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Role of Passive Potassium Fluxes in Cell Volume Regulation in Cultured HeLa Cells

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Summary. Cultured HeLa cells behave as ideal osmometers when subjected to hyperosmolar media, and show no volume regulatory behavior. In hypoosmolar solutions, cell swelling is not as great as predicted, and this is due largely to a loss of intracellular KCl. In hyperosmolar solutions there is a stimulation of the ouabain-insensitive but loop diuretic-sensitive $^{86}Rb^+$ (K^+) pathway. Analysis of the K^+, Na^+ and Cl^- dependency of this K^+ flux pathway demonstrates that the increase is principally due to an increase in its maximal velocity (V_{max}). The sensitivity of this pathway to diuretic inhibition is unchanged in hyperosmolar media. Diuretic-sensitive $^{86}Rb^+$ (K^+) efflux stimulated by hypertonicity shows no marked dependence on external K^+. The K^+ loss observed in hypoosmolar media is distinct from the K^+ transport pathway stimulated by hyperosmolar media on the basis of its sensitivity to furosemide and anion dependence.

Key Words HeLa cells · cell volume · K^+ transport · loop diuretic

Introduction

We have previously demonstrated that a large proportion of the ouabain-insensitive K^+ influx and efflux in cultured HeLa cells is mediated by a loop diuretic-sensitive pathway [2] that shares many characteristics with the loop diuretic-sensitive $Na^+ + K^+ + Cl^-$ "cotransport" system described for other cells, notably avian erythrocytes [23, 38–40] and Ehrlich ascites cells [17]. The importance of this transport system to the overall physiological functioning of the cells in which it is present is still uncertain.

Several groups of workers have demonstrated that, in certain cell types, the diuretic-sensitive "cotransport" system(s) is important in cell volume regulatory behavior, particularly when cells are exposed to hypertonic media [16, 24, 25, 38–40, 43]. In hypertonic conditions the $Na^+ + K^+ + Cl^-$ "cotransport" system can mediate a net salt uptake, allowing cell swelling. Net ion movement is, however, dependent upon the net cation gradient; therefore, under certain circumstances, diuretic-sensitive net salt loss, and thus cell shrinkage, may be observed [24, 38]. This behavior of the "cotransport" system may explain earlier observations of cell volume regulation via cardiac-glycoside insensitive "Na pumps" [34]. Direct measurement of the volume sensitivity of outward furosemide-sensitive $Na^+ + K^+$ "cotransport" into Na^+- and K^+-free media in human red cells, however, has failed to show a volume dependence, other than an uncoupling of Na^+ from furosemide-sensitive K^+ efflux [1].

Cell volume regulation may also be mediated via loop-diuretic insensitive pathways; exposure of cells to hypotonic media often results in a specific increase in passive membrane K^+ permeability which is associated with a net loss of intracellular KCl and subsequent cell shrinkage (volume-regulatory decrease) [5, 9, 10, 24, 35, 41]. Since membrane K^+ conductance may be limited by anion permeability, separate conductive ion channels for both K^+ and Cl^- may be stimulated by cellular swelling [18, 20]. The involvement of diuretic-sensitive transport in volume-regulatory decrease may not be entirely excluded, since in some cells (e.g. sheep red cells, Ehrlich ascites cells, and red cells of the oyster toadfish) exposure to hypotonic media stimulates a furosemide-sensitive and anion-dependent KCl transport [14, 15, 30, 42]. The effect of furosemide upon swelling-induced KCl transport in sheep red cells is of lower affinity than upon "cotransport," suggesting separate molecular entities [15]. Functional relationships exist between Cl^--dependent transport systems; thus volume-sensitive KCl transport and the KCl transport stimulated by N-ethylmaleimide in sheep cells [33] are inhibited by anti-L antibody [15]. Lauf [31] has recently indicated the similarity between NEM-stimulated KCl transport in sheep red cells and the $Na^+ + K^+ + Cl^-$

"cotransport" in other cells. Circumstantial evidence also points to a relationship between KCl transport induced by cell swelling and $Na^+ + K^+ + Cl^-$ "cotransport" in avian red cells, where stimulation of "cotransport" activity by catecholamines is accompanied by disappearance of the swelling-induced KCl transport [22].

The purpose of the present investigation was to define more clearly the volume-regulatory behavior of cultured human carcinoma (HeLa) cells, and in particular to study the involvement of the previously described diuretic-sensitive potassium flux pathway in these cells [2] when cells are exposed to either hypotonic or hypertonic media. Some of the present data has been presented in abstract form [4].

Materials and Methods

Cell Culture

HeLa cells were grown in Eagles basal medium supplemented with 10% new-born calf serum. For experimental purposes, monolayer cultures of cells were grown to confluency in 60 mm 'Nunc' plastic petri-dishes (cell density of 2×10^6 cells per dish).

Solutions

The basic Krebs solution used consisted of (mM): 137 NaCl, 5.4 KCl, 2.8 $CaCl_2$, 1.2 $MgSO_4$, 0.3 Na_2HPO_4, 0.3 K_2HPO_4, 14 Tris, 12 HCl, 5.0 glucose, pH 7.4 at 37°C, supplemented with 1% new-born calf serum. In some experiments a hypotonic solution was made by diluting Krebs solution with distilled water up to 3 parts Krebs : 1 part distilled water. More usually, and for all flux experiments, the basic Krebs solution was made hypotonic with just 85 mM NaCl (final osmolality 211 mosmol/kg) and osmolality was then increased by the addition of mannitol (unless otherwise stated in the figure legends). In some experiments the Na^+, K^+ and Cl^- concentrations of the Krebs solution were varied. For NaCl, equimolar substitutions were made with choline Cl or *n*-methyl-D-glucamine, titrated with HCl; for KCl no substitute cation was used, mannitol being added to maintain isosmolar conditions. To substitute Cl^-, Cl^--free Krebs solution was mixed with the appropriate amount of basic Krebs solution, Cl^--free Krebs solution being made by replacing the Cl^- with the appropriate NO_3^-, isethionate$^-$, gluconate$^-$ salts. All serum used in these experiments was dialyzed against ×40 vol distilled water for at least 24 hr. Osmolalities of experimental solutions were determined directly using a vapor pressure osmometer (Wescor 5100c).

K^+ Flux Measurements

K^+ flux measurements were invariably made using $^{86}Rb^+$ as a tracer for K^+. The suitability of this tracer for K^+ was occasionally checked with $^{42}K^+$ and no significant differences were discernable between either tracer. Full experimental details of the K^+ influx and efflux measurements may be found in Boardman et al. [7] or Aiton and Simmons [3].

Measurement of Intracellular Na^+ and K^+ Ion Contents

Washing in an isotonic sorbitol solution, extraction in double-distilled water and measurement of Na^+ and K^+ by flame photometry was carried out as detailed by Boardman et al. [7] and by Aiton and Simmons [3].

Measurement of HeLa Cell Volume

Two independent methods were used to measure the cell volume of HeLa cells. In the first, electronic cell sizing of cell suspensions prepared by trypsin treatment was performed using a Coulter Counter (model ZF) fitted with a Channelyser (C1000) [7]. Alternatively, the method of Kletzein et al. [27], which utilizes the nonmetabolizable sugar, 3-O-methyl-D-(U-^{14}C)glucose, as an intracellular space marker was used (*see also* [41]).

Determination of Lactate Dehydrogenase (LDH) Activity

To test for HeLa cell integrity in anisotonic media, the release of the cytosolic enzyme lactate dehydrogenase (EC 1.1.1.27) into the supernatant fluid was monitored and compared with distilled water cell extracts or whole cell homogenates [41].

Materials

Wherever possible salts of Analar quality were used. Radiochemicals were obtained from Amersham International. Ouabain was obtained from the Sigma Chemical Company (Poole). Furosemide was a gift from Dr. C. Osborn of Hoechst U.K. (Hounslow).

Statistics

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

Kinetic data were fitted to Michaelis-Menten kinetics by the use of linear regression on the Eadie-Hofstee transformation to produce initial values of the kinetic parameters followed by a nonlinear iterative method using the Gauss-Newton approximation to produce a best fit with minimum values for residual sum of squares [28]. Estimates of kinetic parameters were also obtained for the Hill equation ($V_s = (V^*[S]^H)/([S]_{0.5} + [S]^H)$) where V is the maximal velocity, S is the activating ion concentration and H is the Hill coefficient, by using a linear regression of a form of the Eadie-Hofstee where H is evaluated by minimum sum of squares from a range of initial values of H [28].

Results

Behavior of HeLa Cell Volume in Anisotonic Media

Measurement of cell volume in anchorage-dependent cultured cells is problematic. The present measurements of HeLa cell volume (Fig. 1) were made

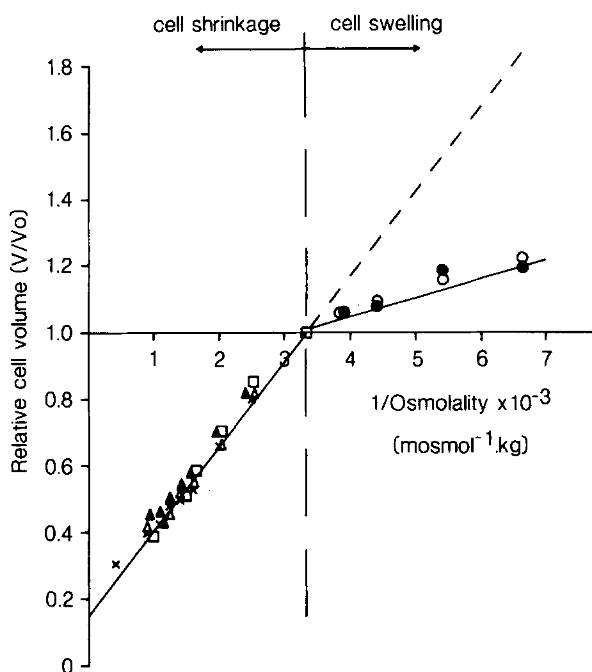

Fig. 1. Change in HeLa cell volume upon suspension in anisotonic media determined by electronic cell sizing of HeLa cell suspensions prepared by trypsinization and a 15-min incubation in normal Krebs. Results are plotted according to the Boyle-Van't Hoff relation $V = \pi_o/\pi \cdot (V_o - b) + b$ where V is the cell volume, π is the osmotic pressure of the suspending solution and b is the nonsolvent space of the HeLa cell, the subscript o denotes values for isotonic media. Cell volumes were calculated from size distributions of at least 10^4 cells. (▲, △) hyperosmolal solutions made by mannitol addition to normal Krebs, cell volumes determined 1 min (▲) or 30 min (△) following mixing. (□, ×) Hyperosmolal solutions made by addition of choline Cl, cell volumes determined 1 min (×) or 30 min (□) following mixing. (●, ○) Hypoosmolal solutions made by diluting normal Krebs with distilled water, determined 1 min (○) or 30 min (●) following mixing. Solid line for cell shrinkage: $V/V_o = 1/\pi \times 0.25 \times 10^{-3} + 0.16$ ($n = 9$, $r = 0.99$, $P < 0.01$). Solid line for cell swelling: $V/V_o = 1/\pi \times 0.06 \times 10^{-3} + 0.82$ ($n = 5$, $r = 0.96$, $P < 0.05$). Osmolality of the experimental solutions was determined using a Wescor osmometer

primarily by electronic cell sizing of cell suspensions made by trypsinization of cell monolayers grown on plastic or glass. Cell suspensions were incubated for 15 min at 37°C in a shaking water bath in isotonic Krebs solution prior to exposure to anisotonic media. To check the validity of using electronic cell sizing of cell suspensions to study the volume behavior of substrate-attached cells, an intracellular space marker, 3-O-methyl-D-(U-^{14}C) glucose, was used to check results obtained by the two methods [27, 41] (Table 1). For HeLa cells, there is good agreement between the two methods for cells in isotonic and anisotonic media (Table 1) substantiating results obtained in other cell-lines [41]. HeLa cells suspended in hypertonic media behave as osmometers (Fig. 1) when mannitol or choline Cl was used to increase medium tonicity. Furthermore, no evidence of volume regulatory behavior was observed under these conditions since cell volumes recorded 1 and 30 min after suspension in hypertonic media are unchanged. It is interesting to note that in some cells, notably mouse lymphoblasts and Ehrlich ascites cells, pre-exposure to hypoosmotic medium is required to demonstrate regulatory increases in cell volume [19, 25]. The intercept on the relative cell volume axis (Fig. 1) gives the nonosmotically active fraction of cell volume [26].

Table 1. Comparison of cell volume data determined by electronic cell sizing of HeLa cell suspensions with cell volumes determined using an intracellular space marker 3-O-methyl-D-(U-^{14}C) glucose, on the same cell cultures (*see* Materials and Methods)[a]

Medium osmolality (mOsm/kg)	Cell volume (cell sizing, μm^3)	Cell water (μl) per plate cell sizing	Cell water (μl) per plate 3-O-methyl glucose
210	2380 ± 26	1.15 ± 0.02 [1.1] (1.16)	1.08 ± 0.04 [1.10] (1.25)
300	2232 ± 19	1.03 ± 0.04 [1.0]	0.98 ± 0.03 [1.0]
396	1965 ± 21	0.95 ± 0.02 [0.92]	0.86 ± 0.02 [0.87]

[a] Cell water per plate by electronic sizing was calculated from the total cell number, and by assuming that 0.16 of the cell volume represents the nonosmotically active fraction of cell volume. Figures in square brackets are the relative cell volumes compared to isotonic (~300 mOsm/kg) media. Figures in parentheses are the predicted values for cell swelling from hypertonic shrinkage (πv = constant). Data are the mean ± SD of five separate determinations.

In contrast to cell shrinkage, cell swelling is associated with markedly nonideal behavior. Cell swelling is not as great as predicted on the basis of extrapolation of the relationship of a perfect osmometer, observed for cell shrinkage (Fig. 1). Since cell sizing in these experiments takes approximately 1 min, this nonideal behavior in hypotonic media is likely to result from a rapid loss of intracellular solute (*see below*). No further change in cell volume is observed on continued incubation for 30 min (Fig. 1).

Cell Swelling Induces a Rapid Leak of Cell K^+

Exposure of HeLa cell monolayers to hypoosmotic media results in a large, rapid and transient stimulation of $^{86}Rb^+$ (K^+) efflux (Fig. 2), which is association with a net loss of intracellular solute (Table 2). That this loss of cell K^+ is due to a specific increase in HeLa cell membrane permeability, rather than to

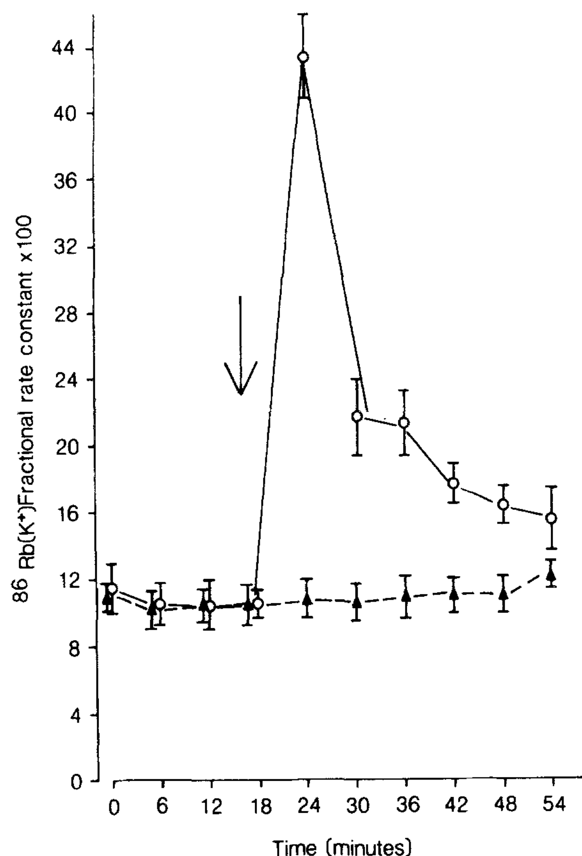

Fig. 2. $^{86}Rb^+(K^+)$ efflux from monolayer cultures of HeLa cells in isosmotic media (85 mM NaCl, plus mannitol to give a final osmolality of 310 mosmol/kg) (▲), or initially in isosmotic media, and then transferred to hypo-osmolal media (85 mM NaCl, 210 mosmolal/kg) at the arrow (○). Each datum is the mean ± SD of three separate experiments

Table 2. Effect of hypoosmotic media upon cation contents of HeLa cells incubated for 1 hr at 37°C[a]

Medium osmolarity (mosm/kg)	Ion contents (nequiv/10^6 cells)	
	K^+	Na^+
206	231.7 ± 5.4[b] $P < 0.1 > 0.05$	16.8 ± 1.1[NS]
247	257.8 ± 8.5	17.8 ± 1.2[c]
289	275.2 ± 5.5	14.5 ± 0.9

[a] Results are the mean ± SE of three separate experiments.
[b] = $P < 0.001$.
[c] = $P < 0.05$.
Significantly different from 289 mOsm/kg media values.
NS = not significant.

cell lysis, is evident from the following data; first, leakage of the cytosolic enzyme LDH into the supernatant fluid is not increased in this range of hypoosmotic exposure, though exposure of cells to distilled water results in a substantial loss of LDH (Table 3); and secondly, intracellular Na^+ contents are not significantly altered in hypoosmotic media (Table 2) (but note the caveat concerning increased Na pump activity in hypotonic media below).

Table 3. Release of lactate dehydrogenase activity (LDH) from HeLa cells by exposure to anisotonic media and by homogenization[a]

Treatment	LDH activity (mU/10^6 cells)
1. Cell homogenization	320.0 ± 45.0
2. 1 hr in distilled water	113.0 ± 25.0
3. 1 hr in hypotonic media (210 mosmol/kg media)	9.7 ± 4.2[NS]
4. 1 hr in isotonic media	10.8 ± 1.0
5. 1 hr in hypertonic media (510 mosmol/kg media)	6.6 ± 5.7[NS]

[a] Results are expressed as the mean ± SD of three separate determinations. NS = not significantly different from LDH activity released in isotonic media.

The stimulation of $^{86}Rb^+$ efflux observed in hypotonic media is unaffected by ouabain or by 0.1 mM furosemide indicating that neither the Na^+-K^+ pump nor the diuretic-sensitive K^+ transporter present in HeLa cells [2] is directly involved in the volume regulatory response to hypotonic media. Measurements of net K^+ loss under these conditions substantiate measurements of $^{86}Rb^+$ efflux (*data not shown*).

The operational definition of the diuretic-sensitive K^+ transport is based upon the use of furosemide, or other loop diuretics, at concentrations giving maximal inhibition of K^+ influx (usually 100 μM) in isotonic media [2]. The dependency of the diuretic-sensitive component upon medium cation and anion composition (NO_3^- does not substitute for Cl^-) may also be used as additional criteria. The increased $^{86}Rb^+(K^+)$ efflux observed in hypotonic media in HeLa cells is attenuated, but not abolished, by replacement of medium Cl^- by NO_3^- (Table 4), in contrast to the results obtained for the diuretic-sensitive component. It should be noted that the difference between control values of fractional $^{86}Rb^+$ loss (Table 4) arises from the use of cell batches at differing cell densities; this in no way alters the response to hypotonic media.

Partition of the $^{86}Rb^+(K^+)$ influx into the ouabain-sensitive and diuretic-sensitive components indicates that the stimulation of total K^+ influx observed in hypotonic conditions, compared to isotonic media (Table 5) is due not only to an increased residual flux (column 4), but also to an increased flux through the Na^+-K^+ pump (Table 5). Measurements of intracellular cation contents at 1 to 5 min following exposure to hypotonic media fail

Table 4. Effect of furosemide (0.1 mM), ouabain, and Cl^--free, NO_3^- media, upon the fractional loss (× 100) of ^{86}Rb from HeLa cells[a]

Time (min)	0	6	12	18	24
Control	10.5 ± 1.3	10.9 ± 0.4	43.4 ± 3.8	21.9 ± 1.9	21.5 ± 2.3
0.1 mM Furosemide	10.2 ± 0.9	10.6 ± 0.2	41.2 ± 2.5	16.9 ± 1.5	14.4 ± 1.1
Control	11.4 ± 1.4	11.6 ± 1.7	47.9 ± 2.1	24.4 ± 1.3	19.7 ± 0.6
1 mM Ouabain	11.1 ± 2.4	10.5 ± 1.6	47.5 ± 2.4	24.4 ± 0.9	20.3 ± 0.9
Control	4.8 ± 0.8	4.9 ± 0.4	30.4 ± 4.1	13.8 ± 3.0	12.1 ± 0.5
Cl^- free, NO_3^- media	2.5 ± 0.5	2.8 ± 0.6	6.7 ± 1.5	7.6 ± 0.6	7.0 ± 0.9

[a] Media was changed from isosmotic to hypotonic at 12 min. Furosemide and ouabain were present from −12 min. All data are the mean ± SD of three separate experiments.

Table 5. Effect of anisotonic media upon K^+ ($^{86}Rb^+$) influx (10-min flux measurement) into HeLa cells[a]

K^+ (^{86}Rb) influx (nmol/10^6 cells · min)						
Medium osmolality mosmolal/kg	Total (1)	$+10^{-4}$ M furosemide (2)	$+10^{-3}$ M ouabain (3)	$+10^{-4}$ M furosemide $+10^{-3}$M ouabain (4)	Furosemide -sensitive influx (1–2) (3–4)	Ouabain -sensitive influx (1–3) (2–4)
210	9.42 ± 0.53[c]	7.35 ± 1.26	2.82 ± 0.38	1.52 ± 0.13[d]	2.05 ± 0.96[NS]	6.58 ± 0.47[d]
					1.31 ± 0.35[b]	5.83 ± 0.86[b]
308	6.71 ± 0.18	4.59 ± 0.41	3.28 ± 0.33	0.83 ± 0.03	2.10 ± 0.52	3.42 ± 0.22
					2.45 ± 0.32	3.77 ± 0.38
410	8.13 ± 0.63[b]	4.28 ± 0.53	3.85 ± 0.43	0.64 ± 0.04[c]	3.84 ± 0.88[c]	4.27 ± 0.39[b]
					3.25 ± 0.48[b]	3.65 ± 0.58[NS]

[a] Data is from a representative experiment where three replicates were made for each datum. Errors are expressed ± SD. Medium osmolality was varied by mannitol addition to a Krebs containing 85 mM NaCl (*see* Materials and Methods). Significantly different from 308 mosmol/kg medium. NS = not significantly different.
[b] $P < 0.05$.
[c] $P < 0.01$.
[d] $P < 0.001$.

to demonstrate an increased cytosolic Na^+ content (*data not shown*). Since cell volume increases, intracellular Na^+ concentration must fall under these conditions. The cause of the increased Na^+-K^+ pump activity cannot be easily explained, though local increases in Na^+ concentration adjacent to the plasma membrane might be postulated. Similar results have been observed by Bui and Wiley [9] in human lymphocytes.

The major conclusion relevant to the present paper concerning the swelling-induced K^+ leak observed in HeLa cells, is that this is unconnected with diuretic-inhibitable K^+ transport previously described for HeLa cells.

Effect of Hypertonic Media Upon $^{86}Rb^+(K^+)$ Fluxes

Diuretic-sensitive "cotransport" (Na^+ + K^+ + $2Cl^-$) flux and diuretic-sensitive K^+ fluxes have been shown to be activated by cell shrinkage on exposure of avian red cells to hypertonic bathing media [38, 43]. Table 5 and Fig. 3 demonstrate a stimulation of diuretic-sensitive $^{86}Rb^+$ fluxes by hypertonic media. The marked stimulation of total $^{86}Rb^+$ influx is predominantly a consequence of an increased diuretic-inhibitable component. There is a smaller stimulation of the ouabain-sensitive $^{86}Rb^+$ influx and a significant reduction in the residual flux. The stimulation of the diuretic-sensitive component is also observed with $^{86}Rb^+$ efflux (Fig. 3*B*) where there is a time-dependent increase in efflux. The progressive stimulation in diuretic-sensitive $^{86}Rb^+$ efflux contrasts to the rapid change in HeLa cell volume subjected to anisotonic media (Fig. 1) and to the effects of the loop diuretic (Fig. 3). The increased $^{86}Rb^+$ efflux observed in hypertonic media is maintained at stimulated plateau values (*see* Figs. 4 and 10) as long as the hypertonic stimulus is maintained and in experiments where the incuba-

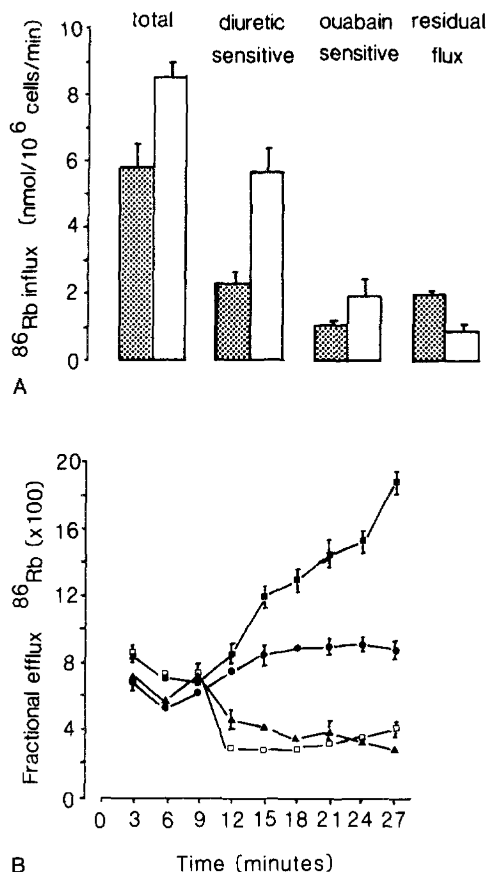

Fig. 3. Effect of exposure of HeLa cells to isosmolal (310 mosmol/kg) and hyperosmolal conditions (510 mosmol/kg mannitol addition to normal Krebs containing 137 mM NaCl, 5.4 mM KCl) upon the components of $^{86}Rb^+$ influx (*A*) and efflux (*B*) measured in the same cell batch. All HeLa cell cultures were preincubated in isotonic media for 3 hr to match cells loaded with $^{86}Rb^+$ for the efflux experiments. (*A*) Influx determinations were measured over 5 min. Stippled histogram bars are for control media. Diuretic-sensitive (0.1 mM furosemide) flux components were determined in the presence of ouabain (1.0 mM). Data are the mean ± SEM ($n = 3$). (*B*) (●) control media, (■) hyperosmolal media, (▲) control media + 0.1 mM furosemide, (□) hyperosmotic media + 0.1 mM furosemide. Using measured values of intracellular K^+ concentration and the rate constants for $^{86}Rb^+$ efflux at 6 min following exposure to hypertonic media in the presence and absence of furosemide the calculated values of efflux (in nmol/10^6 cells · min) were for 310 mosmol/kg media 6.6, plus 0.1 mM furosemide 3.1, 510 mosmol/kg media 8.3, plus 0.1 mM furosemide 2.4(5)

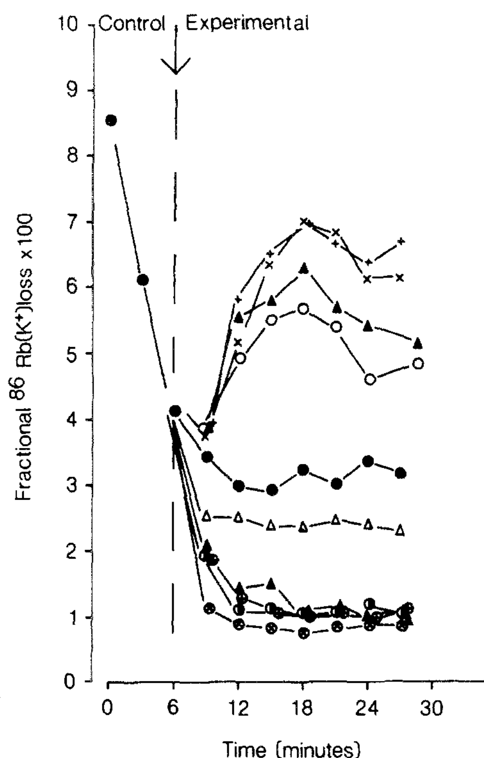

Fig. 4. Stimulation of diuretic-sensitive $^{86}Rb^+$ efflux in hyperosmolal solutions. (●) Control media (310 mosmol/kg, 137 mM NaCl, 5.4 mM KCl). (△) Media plus 50 μM piretanide. (+, ×, ▲, ○) Hypertonic media, 510 mosmol/kg; (+) Na gluconate osmotic additive, (×) mannitol osmotic additive, (▲) choline Cl osmotic additive, (○) NaCl osmotic additive. (⊕, ⊗, ▲, ◑) Hypertonic media plus 50 μM piretanide; (⊕) Na gluconate as osmotic additive, (⊗) mannitol, (▲) choline Cl, (◑) NaCl. Data are the mean of three separate determinations. Error bars are omitted for clarity

tion period has been extended to 45 min (*data not shown*). The stimulated efflux is fully reversible on re-exposure to isosmotic media (*data not shown*). The data in Fig. 3 were obtained with the same cell batch measured under, as far as was possible, identical experimental conditions. Comparison of the total $^{86}Rb^+$ influx and the calculated efflux (*see* legend to Fig. 3) indicates that the cells were in steady state for K^+ exchanges in both isotonic and hypertonic conditions and that flux through the diuretic-sensitive pathway also consisted of equal and opposite unidirectional fluxes of K^+ (an exchange, with no net flux). This point was addressed further by measuring the cation contents of HeLa cells exposed to hypertonic media for prolonged periods of up to 2.5 hr (Table 6). No large change in intracellular cation contents is observed that could compensate for the shrinkage in cell volume induced by the hypertonic stress; indeed the effects were small and not consistently directed. The difference in cellular cation contents between different experiments in Table 6 is due to a density-dependent difference in cell volume, similar to that described for MDCK cells [41]. The data in Table 6 is supportive of the inability of HeLa cells to volume regulate in the hypertonic range (Fig. 1). The change in intracellular K^+ content observed in hypotonic conditions contrasts with the result obtained in hypertonic media.

Table 6. Effect of hyperosmotic media upon cation contents of HeLa cells upon prolonged incubation[a]

Experiment	Incubation time (hr)	Ion contents (nequiv/10^6 cells)			
		310 mosmol/kg media		510 mosmol/kg media	
		K^+	Na^+	K^+	Na^+
1.	1.0	284.1 ± 16.1	13.4 ± 3.1	263.3 ± 5.2[b]	20.2 ± 3.1[b]
2.	2.0	358.3 ± 8.2	38.5 ± 6.8	377.6 ± 12.6[NS]	32.1 ± 5.6[NS]
3.	2.5	259.6 ± 5.3	12.9 ± 1.0	285.6 ± 11.8[c]	12.9 ± 0.8[NS]

[a] Errors are expressed ± SD of each individual experiment, where replication was at least × 3. The control medium (~310 mosmol/kg) contained Na^+ = 140 mmol/liter and K^+ = 5.4 mmol/liter. Significantly different from 310 mosmol/kg media. NS = not significant.
[b] $P = <0.05$.
[c] $P = <0.01$.

Stimulation of the diuretic-sensitive component by hypertonic media is not dependent upon the osmotically active additive to isotonic media. Figure 4 shows that $^{86}Rb^+$ efflux is markedly stimulated compared to control values when either NaCl, Na gluconate, choline Cl or mannitol is used as the osmotic additive. Fractional $^{86}Rb^+$ efflux (×100) is increased from 3.23 ± 0.40 (SD, $n = 3$) in controls to 6.73 ± 0.76 with Na gluconate ($P < 0.05$) as the osmotic additive, or to 4.86 ± 0.70 with NaCl as the osmotic additive ($P < 0.05$). Furthermore, in the presence of 100 μM piretanide, a highly significant reduction of $^{86}Rb^+$ efflux is observed in all hypertonic media [fractional $^{86}Rb^+$ loss × 100 was 1.03 ± 0.21 (SD, $n = 3$) for NaCl as the osmotic additive] compared to isotonic media (fractional $^{86}Rb^+$ loss × 100 was 2.33 ± 0.21, $P < 0.001$). No significant reduction in the magnitude of the increased fractional $^{86}Rb^+$ loss (×100) in hypertonic media is observed at $t = 30$ min [4.86 ± 0.70 (SD, $n = 3$) for NaCl as the osmotic additive] compared to the maximum at 20 min (5.66 ± 0.50, $P > 0.1$). Stimulation of diuretic-sensitive $^{86}Rb^+$ efflux is thus not dependent upon the method used to increase external osmolality. It should be noted that the transmembrane ion gradients for Na^+ and Cl^- in these conditions will be substantially different.

Stimulation of the diuretic-sensitive $^{86}Rb^+$ efflux by mannitol addition is saturable (Fig. 5), saturation being observed at 380 to 400 mosmol/kg. This datum is thus similar to that described by Ueberschar and Bakker-Grunwald [43] for turkey erythrocytes.

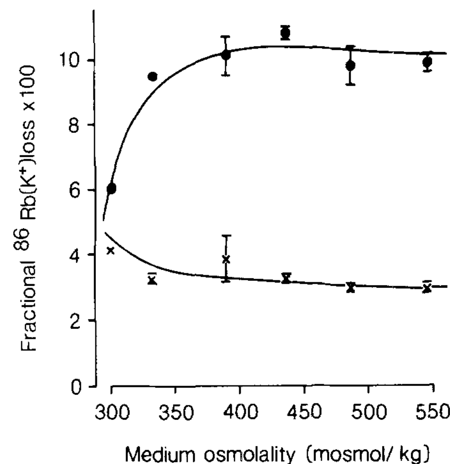

Fig. 5. Effect of increasing medium osmolality upon $^{86}Rb^+(K^+)$ efflux in the absence (●) or presence (×) of 0.1 mM furosemide. Mannitol addition was used to increase medium osmolality. Results are expressed as the mean ± SD; where not shown data points lie within the experimental points

Kinetics of the Diuretic-Inhibitable $^{86}Rb^+$ Influx in Isotonic and Hypertonic Conditions

Previously [2] we have demonstrated that the diuretic-sensitive component of $^{86}Rb^+(K^+)$ influx in HeLa cells displays saturation kinetics with respect to external K^+ and is also Cl^--dependent and Na^+-dependent. Activation of diuretic-sensitive $^{86}Rb^+(K^+)$ flux by medium K^+, Na^+ and Cl^- has been investigated in both isotonic and hypertonic media (Figs. 6–8, Table 7).

Both the K^+ and Na^+ activation of the diuretic-sensitive component of K^+ influx could be fitted by Michaelis-Menten kinetics. For hypertonic media, the main effect was seen to be an increase in the apparent maximal velocity (V_{max}) for both K^+ and Na^+ activation. A significant effect on the K_m for K^+ activation was observed with a decrease in affinity. The residual diuretic-insensitive $^{86}Rb^+$ influx increased linearly as a function of external K^+ in both isotonic and hypertonic media. As previously reported [2] there is an absolute dependence for Na^+ of the diuretic-sensitive K^+ ($^{86}Rb^+$) influx (Fig. 7*A*).

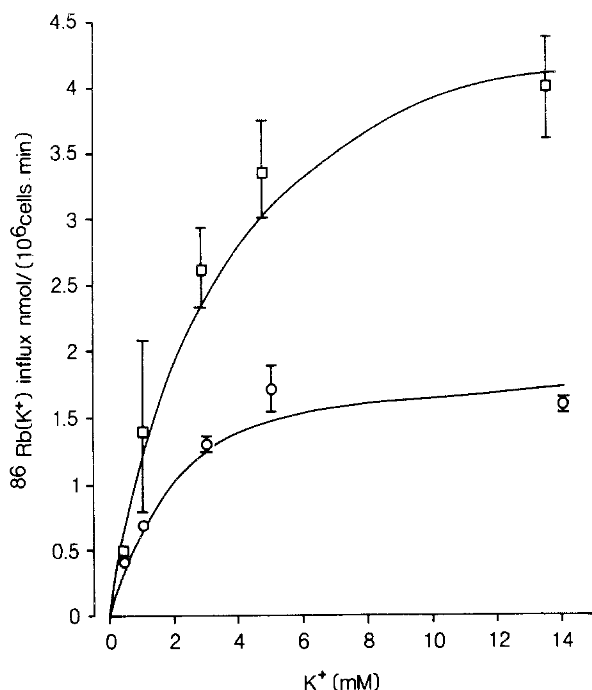

Fig. 6. K^+ activation of the ouabain-insensitive but diuretic (furosemide)-sensitive $^{86}Rb^+(K^+)$ influx in control media (320 mosmol/kg) (○), and hypertonic media (520 mosmol/kg, mannitol addition) (□). The medium Na^+ was 137 mM and the KCl was varied in the range 0.5 to 13 mM (mannitol being used to maintain isosmotic conditions). The curves are the best-fit lines of the data to Michaelis-Menten kinetics fitted as described in Materials and Methods. The kinetic constants V_{max} and K_m were *A*) for 320 mosmol/kg data 1.91 ± 0.12 (SD) nmol/(10^6 cells · min), and 1.49 ± 0.26 (SD) mM, respectively; *B*) for 520 mosmol/kg data, 5.08 ± 0.51 nmol/(10^6 cells · min) and 3.16 ± 0.67 mM, respectively

Also the ouabain and loop diuretic-insensitive K^+ influx was increased in low Na^+ media. This stimulation of the residual potassium flux decreases the precision of measurements of the diuretic-sensitive component, and for this reason we measured the effect of hypertonicity as a function of external Na^+ with two nonpenetrant Na^+ substitutes, choline and *n*-methyl-D-glucamine [6, 32, 37] (Fig. 7*A* and *B*). The main effect of hypertonic media on Na^+-activation was an increase in V_{max}. The K_m for Na^+ activation in choline medium was unchanged by hypertonic medium, though with methyl-D-glucamine a reduction in affinity was observed. For both choline and *n*-methyl-D-glucamine substitutes, a stimulation of the residual ouabain and diuretic-insensitive flux was observed (*data not shown*); in this and other respects (*see* Figs. 3–5, 10) this residual flux demonstrates properties not expected of a passive leak pathway. The stimulation of $^{86}Rb^+$ influx in low Na^+ media is associated with a net loss of Na^+ from the cells; for all other conditions significant changes in internal Na^+ or K^+ in the flux measurement period (5 min) were not present (*data not shown*).

For Cl^- activations of the diuretic-sensitive $^{86}Rb^+$ (K^+) influx we chose three anion substitutes, NO_3^-, isethionate$^-$ and gluconate$^-$ (Fig. 8) as it is now clear that Cl^- activation curves are dependent upon the anion substitute used (*see* [12]). The reason for this is not yet clear, though external Cl^- ion replacements are complicated by change in Cl^- ion concentration on two membrane surfaces, (which is in turn dependent upon the penetrability of the replacement anion) and also there is the possibility of secondary effects arising from changes in the membrane potential or intracellular pH.

For NO_3^- replacements, increasing Cl^- gave a sigmoid curve which could be described by a Hill equation with Hill coefficients close to 2 (Fig. 8, Table 7). Hypertonic media increased the diuretic-sensitive $^{86}Rb^+$ influx at all levels of Cl^- tested, the main effect being judged to be an increase in the V_{max} (Table 7). Similar data were obtained for both gluconate$^-$ and isethionate$^-$ substitutions (Table 7), the sigmoid nature of Cl^- activation being especially obvious in the case of gluconate$^-$ replacement. The residual flux (the ouabain and diuretic-insensitive $^{86}Rb^+$ influx) for the data shown in Fig. 8, was insensitive to Cl replacement (*not shown*).

Sensitivity of the Ouabain-Insensitive $^{86}Rb^+$ Influx to Inhibition by Furosemide

Figure 9 demonstrates that the increased ouabain-insensitive $^{86}Rb^+$ influx in hypertonic media is inhibited by furosemide with a high molar affinity. There is no significant effect either on the Hill slope or the concentration of furosemide giving half-maximal inhibition (Fig. 9).

Effect of External K^+ on $^{86}Rb^+$ Efflux in Hypertonic Media

In duck red cells Haas et al. [23] have demonstrated that for diuretic-sensitive $Na^+ + K^+$ "cotransport" there is no evidence for *trans*-stimulation of "cotransport" by either cation. Thus cation efflux or influx are dependent solely upon the *cis* concentrations of cations. In conditions of zero net flux (exchange), there are thus equal and balanced unidirectional fluxes. In the present data, exposure of HeLa cells to hypertonic media results in an increased diuretic-sensitive influx and efflux of $^{86}Rb^+$ even

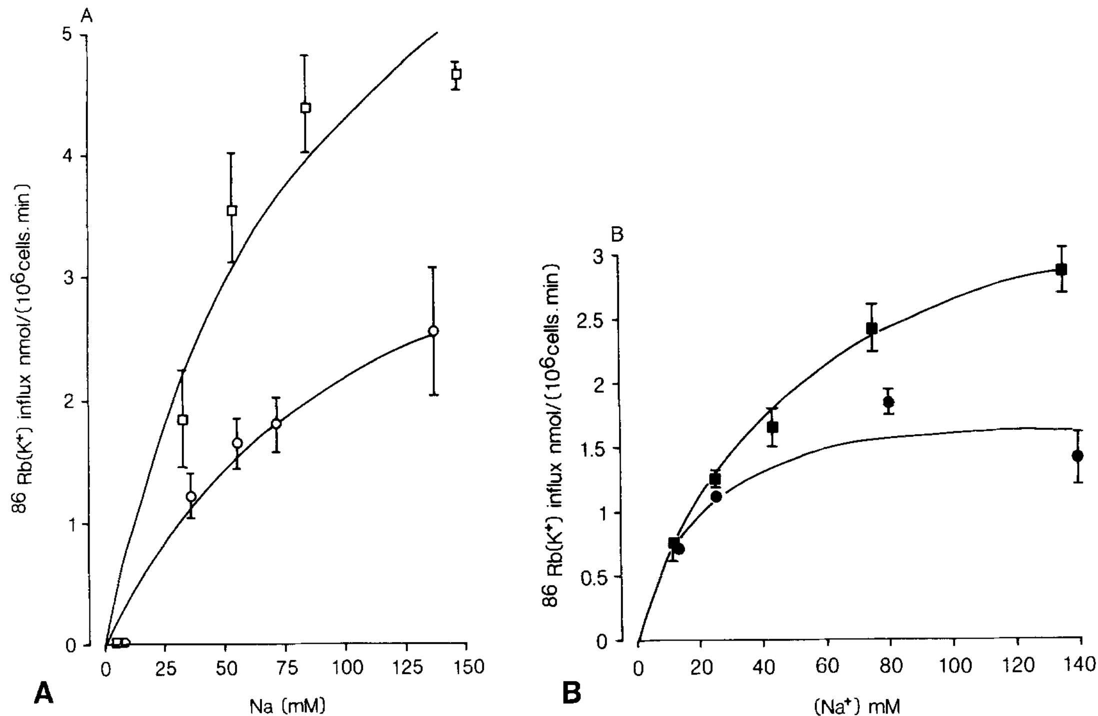

Fig. 7. Na^+ activation of the ouabain-insensitive but diuretic (furosemide)-sensitive $^{86}Rb^+$ influx in (○, ●) control media (310 mosmol/kg) or (□, ■) hypertonic media (510 mosmol/kg, mannitol addition). Medium K^+ was 5.4 mM, NaCl was varied by isosmotic substitution with *A*) choline Cl or *B*) *n*-methyl-D-glucamine titrated to pH 7.0 by HCl. Curves are the best-fit lines of the data for Michaelis-Menten kinetics fitted as in Fig. 6. The kinetic constants V_{max} and K_m for choline substitution were (310 mosmol/kg data) 4.2 ± 0.9 (SD) nmol/(10^6 cells · min) and 95.5 ± 36.1 (SD) mM, and for 510 mosmol/kg data, 8.7 ± 2.1 nmol/(10^6 cells · min) and 102.0 ± 42.8 mM, respectively; for *n*-methyl-D-glucamine substitution 310 mosmol/kg data, V_{max} = 1.8 ± 0.2 nmol/(10^6 cells · min) and the K_m = 15.3 ± 7.6 mM, while for hypertonic data these constants were 4.1 ± 0.3(5) nmol/(10^6 cells · min) and 57.2 ± 10.8 mM, respectively

when external cation concentrations were unchanged (mannitol additions). We were thus interested in determining whether this bidirectional increase in flux represented an obligatory exchange through the diuretic-sensitive transporter. Figure 10 shows the effect of 0.05 and 11 mM KCl upon the diuretic-sensitive component of $^{86}Rb^+$ efflux in isotonic and hypertonic conditions. Reduction of external K^+ to 0.05 mM has no significant effect upon the control fractional $^{86}Rb^+$ efflux measured throughout the experiment (at 21 min fractional $^{86}Rb^+$ efflux × 100 was 4.1 ± 0.7 SD, n = 3, at 0.05 mM KCl compared to 3.2 ± 0.4, n = 3, at 11.0 mM KCl, $P > 0.1$), or upon fractional $^{86}Rb^+$ efflux in the presence of furosemide (which at 21 min was 2.0 ± 0.2, n = 3, at 0.05 mM KCl compared to 2.5 ± 0.3, n = 3, at 11.0 mM KCl, $P > 0.1$). It must be emphasized that at the cytocrit used (< 0.1%), the external K^+ concentration is not significantly altered during the time course of the experiment as a consequence of loss of internal K^+ due to Na^+-pump inhibition.

Table 7. Kinetic constants for Cl^- activation of the ouabain-insensitive but diuretic-sensitive $^{86}Rb^+$ influx in control media and hypertonic media, using NO_3^-, gluconate$^-$ or isethionate$^-$ as the substituent anions[a]

		Medium osmolality (mOsm/kg)	
Substituent Anion for Cl^-		310	510
NO_3^-	V_{max} (nmol/(10^6 cells · min))	2.0	9.9
	$K_{1/2}$ (mM)	111.8	132.0
	H	2.6	1.9
Gluconate$^-$	V_{max} (nmol/(10^6 cells · min))	3.3	6.4
	$K_{1/2}$ (mM)	44.1	70.5
	H	1.9	1.9
Isethionate$^-$	V_{max} (nmol/(10^6 cells · min))	2.9	8.3
	$K_{1/2}$ (mM)	105	123
	H	2.3	1.9

[a] Experimental data and fitted curves are shown in Fig. 4, *A–C*. All data were fitted to the Hill equation with initial estimates of the Hill coefficient (H) between 1.4 and 2.6.

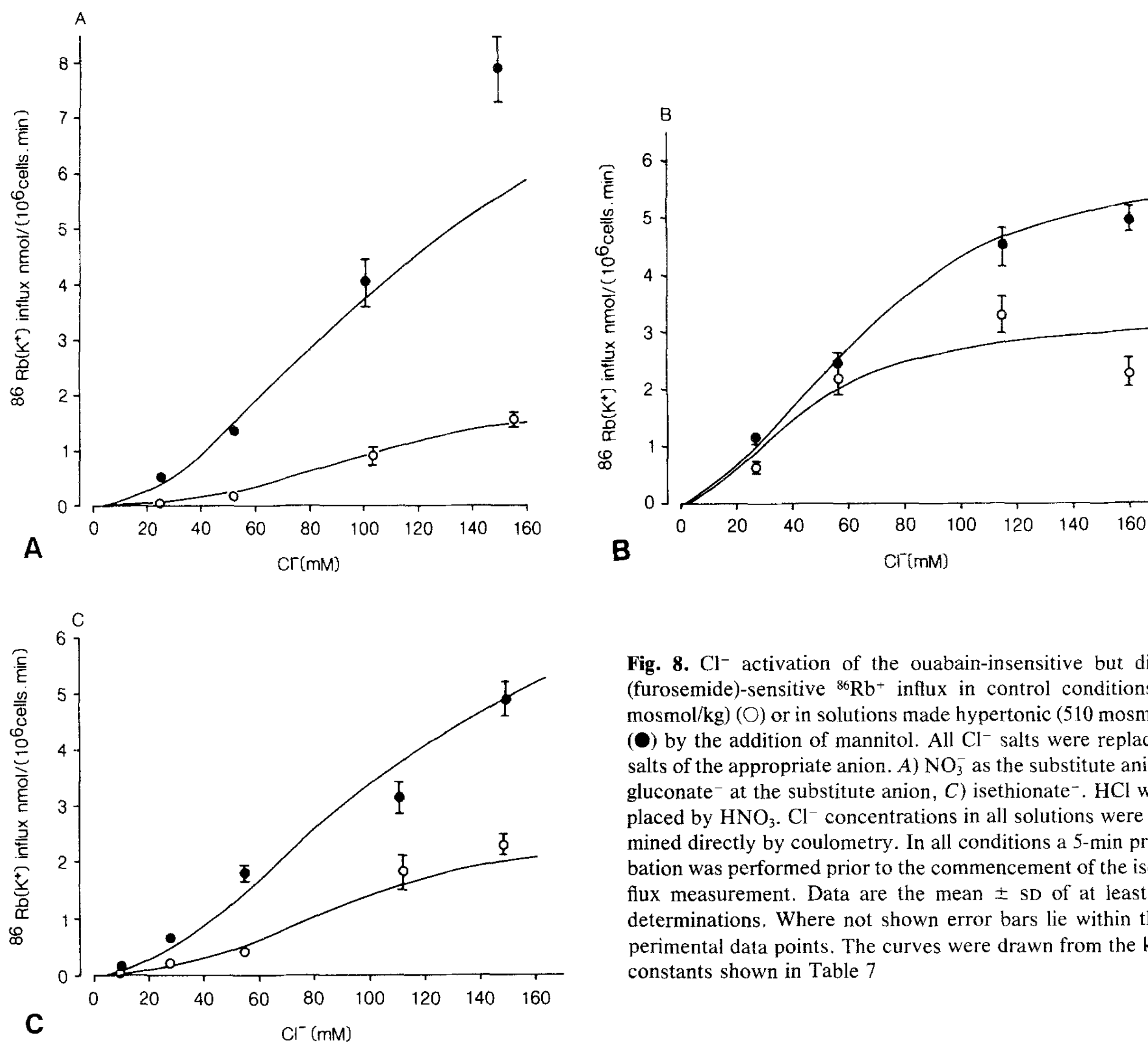

Fig. 8. Cl^- activation of the ouabain-insensitive but diuretic (furosemide)-sensitive $^{86}Rb^+$ influx in control conditions (310 mosmol/kg) (○) or in solutions made hypertonic (510 mosmol/kg) (●) by the addition of mannitol. All Cl^- salts were replaced by salts of the appropriate anion. *A*) NO_3^- as the substitute anion, *B*) gluconate⁻ at the substitute anion, *C*) isethionate⁻. HCl was replaced by HNO_3. Cl^- concentrations in all solutions were determined directly by coulometry. In all conditions a 5-min preincubation was performed prior to the commencement of the isotopic flux measurement. Data are the mean ± SD of at least three determinations. Where not shown error bars lie within the experimental data points. The curves were drawn from the kinetic constants shown in Table 7

Exposure of HeLa cells to hypertonic media at both elevated and reduced external KCl, results in a highly significant stimulation of fractional $^{86}Rb^+$ efflux (Fig. 10). At 21 min in 0.05 mM KCl, fractional ^{86}Rb loss × 100 is 7.6 ± 0.3, $n = 3$, in hypertonic media compared to 4.1 ± 0.7, $n = 3$, in isosmotic media, $P > 0.001$. With 11.0 mM KCl fractional efflux is increased to 9.3 ± 0.6, $n = 3$, in hypotonic media compared to 3.2 ± 0.4, $n = 3$, in isosmotic media, $P > 0.001$. In both elevated and reduced external KCl this increase is sensitive to loop diuretic inhibition (fractional $^{86}Rb^+$ efflux × 100 plus 0.1 mM furosemide in hyperosmotic media measured at 21 min was 1.7 ± 0.5, $n = 3$, for media containing 11.0 mM KCl and 2.9 ± 0.3, $n = 3$, for media plus 0.05 mM KCl, $P > 0.001$, versus data in the absence of diuretic. Since the K_m for K^+ activation of diuretic-sensitive $^{86}Rb^+$ influx is 1 to 6 mM K^+ in isotonic and hypertonic conditions (Fig. 6) it can be concluded that the greater proportion of the diuretic-sensitive fluxes measured in hypertonic conditions (approx. 65%) does not represent obligatory exchange flux through the diuretic-sensitive K^+ transporter.

Discussion

The present results show that anisotonic media and subsequent perturbations in cell volume have a profound effect upon the activity of several membrane transport systems in HeLa cells. The operational definition of these transport systems is primarily made by pharmacological means (the use of ouabain and of loop diuretics such as furosemide and piretanide). Our particular interest was in the behavior of the ouabain-insensitive, but loop diuretic-sensitive K^+ flux component. The use of loop-diuretic

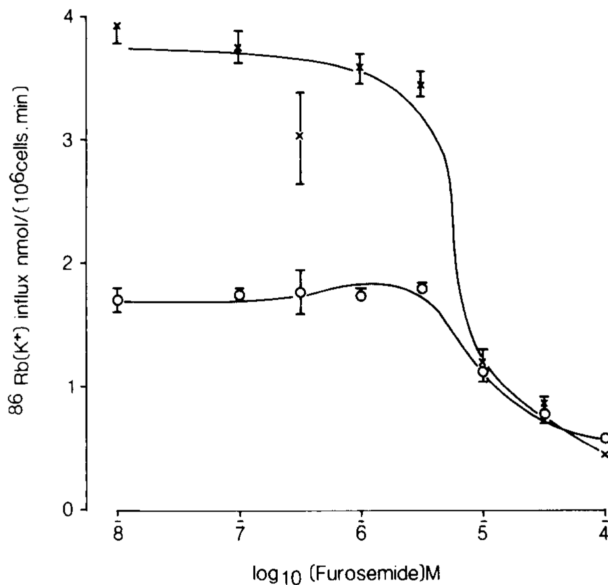

Fig. 9. Sensitivity of the ouabain-insensitive $^{86}Rb^+(K^+)$ influx to inhibition by furosemide in standard control Krebs solution (310 mosmol/kg) (○) or hyperosmolar media (510 mosmol/kg, mannitol addition) (×). The curves were fitted by eye. From linear regression of data from 10^{-6} M furosemide to the Hill equation: $\log(V/(V_{max} - V)) = n \log S - \log k$, where V_{max} is the maximal inhibition by furosemide and S the furosemide concentration, n the Hill coefficient and $k = [S]^n_{0.5}$. For control data $n = 0.99 \pm 0.12$ (SE) and $k = 5.3 \times 10^{-6}$ M, while for hyperosmolar conditions $n = 1.38 \pm 0.15$ (SE) and 6.2×10^{-6} M

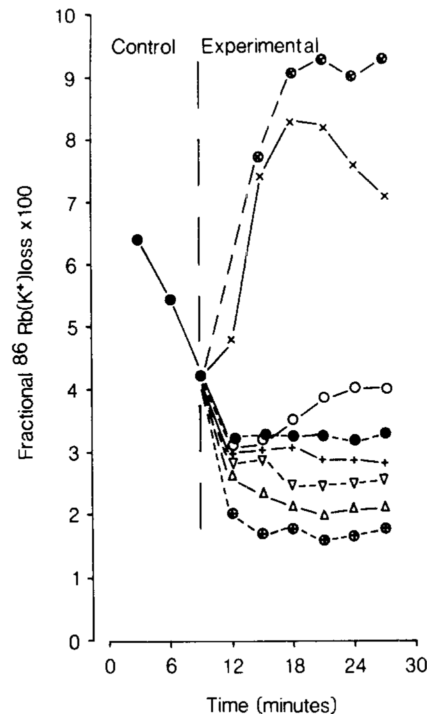

Fig. 10. Stimulation of diuretic-sensitive $^{86}Rb^+(K^+)$ efflux by hypertonic external media (osmolality increased by mannitol) in elevated (11.0 mM KCl) or reduced external KCl (0.05 mM). (●) Control media 320 mosmol/kg, 11.0 mM KCl. (○) Control media, 300 mosmol/kg, 0.05 mM KCl. (⊗) Hyperosmolar media, 520 mosmol/kg, 11.0 mM KCl. (×) Hyperosmolar media 500 mosmol/kg, 0.05 mM KCl. (▽, △, ⊕, +) Media plus 0.1 mM furosemide: (▽, △) 300 to 320 mosmol/kg, 11.0, 0.05 mM KCl, respectively; (⊕, +) 500 to 520 mosmol/kg 11.0, 0.05 mM KCl, respectively. All data are the mean of three separate determinations. Error bars are omitted for clarity

sensitivity as a sole criterion of transport pathway identification is subject to some doubt, in that there are several systems inhibited by loop diuretics, including Cl^--HCO_3^- exchange in human erythrocytes [8] and volume-stimulated K^+Cl^- transport in sheep red cells [14] as well as $Na^+ + K^+ + Cl^-$ "cotransport" in a variety of cell types [36]. The sensitivity to inhibition of these different systems varies, high affinity inhibition being limited to "cotransport" [36]. Additional criteria are necessary, however, since the sensitivity to diuretic inhibition varies with change in medium composition (*see* [21]). For the K^+ flux sensitive to loop diuretic inhibition in HeLa cells we have previously demonstrated that this flux component is dependent upon medium Na^+ and Cl^-. For HeLa cells the anion requirement is extreme, only Br being a partial substitute anion (*see also* [36]). The diuretic-sensitive K^+ flux component in HeLa cells, which is inhibited by loop diuretics with high affinity (Fig. 9, *unpublished data also show that the potency series is bumetanide > piretanide > furosemide*), may thus be regarded as "cotransport" flux. There exist, therefore, additional criteria for ascribing flux components to different membrane transport systems. For the current data on the diuretic-sensitive K^+ flux component we also include a comprehensive analysis of the K^+, Na^+ and Cl^- dependence. It must be stressed, however, that such criteria are entirely arbitrary and do not exclude the possibility that the operational parameters of a single transport system may vary extensively. In HeLa cells treated with ouabain for 1 to 2 hr we have previously shown, using measurements of net cation fluxes, that a fraction (1/5) of the isotopically measured efflux comprises K^+Cl^- transport uncoupled from Na^+ [3].

The data presented in this paper indicate that HeLa cells have a mechanism whereby partial cell volume regulation is possible when cells are hypotonically swollen. This regulation is mainly mediated by a loss of intracellular K^+ via a membrane transport system distinct from the Na^+-K^+ pump and the diuretic-sensitive transport system. The rapid K^+ loss is not abolished, but is attenuated when medium Cl^- is replaced by NO_3^-; this datum suggests that the "cotransport" sensitive to high-affinity loop diuretic inhibition is not responsible;

however, the existence of an anion-dependent K^+ transport cannot be entirely excluded. Further work is needed to further define this system and the possible membrane mechanisms (*see* [11]).

In contrast to cell volume behavior observed in hypotonic media, HeLa cells exposed to hypertonic media are unable to regulate their volumes and behave as perfect osmometers. Similar behavior is observed in Ehrlich cells where a volume regulatory increase, mediated by a diuretic-sensitive "cotransport," is seen only when intracellular K^+Cl^- is first lowered prior to hypertonic exposure [25].

Decreased cell volume does however result in a large stimulation of the ouabain-insensitive but diuretic-sensitive fluxes. Kregenow [29] has also shown in avian red cells that the ouabain-insensitive fluxes of K^+ were stimulated even when no volume regulation is observed at normal values of external K^+ (2.4 mM). The major effect reported in the present data is an increase in the V_{max} for transport, with the major features of K^+, Na^+ and Cl^- activation of the diuretic-sensitive component remaining unchanged. The sensitivity to diuretic inhibition by furosemide is also unchanged. Haas et al. [23] have demonstrated that for $Na^+ + K^+ + Cl^-$ "cotransport" in avian red cells, the net flux mediated by "cotransport" is dependent upon the sum of the chemical activity gradients for Na^+, K^+ and Cl^-.

For the present data, measurement of the diuretic-sensitive component in isotonic conditions (Fig. 3) suggests that no net flux is mediated by the system and that for HeLa cells in these conditions the sum of the chemical activity gradients is zero.

When HeLa cells are shrunken by addition of nonpenetrant inert solute, such as mannitol, then Na_o^+, K_o^+ and Cl_o^- are constant and internal ion concentrations will be increased initially by a factor directly proportional to the ratio of osmolalities (*see* Fig. 1) (*see also* discussion by Ueberschar and Bakker-Grunwald [43]). However, the data in Fig. 3 also show that diuretic-sensitive fluxes are balanced even in shrunken cells, so that a simple summation of the chemical activity gradients cannot be the sole determinant of "cotransport" in HeLa cells in these conditions. The affinities of the internal (cytoplasmic) sites of the transport system for Na^+, K^+ and Cl^- have not been determined in HeLa cells, but outward flux would appear to be saturated with normal intracellular ionic compositions. The interaction between Na^+ and K^+ reported for diuretic-sensitive "cotransport" fluxes in MDCK cells [35] may thus play an important part in determining the normal functioning of the system.

The absence of volume regulation in HeLa cells, where a "cotransport" system exists, apparently differs from the avian system in its response to cell shrinkage. However, even in avian red cells marked change in cell volume is only observed with an elevated extracellular K^+ (for gain of cell water) or markedly reduced medium K^+ (for loss of cell water) [39]. In this respect the diuretic-sensitive "cotransport" system in nonepithelial tissues may not be regarded as a volume regulatory system *per se*, but rather as a system concerned with extracellular K^+ homeostasis [13].

The mechanism which underlies the increased diuretic-sensitive transport is not directly addressed in this report; however, it is noteworthy that we report an absence of a significant *trans* effect of external K^+ for K^+ efflux (as seen for avian red cells [21]). Stimulation of the diuretic-sensitive influx may thus result from an increase in the number of functional transport units in the HeLa cell membrane. If this were the case such acute adaptation of the membrane permeability would be absent or of minor importance in non-nucleated cells such as the human red blood cell.

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