choline raised Na^+ influx when it was a substitute for external K^+. However, there appear to be several discrepancies between the results obtained on red cells in different laboratories.

The function of the diuretic-sensitive passive cation transport pathway has been the subject of much discussion [27]. It has been considered to be a second pump [33], an exchange pathway [34,35] or a cotransport of either ($Na^+ + Cl^-$) or ($Na^+ + K^+ + Cl^-$) [2–4,9,36]. In view of this uncertainty on the nature of the furosemide-sensitive pathway, it is difficult to speculate on its function. Nevertheless, three possibilities are worth mentioning. First, it has been suggested that it serves to regulate cell colume [9] by allowing cell swelling which is opposed by the sodium pump. However, this notion is not fundamentally different from the pump-leak concept [37] and volume regulatory systems in avain red cells [38] which can both swell and shrink. Second, it may account for the elevation of chloride in certain cells and, in turn, secondary transepithelial transport of chloride [39]. The recent observation of Cl^- secretion by MDCK epithelium, which is furosemide-sensitive [40] may allow an unequivocal determination of the role of furosemide-sensitive $Na^+ + K^+ + Cl^-$ co-transport in transepithelial Cl^- secretion. In other cell systems the role of $Na^+ + K^+ + Cl^-$ co-transport intracellular Cl^- accumulation is less clear. There is very little information on the effect of diuretics on smooth muscle [8] and cardiac muscle [41], where a substantial part of the inward chloride movement is in exchange for bicarbonate. In nerve [42] and smooth muscle [43], the furosemide-sensitive uptake of chloride is linked to Na^+ but not to K^+. Also, there is some evidence that K^+ may inhibit Na^+ uptake [7]. The present results obtained with tissue cultured cardiac and smooth muscle cells indicate that this approach will be useful in analysing the role of the $Na^+ + K^+ + Cl^-$ co-transporter in these tissues.

As regards the suggestions that the diuretic sensitive $Na^+ + K^+ + Cl^-$ co-transport may participate in net cation exchanges [9], we have recently observed that in HeLa cells re-establishment of normal cation gradients following a period in K^+-free media is retarded by 0.1 mM furosemide (unpublished data). Clearly the involvement of the co-transport system in non-steady-state conditions deserves further attention.

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References

1 Dunham, P.B., Stewart, G.W. and Ellory, J.D. (1980) Proc. Natl. Acad. Sci. USA 77, 1711–1715
2 Chipperfield, A.R. (1980) Nature 286, 281–282
3 Chipperfield, A.R. (1981) J. Physiol. 312, 435–444
4 Wiley, J.S. and Cooper, R.A. (1974). J. Clin. Invest. 53, 245–755
5 Burg, M., Stoner, L., Cardinal, J. and Green, N. (1973). Am. J. Physiol. 225, 119–124
6 Eveloff, J., Hildmann, B., Kinne, R., Murer, H. (1978). J. Physiol. 285, 9–10P
7 Boyd, C.A.R., Chipperfield, A.R. and Lund, E.K. (1980) J. Physiol. 307, 86P
8 Brading, A.R. (1979). Brit. Med. Bull. 35, 227–234
9 Geck, P., Pietrzyk, C., Burckhardt, B.-C., Pfeiffer, B. and Heinz, E. (1980). Biochim. Biophys. Acta 600, 432–447
10 Stewart, G.W. and Ellory, J.C. (1981). Brit. J. Pharmacol. (in press)
11 Aiton, J.F., Chipperfield, A.R., Lamb, J.F., Ogden, P. and Simmons, N.L. (1980). J. Physiol. 310, 20P
12 Lamb, J.F. and McCall, D. (1972). J. Physiol. 225, 599–617
13 Aiton, J.F. and Lamb, J.F. (1980). Q. J. Exp. Physiol. 65, 47–62
14 Horres, C.R., Lieberman, M., Purdy, J.E. (1977). J. Membrane Biol. 34, 313–329
15 Horres, C.R., Aiton, J.F., Lieberman, M. (1979). Am. J. Physiol. 236, 163–170
16 Cereijido, N., Robbins, E.S., Dolan, W.J., Rotunno, C.A. and Sabatini, D.D. (1978). J. Cell. Biol. 77, 853–880
17 Misfeldt, A.S., Hamamoto, S.R., and Pitelka, D.R. (1976). Proc. Natl. Acad. Sci. USA 73, 1212–1216
18 Simmons, N.L. (1981). J. Membrane Biol., in the press
19 Schubert, D.A., Harris, A.J., Devine, C.E., and Heineman, S. (1974). J. Cell. Biol. 61, 398–413
20 Berman, P.W. and Patrick, J. (1980). J. Exp. Med. 151, 204–223
21 Mauger, J.P., Moura, A.M. and Worcel, M. (1978). Brit. J. Pharmacol. 64, 29–36
22 Boardman, L., Lamb, J.F. and McCall, D. (1972). J. Physiol. 225, 619–635
23 Boardman, L., Huett, M., Lamb, J.F., Newton, J. and Polson, J.M. (1974). J. Physiol. 241, 771–794
24 Aiton, J.F., Lamb, J.F. and Ogden, P. (1981). Brit. J. Pharmacol., in the press.
25 Barker, G., Lamb, J.F., Ogden, P. and Simmons, N.L. (1978). J. Physiol. 285, 46–47P
26 Bakker-Grunwald, T. (1978). Biochim. Biophys. Acta 513, 292–295
27 Lew, V.L. and Beauge, L. (1979) in Membrane Transport in Biology, Vol. II, (Geibisch, G., Tosteson, D.C. and Ussing, M.H., eds.), pp. 81–115, Springer-Verlag, Berlin
28 Sachs, J.R. (1971) J. Gen. Physiol. 57, 259–282
29 Tupper, J.T. (1975). Biochim. Biophys. Acta 394, 586–596
30 Boyd, C.A.R. and Chipperfield, A.R. (1980). J. Physiol. 303, 63P
31 Chipperfield, A.R. and Perring, V.S. (1981). J. Physiol. 310, 21P
32 Funder, J. and Weith, J.O. (1967). Acta Physiol. Scand. 71, 168–185
33 Hoffman, J.F. and Kregenow, F.M. (1966). Ann. N.Y. Acad. Sci. 137, 566–576
34 Dunn, M.J. (1973). J. Clin. Invest. 52, 658–670
35 Lubowitz, M. and Whittam, R. (1969). J. Physiol. 202
36 Heinz, E., Geck, P., Pietrzyk, C., Burkhardt, B.-C. and Pfeiffer, B. (1977). J. Supramol. Struct. 6, 125–133
37 Tosteson, P.C. and Hoffman, J.F. (1960). J. Gen. Physiol. 44, 169–194
38 Kregenow, F.M. (1977) in Membrane Transport in Red Cells (Ellory, J.C. and Lew, V.L., eds), pp. 388–426, Academic Press, New York
39 Frizzell, R.A., Field, M. and Schultz, S.G. (1979) Am. J. Physiol. 236, F1-F8
40 Simmons, N.L. (1979). J. Physiol. 290, 28–29P.
41 Vaughan-Jones, R.D. (1979) J. Physiol. 111–137
42 Russell, J.M. (1979). J. Gen. Physiol. 73, 801–818
43 Widdicombe, J.M. and Brading, A.F. (1980) Pflugers Arch. 286, 35–37