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EFFECT OF OUABAIN UPON DIURETIC-SENSITIVE K^+ TRANSPORT IN CULTURED CELLS

EVIDENCE FOR SEPARATE MODES OF OPERATION OF THE TRANSPORTER

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(1) Unidirectional K^+ (^{86}Rb) influx and efflux were measured in subconfluent layers of MDCK renal epithelial cells and HeLa carcinoma cells. (2) In both MDCK and HeLa cells, the furosemide-inhibitable and chloride-dependent component of K^+ influx/efflux was stimulated 2-fold by a 30 min incubation in $1 \cdot 10^{-3}$ M ouabain. (3) Measurements of net K^+ loss and Na^+ gain in ouabain-treated cells at 1 h failed to show any diuretic sensitive component, confirming the exchange character of the diuretic-sensitive fluxes. (4) Prolonged incubations for 2.5 h in ouabain revealed a furosemide- and anion-dependent K^+ (Cl^-) outward net flux uncoupled from net Na^+ movement. Net K^+ (Cl^-) outward flux was half-maximally inhibited by 2 μM furosemide. (5) After 2.5 h ouabain treatment, the anion and cation dependence of the diuretic-sensitive K^+ influx/efflux were essentially unchanged when compared to untreated controls.

Introduction

There is now considerable evidence to suggest that in a variety of cell types, a large proportion of the ouabain-insensitive cation fluxes is mediated by an electroneutral cotransport with Cl^- (i.e., $Na^+ + Cl^-$, $K^+ + Cl^-$ or $Na^+ + K^+ + 2Cl^-$). This is sensitive to inhibition by loop diuretics such as furosemide and bumetanide [1–8] and is unrelated to passive fluxes which may be mediated by the (Na^+-K^+)-pump [9]. Measurements of the coupling between diuretic-sensitive net ion flows in ascites tumour cells and in red cells from avian species, indicate that this is extremely tight, with stoichiometries of $1Na^+$ to $1K^+$ ion transported [10] or $1Na^+$ to $1K^+$ to $2Cl^-$ ions transported [3,6]. In principle, it has also been demonstrated that the overall direction of diuretic-sensitive net transport is dependent upon the overall sum of transmembrane chemical activity gradients for Na^+, K^+ and Cl^- [6].

We have previously demonstrated, by measurements of isotopic K^+(^{86}Rb) influx in HeLa cells and MDCK epithelial cells, a ouabain-insensitive but diuretic-inhibited flux component possessing many features consistent with $Na^+ + K^+ + 2Cl^-$ cotransport [4,11]. Indeed, for MDCK cells, measurements of diuretic-sensitive isotopic Na^+, K^+ and Cl^- influxes are entirely consistent with net measurements in other systems [7,8]. Dunham et al. [1] have observed, however, that the diuretic sensitive Na^+ influx in human red cells was significantly lower than the diuretic-sensitive K^+ influx, or efflux, suggesting the existence of Na-coupled and Na-independent pathways for diuretic-sensitive K^+ transfer (exchange). Stimulation of diuretic and anion-sensitive K^+ exchanges fluxes has been described as a secondary consequence of (Na^+-K^+)-pump inhibition in ascites cells [5] and 3T3 cells [12]. However, net cation fluxes under these conditions have not been measured [12], and the question of the degree of coupling between

fluxes (Na^+, K^+ and Cl^-) under these conditions remains unexplored. In the present paper, we describe measurements on cultured HeLa and MDCK cells which provide evidence for stimulation of an ouabain-insensitive K^+ exchange flux sensitive to diuretic inhibition and to replacement of Cl^- by NO_3^-, similar to results reported for 3T3 cells and ascites tumour cells [5,12]. In addition, we have examined coupling between net cation movements in ouabain-treated cells; this reveals a furosemide-sensitive loss of cellular K^+ (Cl^-), sensitive to medium Cl^-, replacement by NO_3^-, but uncoupled from net Na^+ movement.

Materials and Methods

Cell culture

HeLa cells. HeLa cells were grown in Eagle's basal medium supplemented with 10% new-born calf serum [11]. For experimental purposes, monolayers of cells were grown to confluency in 60-mm Sterilin plastic-petri dishes (approx. $2 \cdot 10^6$ cells/plate final cell density).

MDCK cells. MDCK cells at 60 serial passages were obtained from Flow Labs (Irvine, U.K.) and maintained in serial culture in Roux flasks in Eagle's minimum essential medium supplemented with non-essential amino acids, 2 mM glutamine, 1 $\mu g/cm^3$ kanamycin antibiotic, 8% (v/v) horse donor serum and 2% (v/v) foetal bovine serum [4]. For experiments cells were grown in 60-mm Sterilin plastic dishes to form subconfluent cell monolayers ($(0.5-1.0) \cdot 10^6$ cells/plate).

Experimental measurements

The techniques used in this study have been described in detail [4,11] and are summarised below. All experiments were at 37°C.

(a) K^+ influx measurement. ^{86}Rb was used as a tracer in all experiments [4,11]. ^{86}Rb influx into cell layers was from a Krebs' solution (composition, see below) supplemented with 1% (v/v) serum and 0.2 $\mu Ci/cm^3$ ^{86}Rb for a 10 min (HeLa) or 5 min (MDCK) period; cell layers were then rinsed (four periods of less than 20 s) in ice-cold Krebs' solution to remove extracellular isotope and trypsinised to form a single 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.

(b) K^+ efflux measurements. Cell layers were preloaded with ^{86}Rb (0.75 $\mu Ci/cm^3$) for 3 h, then briefly rinsed in Krebs' solution to remove extracellular isotope. ^{86}Rb efflux was then determined by successive addition and collection of aliquots of Krebs' solution. The ^{86}Rb content of the cells at the end of the experimental period was determined from aliquots of a cell suspension prepared by trypsinisation (see above).

(c) Estimation of intracellular Na^+ and K^+ contents. Following incubation, cell layers were washed (four periods of less than 20 s each) in ice-cold isotonic sorbitol or choline chloride solution and extracted in double-glass-distilled water for 2 h at 20°C. Na^+ and K^+ were then measured by flame photometry. Cell numbers of identical plates in the same batch were determined using the Coulter Counter (see above).

(d) Estimation of cell volume by 3-O-methylglucose equilibration. Cell volume was measured by a modification of the method of Kletzien et al. [13] using 3-*O*-methyl-D-[^{14}C]glucose as an intracellular space marker. Cell layers were incubated in standard Krebs' solutions with 1 $\mu Ci/cm^3$ of 3-*O*-methyl[^{14}C]glucose for at least 1 h to ensure equilibrium. Cell layers were washed and extracted as in (a) above. Aliquots of cell suspension were then counted for their ^{14}C activity in 10 ml Triton-toluene-Scintol 2 (New England Nuclear) (4.5 : 4.5 : 1, v/v) liquid scintillation cocktail in a Packard Model 3255 liquid scintillation spectrometer.

Solutions

The normal Krebs' solution contained 137 mM NaCl, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.3 mM NaH_2PO_4, 0.3 mM KH_2PO_4, 15 mM Tris, 12 mM HCl and 10 mM glucose (pH 7.4).

A sodium-free medium was obtained by isosmotic replacement of NaCl by choline chloride or LiCl and omitting NaH_2PO_4. A low-Cl^- medium was obtained by isosmotic replacement of NaCl by either $NaNO_3$, NaBr, NaI, sodium isethionate or

Na_2SO_4 (+mannitol to maintain isotonicity). All serum used in Na-free or low-Cl^- media was dialysed overnight against two changes of 50 vol. distilled water.

Materials

^{86}Rb and 3-*O*-methyl[^{14}C]glucose were from Amersham International (Amersham, U.K.). Tissue culture supplies were obtained from Flow Laboratories (Irvine, U.K.) and Gibco-Biocult Ltd. (Paisley, U.K.). Ouabain was from the Sigma Chemical Co. (Poole U.K.). Furosemide was a gift from Dr S. Dombey of Hoechst Pharmaceuticals (Hounslow, U.K.).

Statistical methods

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

Results

Stimulation of furosemide-sensitive K^+ exchange by ouabain

K^+ exchanges in HeLa and MDCK cells consists of three main components: an ouabain-sensitive influx mediated by the Na^+ pump, an ouabain-insensitive but furosemide-sensitive K^+-exchange flux mediated by a cotransport system, and a residual leak flux [4,11]. Incubation in 1 mM ouabain causes a progressive increase in K^+ efflux (measured by ^{86}Rb [4,11]) (Fig. 1) which is maximal after 18 min incubation. (Na^+-K^+)-pump inhibition under these experimental conditions ($1 \cdot 10^{-3}$ M ouabain, 5.4 mM external K^+ concentration) is judged to be virtually instantaneous [14,15]. The increase in K^+ efflux resulting from ouabain treatment is not only observed in cultured cells but has also been described for certain epithelia [16] where the result has been interpreted as evidence of K^+ recapture by the Na^+-pump in occluded epithelial lateral spaces [16]. That this interpretation is incorrect for the present data is shown in Table I, where the stimulation of ^{86}Rb efflux is shown to be sensitive to the diuretic, furosemide (0.1 mM), and to medium Cl^- replacement by NO_3^- in both the epithelial cell-line, MDCK, and in HeLa cells (compare columns 5 and 6) with columns 3 and 4, respectively). These results are, therefore, consistent with these observed in ascites cells [5,17] and in SV-3T3 cells [12], indicating that this phenomenon may be more general than previously considered [12].

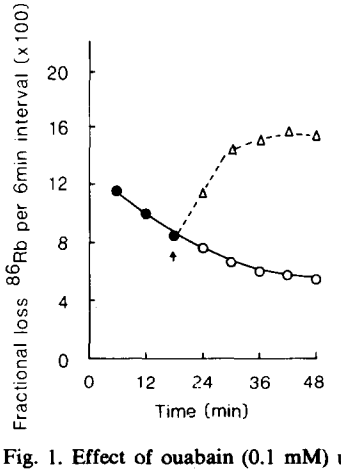

Fig. 1. Effect of ouabain (0.1 mM) upon fractional ^{86}Rb loss from sub-confluent MDCK cells. ●, controls $n = 8$; ○, controls $n = 4$, △, +0.1 mM ouabain.

Previously we reported that the effects of ouabain and of furosemide were approximately additive upon K^+ influx [4,11] in HeLa and MDCK cells; occasionally, however, we observe that the diuretic-sensitive flux measured in the presence of ouabain exceeds that measured in its absence by 20–30% [18]. Such variable findings are due to the time-dependent stimulation of the diuretic-sensitive flux; thus if cells are preincubated in media in the presence and absence of ouabain (Table II) the apparent ouabain-sensitive flux is reduced due to stimulation of the ouabain-insensitive flux. That this is due to increased activity of the furosemide-sensitive component is shown in Table III, where a prolonged flux measurement period of 10 min in the presence of ouabain (compared to the usual 5 min period) results in an apparent increase in K^+ influx in ouabain-containing media. Moreover, cotransport flux is greater when estimated in ouabain-containing media using furosemide, or NO_3^- media for either 10 min or 30 min incubations as compared to estimates of cotransport flux in the absence of ouabain (Table III).

The increased influx and efflux of K^+ mediated by the cotransport system in the presence of

TABLE I

THE EFFECT OF FUROSEMIDE ($1 \cdot 10^{-4}$ M) AND OF Cl^- REPLACEMENT BY NO_3^- UPON THE STIMULATION OF FRACTIONAL ^{86}Rb EFFLUX IN BOTH HeLa AND MDCK CELLS

Results are expressed as the ratio of fractional effluxes (T_{20}/T_0) at times immediately prior to, and 20 min subsequent to change of experimental media (for HeLa cells, this ratio was T_{30}/T_0). All results are expressed as the mean ± S.E. The numbers in parentheses represent the number of separate experimental determinations made.

Cell type	Ratio of fractional effluxes T_x/T_0					
	Control	10^{-3} M Ouabain	10^{-4} Furosemide	NO_3^- media	10^{-3} M Ouabain + 10^{-4} M furosemide	10^{-3} M ouabain in NO_3^- media
MDCK	0.78 ± 0.04 (10)	1.55 ± 0.06 [c] (10)	0.41 ± 0.05 [c] (8)	0.35 ± 0.06 [c] (8)	0.58 ± 0.08 [a,x] (5)	0.42 ± 0.12 [b,x] (5)
HeLa	0.69 ± 0.01 (3)	1.61 ± 0.07 [c] (3)	0.36 ± 0.03 [c] (3)	0.53 ± 0.02 [c] (3)	0.20 ± 0.01 [c,x] (3)	0.20 ± 0.01 [c,x] (3)

Significantly different from control values:
[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$.
Significantly different from ouabain-treated values:
[x] $P < 0.001$.

ouabain indicates that this represents primarily an exchange flux of K^+.

Effect of furosemide upon dissipation of normal intracellular ion gradients in the presence of ouabain

Fig. 2 shows the time-dependence of the effect of furosemide upon intracellular Na and K contents in the presence of ouabain in both MDCK and HeLa cells. For both MDCK and HeLa, furosemide, in the absence of ouabain, has no discernible effect on intracellular monovalent ca-

TABLE II

EFFECT OF PREINCUBATION IN KREBS' SOLUTION IN THE PRESENCE AND ABSENCE OF 10^{-3} M OUABAIN UPON ^{86}Rb INFLUX INTO MDCK CELLS

Total influx time was 5 min. Apparent ouabain-sensitive influx is the difference between time-matched controls ± ouabain. All results are the mean ± S.E. of at least three separate determinations.

Preincubation time (min)	Apparent ouabain sensitive influx (nmol/10^6 cells per min)	Ouabain-insensitive K^+ influx as % of total influx
0	4.10 ± 0.08	64
15	1.32 ± 0.06 [a]	86
30	0.14 ± 0.04 [a]	94

[a] Significantly different from zero preincubation time, $P < 0.001$.

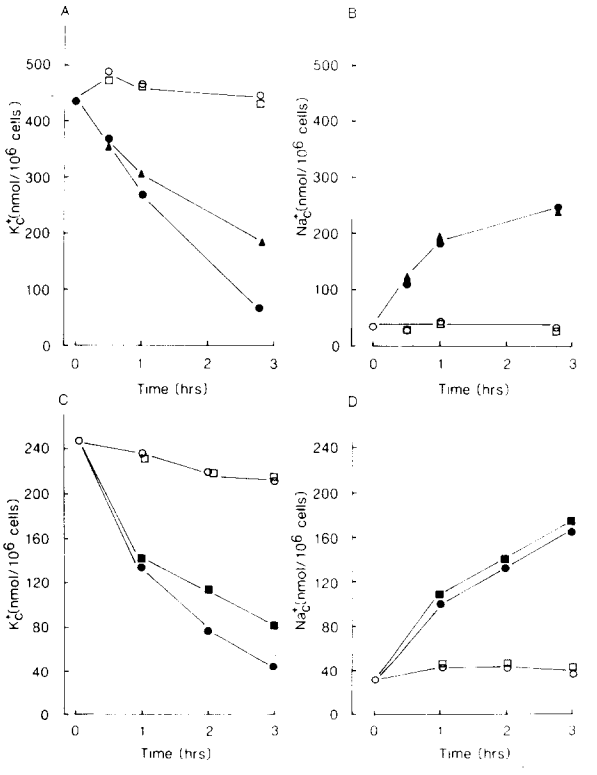

Fig. 2. Dissipation of normal intracellular K^+ (A) and Na^+ (B) contents in HeLa cells or in MDCK (C, K^+; D, Na^+) in media containing 10^{-3} M ouabain (●), or 10^{-3} M ouabain together with 10^{-4} M furosemide (▲, ■) Control data are for normal Krebs' soln (○) or Krebs' soln. containing 10^{-4} M furosemide (□).

TABLE III

EFFECT OF FUROSEMIDE AND REPLACEMENT OF MEDIUM Cl^- BY NO_3^- UPON ^{86}Rb INFLUXES INTO MDCK CELLS AFTER PREINCUBATION IN OUABAIN-FREE OR OUABAIN-CONTAINING MEDIA FOR 30 MIN

Influxes were determined over a 10-min incubation period. Results are the mean ± S.E. of four determinations.

Preincubation	^{86}Rb influx: total flux (mmol/l cell H_2O per min)		(Na-K)-pump flux	Cotransport flux
Krebs' solution	Krebs' soln.	4.31 ± 0.31	–	–
	10^{-3} M ouabain	4.96 ± 0.34 [ns]	-0.65 ± 0.36 (Krebs'-ouabain)	–
	$+10^{-4}$ M furosemide	2.22 ± 0.24 [a]	–	2.09 ± 0.33 (Krebs'-furosemide)
	NO_3^- Krebs' soln.	2.16 ± 0.09 [a]	–	1.97 ± 0.32 (Krebs'-NO_3^-)
	10^{-3} M ouabain $+10^{-4}$ M furosemide	0.59 ± 0.02 [b]	1.63 ± 0.25 (furosemide-furosemide/ ouabain)	4.37 ± 0.35 (ouabain-ouabain /furosemide)
	NO_3^- Krebs' soln. $+10^{-4}$ M furosemide	0.24 ± 0.02 [b]	1.92 ± 0.11 (NO_3^--NO_3^- / ouabain)	4.72 ± 0.35 (ouabain-NO_3^- / ouabain)
Krebs' soln. + ouabain	10^{-3} M ouabain	3.78 ± 0.23 [ns]	–	
	$+10^{-3}$ M ouabain $+10^{-4}$ M furosemide	0.35 ± 0.01 [b]	–	3.43 ± 0.24 (ouabain-ouabain/ furosemide)
	$+10^{-3}$ M ouabain + NO_3^--Krebs' soln.	0.47 ± 0.01 [b]	–	3.31 ± 0.24 (ouabain-ouabain/NO_3^-)

Significantly different from incubation in Krebs' soln.:
[ns] = not significant.
[a] $P < 0.01$.
[b] $P < 0.001$.

tion contents. This agrees with previous observations [4,11]. Incubation in 1 mM ouabain causes a progressive decrease in K^+ contents and an increase in Na^+ cell content. The effect of furosemide on both HeLa and MDCK cells is to retard the net outward loss of intracellular K^+ (Fig. 2A, C). The increased bidirectional K^+ flux seen in the presence of ouabain does not result in an initial net increase in K^+ loss (see above). An important observation is that the net loss of K^+ which is sensitive to furosemide inhibition noted at 3 h is dissociated from a retardation of net Na^+ gain sensitive to furosemide (Fig. 2, Table IV). Such a retardation would be predicted upon the basis of a tightly coupled Na + K + 2Cl (or Na + K) cotransport mediating net outward transport [3,6]. Conversely, the diuretic-sensitive Na-K exchange in ouabain-containing media, observed by Jayme et al. [19] in mouse fibroblasts, is absent.

We measured changes in cell volume directly so as to determine whether the furosemide-inhibitable net loss of K^+, seen in ouabain-poisoned cells, was due to the operation of a K^+ channel separate from the normal cotransport system and instead stimulated by cell swelling. Using the method of Kletzien et al. [13], in which 3-*O*-methyl[^{14}C]glucose is used as an intracellular space marker, the volume of control cells was determined; in MDCK cells, the control cell volume was 1.93 ± 0.10 $\mu l/10^6$ cells; this was reduced to 1.49 ± 0.03

TABLE IV

EFFECT OF FUROSEMIDE

Effect of furosemide in normal Krebs' soln., Cl^--depleted NO_3^- media, and in Na^+-depleted choline media upon the ouabain-dependent changes in intracellular Na^+ and K^+ contents (ΔK^+c and ΔNa^+c) in HeLa cells and in MDCK cells measured at 3 h. Data for MDCK cells in choline media are omitted due to nonspecific effects of long-term choline incubations noted in control data on K^+ contents. All values in nmol/10^6 cells. Figures in parentheses represent the number of separate determinations.

	HeLa cells		MDCK cells	
	$-\Delta$[K]c	$+\Delta$[Na]c	$-\Delta$[K]c	$+\Delta$[Na]c
Normal Krebs' soln.				
ouabain 10^{-3} M	242 ± 11 (3)	163 ± 6 (3)	166 ± 5 (10)	115 ± 6 (10)
ouabain 10^{-3} + furosemide 10^{-4} M	150 ± 6 [b] (5)	165 ± 9 [ns] (3)	136 ± 5 [b] (10)	127 ± 7 [ns] (10)
NO_3^- media				
ouabain 10^{-3} M	94 ± 4 (3)	120 ± 8 (3)	134 ± 4 (4)	116 ± 5 (4)
ouabain 10^{-3} M + furosemide 10^{-4} M	107 ± 6 [ns] (3)	128 ± 12 [ns] (3)	134 ± 6 [ns] (4)	125 ± 9 [ns] (4)
Choline media				
ouabain 10^{-3} M	136 ± 7	–	–	–
ouabain 10^{-3} M + furosemide 10^{-4} M	68 ± 12 [a] (3)			

Significantly different from media minus furosemide

[ns] = not significant.

[a] $P < 0.01$.

[b] $P < 0.001$.

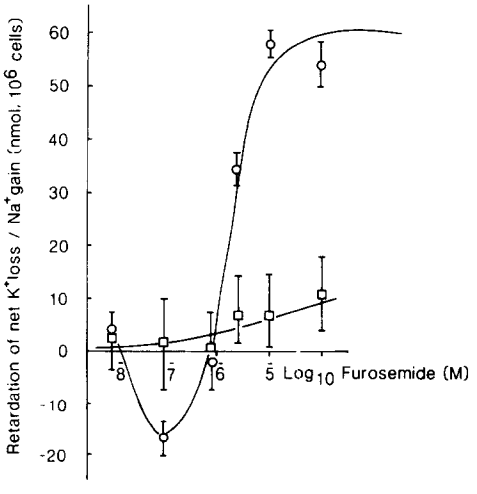

Fig. 3. Dose-response curve for furosemide action upon retardation of K^+ loss in (○) ouabain-containing media in HeLa cells. Furosemide is without effect upon net Na^+ gain (□) at all furosemide concentrations tested. Each point is the mean ± S.E. of at least five separate determinations. Solid lines are drawn by eye.

$\mu l/10^6$ cells after 3 h ouabain treatment. Equivalent data were obtained for HeLa cells (control volume, 0.72 ± 0.05 $\mu l/10^6$ cells after 3 h ouabain treatment, 0.65 ± 0.06 $\mu l/10^6$ cells). Since the 3-*O*-methyl-D-glucose technique does not have sufficient time resolution to allow measurements of rapid volume changes, the effect of ouabain on cell volume was also determined by electronic cell sizing (Coulter) of a suspension of HeLa cells. No transient cell swelling was observed in the presence of ouabain after incubations for up to 2 h (control cell volume minus ouabain = 2933 ± 76 μm^3 (S.D.); after 10, 20, 60, and 120 min in the presence of ouabain the cell volumes were 2977 ± 80, 2928 ± 32, 2855 ± 26 and 2699 ± 7 μm^3, respectively). The cell swelling which occurs in both HeLa cells and in MDCK cells on exposure to hypotonic medium does induce a net KCl loss, similar to that seen in human red cells [24], but this is insensitive to furosemide inhibition and is therefore separate from the cotransport system (Simmons, N.L., unpublished data).

The furosemide-sensitive loss of K^+ is itself

sensitive to the anion composition of the bathing medium; replacement of medium Cl^- by the permeant anion NO_3^- [8] abolishes the furosemide-dependent retardation of net K^+ loss (Table IV). For a series of anion substituents, tested in HeLa cells, it was found that a furosemide-sensitive K^+ loss was observed only in Cl^-- and Br^--containing media, whereas NO_3^--, thiocyanate- and SO_4^{2-}-containing media were unable to support the furosemide-sensitive K^+ loss. These findings are thus similar to the anion dependency of ^{86}Rb exchange flux noted previously in HeLa cells [11].

In contrast to the results obtained when changing the anion composition of the medium, replacement of medium Na^+ by choline has no effect upon the furosemide-dependent loss of K^+, even though intracellular Na^+ falls to non-detectable levels (Table IV).

Fig. 3 shows that the action of furosemide on net K^+ loss in the presence of ouabain is dose-dependent, half-maximal retardation being observed at 2 μM. An acceleration of net K^+ loss is observed at 10^{-7} M furosemide. The dose of furosemide required to inhibit net K^+ loss is similar to that observed for furosemide inhibition of ^{86}Rb influx [11] in HeLa cells. Table V demonstrates that the pharmacological sensitivity of the net K^+ los sis also similar to inhibition of ^{86}Rb influx, K^+ loss being retarded by furosemide and piretanide but unaffected by the potent anion transport inhibitor, SITS [11]. Moreover, a Gardos-type effect [23] may be discounted by the fact that quinine is unable to retard K^+ loss (Table V).

TABLE V

PHARMACOLOGICAL SENSITIVITY OF THE DIURETIC-SENSITIVE NET K^+ LOSS FROM OUABAIN-TREATED HeLa CELLS

Results are expressed as the mean ± S.E.

Treatment	n	Retardation of K^+ loss compared to ouabain-treated controls (nequiv/10^6 cells)
10^{-4} M furosemide	4	54.4 ± 5.4
10^{-4} M piretanide	4	64.9 ± 6.4 ^{ns}
10^{-5} M SITS	4	−3.8 ± 4.9 ^{a,ns}
10^{-4} SITS	4	−13.9 ± 3.5 ^{a,x}
10^{-4} M SITS + 10^{-4} M furosemide	4	61.2 ± 5.8 ^{ns,y}
10^{-4} M quinine	4	10.1 ± 7.5 ^{a,ns}
10^{-4} M quinine + 10^{-4} M furosemide	4	54.4 + 6.8 ^{ns,y}

Significantly different from furosemide-containing media:
^{ns} = not significant.
^{a} $P < 0.001$.
Significantly different from zero retardation:
^{ns} = not significant.
^{x} = $P < 0.05$.

Inhibition of the (Na^+-K^+)-pump in low K^+-containing media

In order to demonstrate that the diuretic-sensitive K^+ loss, uncoupled from Na^+, is not the result of a direct pharmacological action of ouabain, (Na^+-K^+)-pump activity was inhibited by a reduction in the external bathing medium K^+ [20]. HeLa cells in reduced K^+ media (i.e., 1.0 mM K^+) lose intracellular K^+ and gain Na^+ (Table V). As with ouabain inhibition of (Na^+-K^+)-pump activity (see above), 0.1 mM furosemide retards net K^+ loss in reduced medium K^+, without effecting net cellular Na^+ uptake (Table VI).

TABLE VI

EFFECT OF LOW-K^+-CONTAINING KREBS' SOLUTION UPON INTRACELLULAR Na^+ AND K^+ CONTENTS IN THE PRESENCE AND ABSENCE OF FUROSEMIDE

Monolayers of HeLa cells were incubated for 2.5 h at 37°C. Each datum is the mean ± S.D. of four observations.

Medium K^+ (mM)	Furosemide (10^{-4} M)	Intracellular cation contents (nmol/10^6 cells)	
		K_c^+	Na_c^+
4.95	−	326 ± 26	19 ± 2
	+	327 ± 21 ^{ns}	20 ± 2 ^{ns}
1.04	−	288 ± 13	26 ± 3
	+	306 ± 18 ^{ns}	28 ± 4 ^{ns}
0.54	−	269 ± 29	56 ± 5
	+	334 ± 8 ^{b}	64 ± 3 ^{a}
0.29	−	158 ± 5	83 ± 3
	+	255 ± 11 ^{c}	89 ± 10 ^{ns}
0.14	−	88 ± 3	117 ± 12
	+	168 ± 22 ^{b}	144 ± 24 ^{ns}

Significant effect of furosemide upon ion contents:
^{ns} = not significant.
^{a} $P < 0.05$.
^{b} $P < 0.01$.
^{c} $P < 0.001$.

Effect of variation in the external [K^+] upon net K^+ loss via the diuretic-sensitive pathway

For a tightly coupled $Na^+ + K^+$ cotransport, sensitive to furosemide, variation in the net chemical gradient for K^+ should produce quantitatively similar effects on both net Na^+ and K^+ transport [3,6]. It is clear that, whereas enhancement of the outward chemical gradient for K^+ (by a reduction of the external K^+ concentration) markedly increases the furosemide-sensitive net loss of K^+ in a linear fashion (Fig. 4), there is no stoichiometric reduction in net Na^+ gain. Indeed, in the presence of furosemide, net Na^+ gain is independent of the magnitude of the outwardly directed K^+ gradient (Fig. 4). These data provide, therefore, further support for the notion that the diuretic-sensitive outward K^+ (Cl^-) transport is uncoupled from net Na^+ movement.

Diuretic-sensitive K^+(^{86}Rb) influx / efflux in ouabain-treated cells: effect of medium cation and anion composition

The demonstration of outward diuretic-sensitive K^+ transport uncoupled from Na^+ poses the question as to whether this represents the normal mode for outward transport, or whether ouabain treatment alters the functioning of the diuretic sensitive pathway via secondary effects upon cellular cations (e.g., Na^+, K^+, Ca^{2+}, Mg^{2+}). In untreated cells, we have previously demonstrated the dependence of furosemide-sensitive K^+ influx upon medium cation and anion composition [4,11]. Table VII demonstrates that the dependence of ^{86}Rb influx upon medium Na^+ (choline as substituent) and upon Cl^- (NO_3^- as substituent) is maintained. Furthermore, the ability of Li^+ and of Br^- to substitute partially for Na^+ and Cl^-, respectively, is identical to the behaviour of the furosemide-sen-

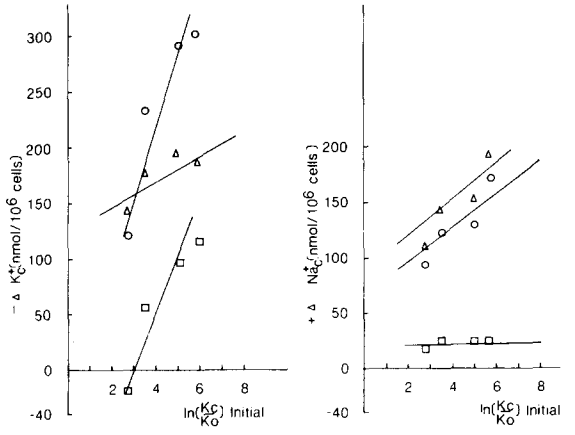

Fig. 4. Effect of the initial K^+ gradient (plotted as $\ln K_o^+/K_c^+$ where K_o^+ = external K^+ concentration and K_c^+ = initial cellular K^+ concentration) upon the changes in intracellular Na and K contents (loss of intracellular K^+, $-\Delta K_c^+$ or gain in intracellular Na^+, $+\Delta Na_c^+$) in ouabain containing media for HeLa cells. (○) changes in the presence of 1 mM ouabain. (△) changes in the presence of 1 mM ouabain and 10^{-4} M furosemide. (□) furosemide-dependent changes. $n = 4$ for all data points and standard error of the mean was 10% of mean values for all data. Lines are fitted by eye.

TABLE VII

EFFECT OF MEDIUM CATION (A) AND (B) COMPOSITION UPON ^{86}Rb INFLUX INTO OUABAIN-PRETREATED HeLa CELLS

Cells were treated with ouabain for 2.5 h in the normal Krebs' solution. For details of solutions see methods. All data are the mean ± S.E. of at least four determinations.

Salt	Furosemide (0.1 mM)	^{86}Rb influx (mmol/l cell water per min)	Furosemide-sensitive influx (mmol/l cell water per min)
A. NaCl	−	0.58 ± 0.02	0.41 ± 0.03
	+	0.17 ± 0.02	
LiCl	−	0.37 ± 0.02 [a]	0.13 ± 0.02 [a]
		0.24 ± 0.02 [ns]	
Choline chloride	−	0.38 ± 0.03 [a]	−0.03 ± 0.03 [b]
	+	0.41 ± 0.03 [b]	
B. NaCl	−	0.85 ± 0.02	0.69 ± 0.03
	+	0.16 ± 0.01	
NaBr	−	0.81 ± 0.05 [ns]	0.64 ± 0.05 [ns]
	+	0.16 ± 0.02 [ns]	
NaI	−	0.29 ± 0.02 [b]	0.11 ± 0.04 [b]
	+	0.18 ± 0.02 [ns]	
$NaNO_3$	−	0.17 ± 0.04 [b]	0.06 ± 0.03 [b]
		0.11 ± 0.02 [ns]	
Sodium isethionate	−	0.12 ± 0.02 [b]	0.02 ± 0.02 [b]
	+	0.11 ± 0.01 [ns]	
Na_2SO_4	−	0.05 ± 0.02 [b]	−0.01 ± 0.02 [b]
	+	0.06 ± 0.02 [a]	

Significantly different from NaCl media:
[ns] = not significant.
[a] $P < 0.01$.
[b] $P < 0.001$.

TABLE VIII

THE EFFECT OF FUROSEMIDE ($1 \cdot 10^{-4}$ M) Cl^- REPLACEMENT BY NO_3^-, AND OF Na^+ REPLACEMENT BY CHOLINE IN THE EXTERNAL MEDIUM UPON FRACTIONAL ^{86}Rb EFFLUX IN MDCK CELLS

Results are expressed as the ratio of fractional effluxes (T_{15}/T_0) at times immediately prior to, and 15 min subsequent to, change of experimental media. All results are expressed as the mean ± S.E. of at least three determinations. During the 20 min incubation in Na^+-free media, intracellular Na^+ levels fall to 5% of initial values.

Treatment	Ratio of fractional effluxes T_{15}/T_0				
	Control	Furosemide	NO_3^- media	$Choline^+$ media	choline media + $1 \cdot 10^{-4}$ M furosemide
Control	0.79 ± 0.10	0.20 ± 0.06 [a]	0.18 ± 0.0	0.84 ± 0.01	0.72 ± 0.14 [ns]
Ouabain-preincubated (2.5 h)	0.99 ± 0.02	0.32 ± 0.01 [a]	0.39 ± 0.14	0.35 ± 0.07	0.23 ± 0.02 [ns]

Significant effect of furosemide:
[ns] = not significant.
[a] $P < 0.001$.

sitive ^{86}Rb influx pathway of untreated cells [11]. The sensitivity of the diuretic-inhibitable ^{86}Rb influx pathway to replacement of medium Na^+ by choline is also observed at intermediate times following ouabain inhibition. Thus after a preincubation in ouabain-containing media for 15 min the furosemide-sensitive ($1 \cdot 10^{-4}$ M) component of K^+ influx (0.93 ± 0.24 (S.E.) mmol/cell water per min) observed in Na^+-containing media is abolished in Na^+-free choline media (−0.01 ± 0.13 (S.E.) mmol/l cell water per min). The ^{86}Rb efflux also shows a similar dependence upon medium Cl^- and Na^+; after a 20 min incubation in the presence of a zero Na^+ medium ($choline^+$ as substituent cation) no furosemide-sensitive component is observed in control or ouabain-pretreated cells (Table VIII). This result demonstrates that the diuretic-sensitive pathway is predominantly one of K-K exchange and that this exists concurrently with a diuretic-sensitive Na^+-uncoupled outward K^+ (Cl^-) net flux mechanism.

Discussion

In common with a variety of other cells (Erlich ascites cells [3,5] erythrocytes [1,2,10]), cultured HeLa cells and MDCK renal epithelial cells possess a diuretic-sensitive K^+ influx pathway which comprises the greater proportion of the ouabain-insensitive K^+ influx [4,11]. Several features of the diuretic-sensitive K^+ influx pathway, including its dependence upon medium Na^+ and Cl^- ions, are consistent with the existence of an electroneutral cotransport system for $Na^+ + K^+ + 2Cl^-$ [4,11].

For cells in which the (Na^+-K^+)-pump remains functional, application of 0.1 mM furosemide, a concentration known to inhibit the cotransport mechanism completely, is without effect on intracellular Na^+ and K^+ concentrations, suggesting that the diuretic-sensitive cotransport is involved solely in exchange fluxes of Na^+ K^+ and Cl^-. (Na^+-K^+)-pump inhibition by ouabain leads to a 2–3-fold increase in diuretic-sensitive K^+ fluxes, and since initial changes in internal Na^+ and K^+ concentrations are unaffected by diuretic application, these diuretic-sensitive K^+ fluxes must be primarily exchange fluxes.

For avian erythrocytes, where the properties of the ouabain-insensitive but diuretic-inhibitable transport system have been extensively studied [16], the absence of net cation transfer via the furosemide-sensitive pathway would indicate that the net sum of the transmembrane chemical activity gradients for participant ions in this tightly coupled system (Na^+, K^+ and Cl^-) is zero [6]. Experimental imposition of a sufficiently large ion gradient for a single participant ion, e.g., K^+, in ouabain-poisoned avian cells will drive the net flux of the obligatory coupled ions (Na^+ and Cl^-) with a stoichiometry of $1Na^+$, $1K^+$, $2Cl^-$ [6]. The dissipation of the normal cation gradients of both HeLa and MDCK cells which occurs after ouabain

TABLE IX

COMPARISON OF OUTWARD K^+ TRANSPORT SENSITIVE TO DIURETIC INHIBITION WITH DIURETIC-SENSITIVE ^{86}Rb INFLUX (EXCHANGE) [11]

		Outward K^+ (Cl^-) (net) transport	^{86}Rb influx (exchange)
1	Sensitivity to furosemide K_i (μM)	2.0	3–5
2	Sensitivity of the anion transport inhibitor SITS	insensitive	insensitive
3	Anion dependence	$Cl^- \geqslant Br^- \gg NO_3^-, SO_4^{2-}$	$Cl^- \gg Br^- \gg NO_3^-, SO_4^{2-}$
4	Na^+ dependence	–	$Na^+ > Li^+ \gg$ choline$^+$

treatment provides no evidence for linked diuretic-sensitive net fluxes of Na and K, nor for diuretic-sensitive Na-K exchange [19]. A net diuretic-sensitive K^+ (Cl^-) efflux is noted, but this occurs only at lowered internal cellular K^+ contents and is clearly dissociated from net Na^+ gain by the cells. Table IX compares the properties of the outward K^+ (Cl^-) transport demonstrated in this paper with the isotopically measured (^{86}Rb) K-K exchange. The striking similarities in the affinity for furosemide inhibition, insensitivity to the anion transport inhibitor SITS and the anion dependence of net K^+ movements suggest that these are all measures of the operation of a single, highly complex transport system rather than separate transport systems. The possibility that the increased $K^+(Cl^-)$ net loss which is inhibited by furosemide is via a Gardos-type channel may be discounted, since Ca^{2+}-activated K^+ transport in MDCK cells is not inhibited by 0.1 mM furosemide [23]; moreover, 0.1 mM quinine has no effect upon the diuretic-sensitive K^+ (Cl^-) loss (Results). Since the properties of the K-K exchange pathway are unaltered following ouabain treatment, both modes of operation must exist simultaneously (see Fig. 5).

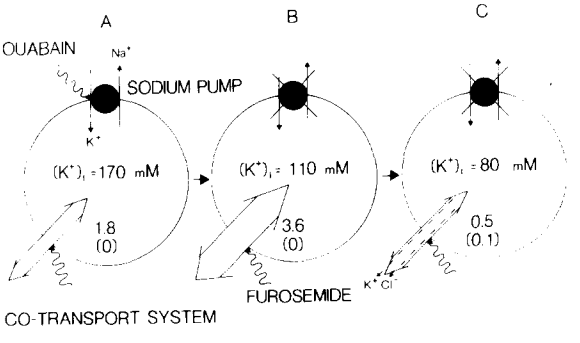

Fig. 5. Schematic drawing showing the changes which occur in the cotransport K^+ flux of HeLa cells in (A) control conditions and (B) following a 30 min or (C) 2 h exposure to 10^{-3} M ouabain. Figures show the K^+ cotransport exchange flux. Figures in brackets show the net outward K(Cl^-) flux which is furosemide sensitive (all fluxes expressed as mmol/l cell water per min).

Although the stoichiometry of diuretic-sensitive K^+/Cl^- net fluxes has not been directly measured, it is likely that this transport is electroneutral, since the anion transport inhibitor SITS is without effect, as would be predicted if membrane Cl^- permeability were significantly reduced.

Coupling between Na^+ and K^+ movements in diuretic-sensitive systems may be extremely tight. In avian erythrocytes and Ehrlich ascites cells the stoichiometry is $1Na^+ : 1K^+ : 2Cl^-$ [6,3]. In other systems, although Cl^- appears to be specifically coupled to cation flux, the stoichiometry between Na^+ and K^+ may vary; for example, in human red cells the anion-dependent, furosemide-sensitive K^+ influx exceeds the anion-dependent diuretic inhibitable Na^+ influx 5-fold [1]. In sheep erythrocytes, the diuretic-sensitive and anion-dependent K^+ fluxes which may be stimulated by volume enlargement [20] and by *N*-ethylmaleimide [21] are independent of Na^+ movements [20–22]. For HeLa and MDCK cells, the mechanism of stimulation of the outward KCl transport and the putative uncoupling from Na^+ movements are unknown, though activation by an increased cell volume may be discounted (see Results).

The stimulation of furosemide-sensitive exchange fluxes of K^+ in ouabain-containing media, demonstrated in this paper, indicates that estimation of ouabain-sensitive flux via the (Na^+-K^+)-pump may be in serious error when long flux-measurement times are used and stimulation of the cotransport system occurs. It is highly recommended that ouabain-sensitive fluxes be determined in NO_3^--containing media rather than Cl^- media in order to eliminate possible effects upon cotransport-mediated fluxes. The mechanism of stimulation of K-K exchange is unknown, but it is most probably a secondary consequence of (Na-K^+)-pump inhibition [12]. The present results exclude the possibility that K-K exchange is stimulated by an increase in cell volume [12].

Although changes in cell volume are known to affect the operation of the cotransport system in a variety of cell types, e.g., turkey erythrocytes [25], ascites cells [26] and lymphocytes [27]. We feel that this cannot account for the stimulation of diuretic-sensitive K-K exchange seen after ouabain treatment. Measurements of net cation balance (Na and K) over the first 30 min of incubation in ouabain do not demonstrate sufficient loss of intracellular solute to cause cell shrinkage and the consequent activation of K-K exchange. Direct measurement of changes in cell volume confirm this view.

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