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KINETIC PARAMETERS AND MECHANISM OF ACTIVE CATION TRANSPORT IN HeLa CELLS AS STUDIED BY Rb^+ INFLUX

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On incubation of HeLa cells in chilled isotonic medium, intracellular Na^+ (Na_c^+) increased and K^+ (K_c^+) decreased with time, reaching steady levels after 3 h. The steady levels varied in parallel with the extracellular cation concentrations ($[Na^+]_e$, $[K^+]_e$). The cell volumes and the protein and water contents, respectively, of cells kept for 3 h in chilled media of various $[Na^+]_e$ and $[K^+]_e$ were not significantly different. Ouabain-sensitive Rb^+ influx took place at the initial rate for a certain period which depended on $[Na^+]_c$ at the beginning of the assays. The existence of two external K^+ loading sites per Na^+/K^+-pump was demonstrated. The affinities of the sites for Rb^+ as a congener of K^+ were almost the same. Na_e^+ inhibited ouabain-sensitive Rb^+ influx competitively, whereas K_c^+ was not inhibitory. Kinetic parameters were determined: the $K_{1/2}$ for Rb_e^+ in the absence of Na_e^+ was 0.16 mM and th K_i for Na_e^+ was 36.8 mM; the $K_{1/2}$ for Na_c^+ was 19.5 mM and the K_i for K_c^+ seemed to be extremely large. The rate equation of the ouabain-sensitive Rb^+ influx suggests that Na^+ and K^+ are exchanged alternately through the pump by a binary mechanism.

Introduction

Red cells and their ghosts and giant axons are excellent for kinetic studies on the Na^+/K^+-pump. These cells are useful because it is easy to regulate their intracellular ion concentration or replace their cytoplasm by artificial media. From studies on these cells, various transport rate equations have been proposed to describe cation movements through the pumps, in connection with cation environments. On the other hand, the kinetic studies have not extensively been made in other types of cells. For example, there have been only a few studies on the number of cation sites in Ehrlich ascites tumor cells [1] and CHO cells [2]. One disadvantage of such cells is that monovalent cations become unevenly distributed in the cells: Na^+ is preferentially accumulated in the nucleus, whereas K^+ and Cl^- are more highly concentrated in the cytoplasm of Ehrlich ascites tumor cells [3]. Moreover, there are two different K^+ compartments in cultured neuroblastoma cells [4] and CHO cells [5], and such intracellular compartmentation of cation makes analyses of transport mechanisms difficult. Nevertheless, virtually all the K^+ exchange in isolated smooth muscle cells occurs in a manner that can be explained by supposing that the K^+ is distributed in a single intracellular compartment [6]. Rubidium ions also become distributed in a single compartment that kinetically occupies the major part (95%) of the intracellular K^+ compartment in HeLa cells [7]. K^+ and Rb^+ have been shown to accumulate inside various cultured cells at constant rates for certain

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periods after the beginning of assays [2,8–12]. Therefore, data on active Rb^+ influx seem to be available for quantitative purposes in elucidating transport kinetics. In addition, the high density of the Na^+/K^+-pump sites in various cultured cells [13] implies that these cells are suitable for use in a sensitive assay of pump activity.

In the present study we have attempted to derive a rate equation for active Rb^+ transport and to provide kinetic constants for cation transport in HeLa cells.

Materials and Methods

Cell culture. HeLa cells (strain S3) were maintained by serial culture in glass flasks (40 × 55 × 150 mm, Ikemoto Sci. Co.), containing 10 ml of modified minimum essential medium supplemented with 10% (v/v) calf serum, as reported elsewhere [14]. Hepes buffer was omitted from the culture medium. Cells in growing cultures were detached from the flasks with 0.5% trypsin and suspended in the culture medium. They were reinoculated into plastic culture dishes (60 mm diameter, Corning Glass Works) containing 6 ml of medium at a density of $6 \cdot 10^4$ cells/ml and then kept at 37 °C for 48 h in a CO_2-incubator in a humid atmosphere of 5% CO_2 in air.

Cell chilling. The culture medium was replaced by the modified minimum essential medium containing 5.6 mM glucose and 20 mM Hepes (pH 7.2) without serum. The cells were reincubated for 15 min at 37 °C with or without 0.1 mM ouabain, and then the medium was replaced by cold mixtures of an isotonic Na medium and K medium as described below. The Na medium contained 116 mM NaCl, 1 mM NaH_2PO_4 and 21 mM $NaHCO_3$; whereas the K medium contained the corresponding concentrations of KCl, KH_2PO_4 and $KHCO_3$. In addition, both media commonly contained 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5.6 mM glucose and 20 mM Hepes. As the pH of the stock solutions of the Hepes buffer (1 M) was adjusted to 7.2 with NaOH or KOH, the final concentration of Na^+ or K^+ in the media became 140 mM. These media were mixed so that the mixtures contained various concentrations of the monovalent cations as shown in the Results. The cultures were placed in an ice bath for 3 h, unless otherwise stated. The Hepes buffer was essential for preventing detachment of the cells from the culture dishes.

Assay of cation concentration. At the end of the chilling process, cells were washed once and the media were replaced by similar media at 37 °C containing appropriate concentrations of RbCl instead of KCl and various concentrations of NaCl as shown in Results. The isotonicity of the media was maintained by adding different concentrations of choline chloride, so that the sum of the concentrations of sodium, potassium and choline salts was 140 mM. Ouabain-sensitive Rb^+ influx was determined by subtracting the amount of Rb^+ accumulated in the cells in the presence of ouabain from that in the absence of ouabain. As ouabain-sensitive Rb^+ influx occurred at a constant rate for at least 6 min (Fig. 3), influx was normally assayed for 5 min and is expressed in nmol/mg cell protein per min. When the Rb^+ uptake was complete, the media were discarded. The procedures used for washing the cells and assaying the concentrations of alkali metal cations were as reported previously [12], except that another type of flame-spectrophotometer (Model 170-30, Hitachi Ltd.) was used. For cation assay, 15 mM LiCl was used as an internal standard.

Other assays. The diameters of the cells were measured by microscopy and the cell volumes were calculated from the diameters (Table I). The water content of cells was determined by incubation of the cells with ^3H_2O (5 mCi/ml, Amersham International) and inulin-[^{14}C]carboxylic acid (5 mCi/mmol, Amersham International) dissolved in the isotonic media at 5 μCi/ml and 0.5 μCi/ml, respectively. Cell protein was dissolved in 0.5 M NaOH and assayed by the method of Lowry et al. [15] with serum albumin as a standard.

Reagents and other substances. RbCl (Suprapur) was purchased from Merck; specially pure grade KCl, NaCl, LiCl, other inorganic salts and Folin-phenol reagent were from Wako Pure Chem. Co.; ouabain, bovine serum albumin (fraction V) and Hepes were from Sigma Chem. Co.; trypsin (1 : 250) was from Difco Lab.; calf serum was from Nakarai Chem. Ltd. Concentrated solutions of vitamins (100 ×) and amino acids (50 ×) from Flow Laboratories were used in preparation of the modified minimum essential medium.

Results

Effects of cell chilling

Changes in intracellular Na^+ and K^+ (Na_c^+ and K_c^+) with time on incubation at 0 °C in the isotonic media of two different cation compositions are shown in Fig. 1. In medium of high K^+ concentration (100 mM), Na_c^+ increased slightly and became constant at about double the control value after 3 h. K_c^+ also increased slightly in the first 30 min and then remained steady. In K^+-free medium, Na_c^+ increased rapidly, reaching to similar level as K_c^+ in control cells after 3 h. K_c^+ decreased faster than Na_c^+ increased and reached an extremely low level. Therefore, both Na_c^+ and K_c^+ tended to reach constant levels within 3 h at 0 °C, regardless of the cation composition of the medium. Next, we measured Na_c^+ and K_c^+ after chilling the media of various cation compositions for 3 h (Fig. 2). Na_c^+ and K_c^+ tended to change in parallel with the extracellular concentrations of Na^+ and K^+ ($[Na^+]_e$ and $[K^+]_e$). The sum of the contents of Na_c^+ and K_c^+ became nearly the same as that in control cells when $[Na^+]_e$ was higher than 100 mM. With increasing $[K^+]_e$, the sum of Na_c^+ and K_c^+ first increased above the control level and then reached a steady level for $[K^+]_e \geqslant$ 120 mM. As the cell volume was not significantly different after chilling the cells in media of various cation compositions (Table I), this higher level implies that the sum of the intracellular concentrations of these cations tends to increase as $[K^+]_e$ increases. The cell volume and cell water and protein contents were measured after chilling the cells in the media of various cation compositions for 3 h (Table I). There were no significant differences in the respective values. However, the cell volume may change temporarily during chilling, because there is a discrepancy between the rates of change in Na_c^+ and K_c^+ before reaching the steady levels (Fig. 2), and the cations must move with water osmotically. Based on a report of Lamb and MacKinnon [16] that the cell volume did not alter after trypsinization, we measured the diameters of cells suspended in cold media. Cells tended to become round on chilling and so were easily detached from the culture dishes like spheroid or ellipsoid particles. The mean cell volume was 3.06 pl and cell water occupied 82.4% of the total cell volume. These values were consistent with those of 3.08 pl and 79% reported for the same cells [17]. The constancy of the cell volume and cell water and protein contents indicates that the cation contents expressed on the basis of the amount of protein correspond to their intracellular concentrations ($[Na^+]_c$ and $[K^+]_c$) expressed on the basis of the water volume. The chilling technique was used to regulate intracellular cation concentrations in the following experiments.

Time-course of ouabain-sensitive Rb^+ influx

The accumulation of Rb^+ by ouabain-sensitive

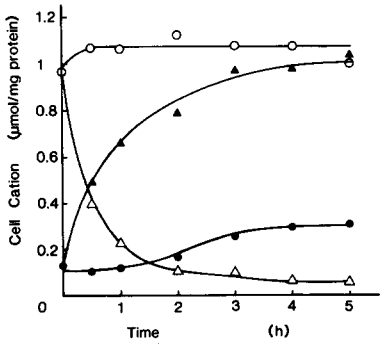

Fig. 1. Time-dependent changes in monovalent cation contents in HeLa cells on chilling at 0 °C for 3 h in isotonic media of different cation compositions. Cation concentrations in the media: circles, 40 mM Na^+ and 100 mM K^+; triangles, 140 mM Na^+ free of K^+. Intracellular cations: closed symbols, Na^+; open symbols, K^+. Points are the means for four samples.

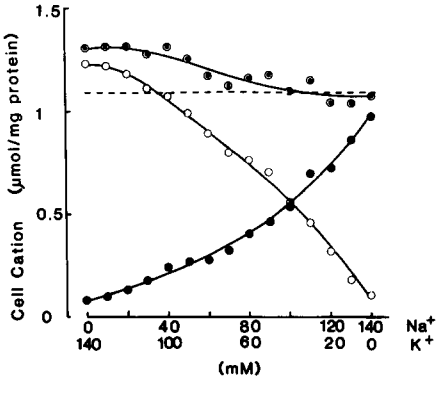

Fig. 2. Changes in intracellular cation contents of HeLa cells on chilling at 0 °C for 3 h in isotonic media of various cation compositions. Cations: ●, Na^+; ○, K^+; and ⊙, Na^+ plus K^+. the broken line indicates the level of Na^+ plus K^+ in control cells. Points are the means for four samples.

TABLE I

CELL VOLUME AND CELLULAR PROTEIN AND WATER CONTENTS OF HeLa CELLS CHILLED FOR 3 H IN ISOTONIC MEDIA OF DIFFERENT CATION COMPOSITIONS

The diameters of the cells were measured by microscopy and the cell volumes were calculated. The water content was determined by incubation of the cells with ^3H_2O (5 μCi/ml) and inulin-[^{14}C]carboxylic acid (0.5 μCi/ml) in the medium. The number of cells used for measuring diameters was 208–411, and the number of culture dishes used for determination of cellular protein and water contents was four.

Cation in medium (mM)		Volume (pl)		Protein (ng/cell)		Water (% of volume)
Na^+	K^+	Expt. A	Expt. B	Expt. C	Expt. D	Expt. E
140	0		2.94	0.515	0.513	83.1
120	20	2.99				
100	40	2.96	2.88	0.484	0.496	82.7
80	60	3.03				
60	80	3.12				
40	100	3.07	3.24	0.465	0.491	80.8
20	120	3.32				
0	140	3.15	2.98	0.475	0.488	82.8
Mean ± S.D.		3.06 ± 0.13		0.491 ± 0.016		82.4 ± 1.0

uptake in cells with different initial Na^+ concentrations, and the changes in $[Na^+]_c$ and $[K^+]_c$ were investigated as functions of the time after rewarming to 37 °C (Fig. 3). Cells with high initial $[Na^+]_c$ accumulated Rb^+ linearly with time for at least 20 min. Although $[Na^+]_c$ decreased with time, the cation was still maintained at 0.14 μmol/mg protein, i.e., 33 mM, after 20 min. The value seems to be above the $K_{1/2}$ for Na_c^+, i.e., 19.5 mM in Table III, suggesting that the decrease in $[Na^+]_c$ may not significantly limit the rate of Rb^+ influx. Active Rb^+ uptake into cells with as low an initial $[Na^+]_c$ as that of control cells continued at a low rate for at least 10 min and then gradually decreased with time. The concentration of Na_c^+ decreased insignificantly. The Rb^+ influx into cells with intermediate initial $[Na^+]_c$, i.e., 0.218 μmol/mg of protein, which was slightly higher than the control level, occurred at an intermediate rate for only 6 min, and then decreased like that in cells with a low $[Na^+]_c$. In contrast to $[Na^+]_c$, $[K^+]_c$ increased with time in cells with a high $[Na^+]_c$, whereas it remained at a constant high level when $[Na^+]_c$ was low or intermediate.

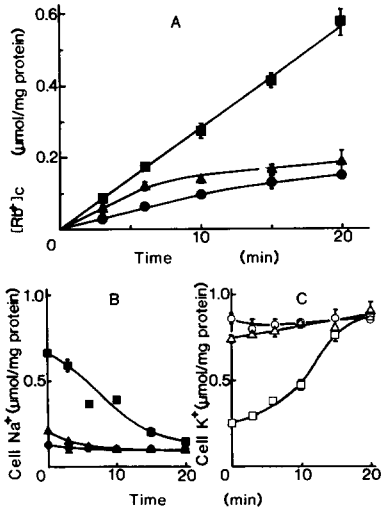

Fig. 3. Time-dependent changes in cation contents in HeLa cells chilled in cold isotonic media of different cation compositions for 3 h. (A) Accumulation of Rb^+ due to ouabain-sensitive influx. (B) Intracellular Na^+. (C) Intracellular K^+. Initial intracellular Na^+ contents: ● and ○, 0.131 ± 0.015 μmol/mg protein (26 ± 3 mM based on 5.13 μl water per mg of protein); ▲ and △, 0.218 ± 0.040 μmol/mg protein (42 ± 8 mM); ■ and □, 0.664 ± 0.017 μmol/mg protein (129 ± 3 mM). Points and bars are the means ± S.D. for four samples.

Determination of kinetic parameters

The effect of Na_e^+ on ouabain-sensitive Rb^+ influx is shown by the double-reciprocal plots in Fig. 4A. There are two sets of lines. Each set consists of three lines obtained for cells with the same $[Na^+]_c$ at three different $[Na^+]_e$ values. The

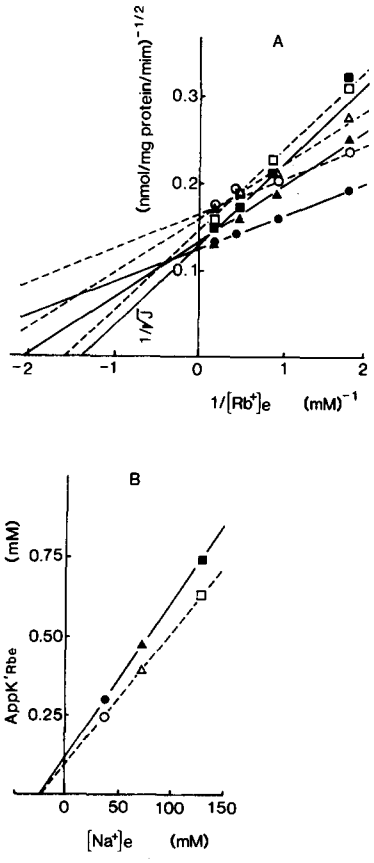

Fig. 4. Effect of extracellular Na^+ on ouabain-sensitive Rb^+ influx in HeLa cells with different Na^+ contents (A) Double-reciprocal plots of the square root of the influx vs. the extracellular Rb^+ concentration. Initial intracellular Na^+ contents: open symbols, 0.324 ± 0.063 μmol/mg protein (63 ± 12 mM); closed symbols, 0.953 ± 0.058 μmol/mg protein (186 ± 11 mM). Extracellular Na^+ concentrations; ● and ○, 36 mM, ▲ and △, 72 mM: and ■ and □, 131 mM. (B) Reciprocals of the intersections of the three lines belonging to each set in (A) with the abscissa, i.e. K'_{Rbe}, as a function of the extracellular Na^+ concentration. $K'_{Rbe} = 0.111$ and 0.128 mM (open and closed symbols, respectively), and $K_{Nae} = 28$ mM.

lines seem to cross at a point close to or on the ordinate. From the intersections of the lines on the abscissa the apparent $K_{1/2}$ values for Rb_e^+ (appK'_{Rbe} values) were calculated and are plotted against $[Na^+]_e$ (Fig. 4B). The three points belonging to each set are obviously on a straight line and the intersections of the line with the abscissa and ordinate represent K'_{Rbe} and K_{Nae}. Thus Eqn. A2 in Appendix 1 was modified taking the relation described above into consideration:

$$1/J = (1/\text{app}J_{max})\{1 + (1 + [Na^+]_e/K_{Nae})K'_{Rbe}/[Rb^+]_e\}^2 \quad (1)$$

Eqn. 1 reveals that Na_e^+ competes with Rb_e^+. Fig. 4B shows two straight lines which converge at a point, i.e. K_{Nae}, on the abscissa. Therefore, K_{Nae} is independent of $[Na^+]_c$ and represents the K_i for Na_e^+. A family of three parallel lines is seen in a similar double-reciprocal plot for cells with different $[Na^+]_c$ values, except in cases where $[Rb^+]_e$ exceeds 2 mM when $[Na^+]_c$ is relatively low (Fig. 5A). In addition to the relation between Na_e^+ and Rb_e^+ expressed by Eqn. 1, the functional relation of J to Na_c^+ and Rb_e^+ can be expressed by the

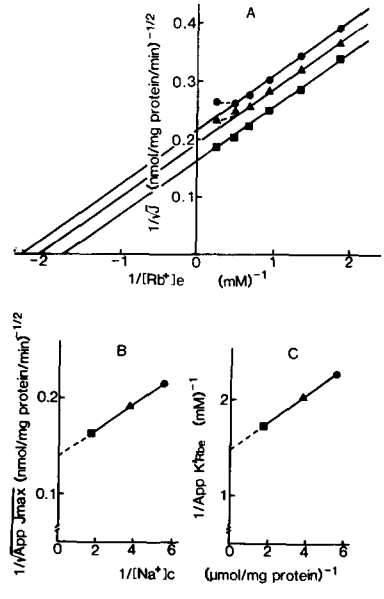

Fig. 5. Stimulatory effect of intracellular Na^+ on ouabain-sensitive Rb^+ influx in HeLa cells as a function of the extracellular Rb^+ concentration. (A) Initial intracellular Na^+ content: ●, 0.180 ± 0.025 μmol/mg protein (35 ± 5 mM); ▲, 0.266 ± 0.018 μmol/mg protein (52 ± 4 mM); ■, 0.584 ± 0.020 μmol/mg protein (113 ± 4 mM). Points are the means for four samples. (B) Intersections of the three lines in (A) with the ordinate plotted against the reciprocals of the initial intracellular Na^+ content values. (C) Intersections of the three lines with the abscissa plotted against the reciprocal of the initial intracellular Na^+ content values. The reciprocals of the Na^+ content values are 5.56, 3.76 and 1.71 mg protein/μmol, respectively. $J_{max} = 51.8$ nmol/mg protein per min, and $K'_{Rbe} = 0.674$ mM.

following equation:

$$1/J = (1/J_{max})\{1+(1+[Na^+]_e/K_{Nae})K_{Rbe}/[Rb^+]_e + appK_{Nac}/[Na^+]_c\}^2 \quad (2)$$

where $appK_{Nac}$ is the apparent $K_{1/2}$ for Na_c^+.

From Eqn. 2

$$1/appJ_{max} = (1/J_{max})\{1+appK_{Nac}/[Na^+]_c\}^2 \quad (3)$$

and

$$1/appK'_{Rbe} = (1/K_{Rbe})(1+appK_{Nac}/[Na^+]_c)/(1+[Na^+]_e/K_{Nae}) \quad (4)$$

Replots of the data in Fig. 5A demonstrate the validity of Eqns. 3 and 4, because, the intersections of the three lines with the ordinate and abscissa, when plotted as functions of the reciprocals of $[Na^+]_c$, are obviously situated on straight lines (Figs. 5B and 5C). Extrapolation of the lines to the ordinates gives estimates of J_{max} and $(1+[Na^+]_e/K_{Nae})K_{Rbe}$, i.e. the apparent $K_{1/2}$ for Rb_e^+. In another experiment, cells with various initial Na^+ concentrations were incubated in media containing 140 mM Na^+ and three different Rb^+ concentrations. With these cells, another family of three parallel lines was obtained by double-reciprocal plots of $\sqrt{J}$ vs. $[Na^+]_c$ (Fig. 6A), which is also well described by Eqn. 2. The values of J_{max} and $appK_{Nac}$ can be calculated from the intersections with the ordinate in Figs. 6B and 6C of the lines obtained by replotting the data in Fig. 6A. Assuming that K_c^+ inhibits ouabain-sensitive Rb^+ influx by competing with Na_c^+, Eqn. 3 can be rewritten as

$$(\sqrt{J_{max}/appJ_{max}}-1)[Na^+]_c = K_{Nac}+(K_{Nac}/K_{Kc})[K^+]_c \quad (5)$$

where K_{Kc} is the K_i for K_c^+.

The left-hand side of the equation is identical with $appK_{Nac}$ and can be calculated, since J_{max} has been obtained (Table III). The $appK_{Nac}$ obtained from the data in Fig. 6C is also directly applicable

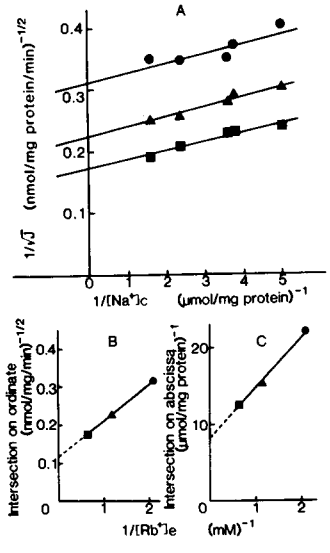

Fig. 6. Stimulatory effect of extracellular Rb^+ on ouabain-sensitive Rb^+ influx in HeLa cells as a function of the initial intracellular Na^+ content. (A) Extracellular Rb^+ concentration: ●, 0.48 mM; ▲, 0.86 mM; and ■, 1.60 mM. Points are the means for four samples. (B) The intersections of the three lines in (A) with the ordinate plotted against the reciprocal of the extracellular Rb^+ concentration. (C) A similar plot of the intersections of the lines with the abscissa. The reciprocals of the Rb^+ concentrations are 2.08, 1.16 and 0.63 mM^{-1}, respectively.

TABLE II

KINETIC PARAMETERS FOR EXTRACELLULAR CATIONS IN HeLa CELLS

Expt.	J_{max} (nmol/mg per min)	K_{Nae} (mM)	Apparent K_{Rbe} (mM)	K_{Rbe} (mM)
F		53		
G	78.3		1.046	
H	58.3		0.599	
I		30		
J		36		
K		28		
L	73.7			
M	47.6		0.551	
N	98.0		0.947	
O	51.8		0.674	
Mean ± S.D.	68.0 ± 17.4 (13.3 ± 3.4 [a])	36.8 ± 9.8	0.763 ± 0.197	0.159 [b]

[a] J_{max} expressed in mmol/l water per min.

[b] K_{Rbe} calculated as $appK_{Rbe}/(1+[Na^+]_e/K_{Nae})$ taking $[Na^+]_e = 140$ mM.

TABLE III

KINETIC PARAMETERS FOR INTRACELLULAR CATIONS IN HeLa CELLS

Expt.	$[K^+]_c$	μmol/mg protein	
		Apparent K_{Nac}	K_{Nac}
H	0		0.088 [b]
	0.378	0.091	
	1.234	0.083	
	1.379	0.089	
L		0.122 [a]	
M	0		0.112 [b]
	0.574	0.111	
	0.818	0.112	
	0.943	0.112	
O	0		0.099 [b]
	0.534	0.097	
	0.898	0.101	
	1.007	0.098	
Mean ± S.D.		0.102 ± 0.012	0.100 ± 0.010 (19.5 ± 2.0 [c])

[a] Value obtained in the experiment shown in Fig. 6.

[b] Value obtained by extrapolation of values of appK_{Nac} to $[K^+]_c = 0$.

[c] Value expressed in mmol/l water.

to the equation. Table III shows that the values of appK_{Nac} are almost constant, regardless of change in $[K^+]_c$, indicating extremely large values of K_{Kc} compared with those of K_{Nac}. Therefore, the effect of K_c^+ as an inhibitor of the Na^+/K^+-pump can be ignored, at least when its concentration does not exceed the control level. The value of K_{Kc} cannot be determined exactly until techniques are available to control $[Na^+]_c$ at constant $[K^+]_c$, and vice versa. The various kinetic constants obtained so far are listed in Tables II and III with respect to extracellular and intracellular cations.

The value of K_{Rbe} is calculated from the mean of the apparent K_{Rbe} by substituting the mean of K_{Nae} and taking $[Na^+]_e$ as 140 mM. Finally, we obtain the following rate equation from experimental results for ouabain-sensitive Rb^+ influx:

$$1/J = (1/J_{max})\{1 + (1 + [Na^+]_e/K_{Nae})K_{Rbe}/[Rb^+]_e + K_{Nac}/[Na^+]_c\}^2 \quad (6)$$

If K_c^+ acted as an uncompetitive inhibitor of the pump, then a similar set of parallel lines to that in Fig. 5A should be obtained. However, this was not

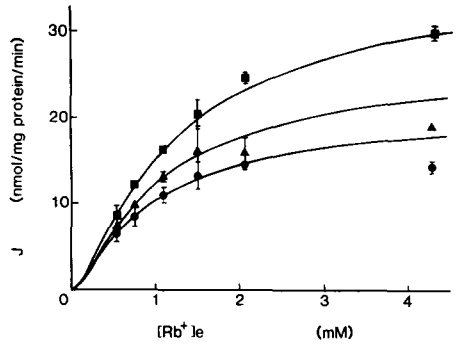

Fig. 7. Relation of ouabain-sensitive Rb^+ influx to the extracellular Rb^+ concentration in HeLa cells with different Na^+ contents. Data are the same as for Fig. 5. The curves were drawn according to Eqn. 6 taking J_{max} as 51.76 nmol/mg protein per min, K_{Rbe} as 0.14 mM, K_{Nae} as 36.8 mM, and K_{Nac} as 99 nmol/mg protein (19 mM). For symbols, see Fig. 5. Points and bars are the means ± S.D. for four samples.

the case, because plots of the reciprocal of $\sqrt{\text{app}J_{max}}$ and of appK'_{Rbe} against $[K^+]_c$ did not give straight lines (data not shown). Eqn. 6 was found to describe fairly well experimental results in cells with high $[Na^+]_c$ over a wide range of $[Rb^+]_e$ and in cells with low $[Na^+]_c$ when $[Rb^+]_e$ did not exceed 2 mM (Fig. 7).

Discussion

There have been few studies on changes in Na_c^+ and K_c^+ on chilling cells in cold isotonic media of various cation concentrations in any other cells than red cells. Increase in Na_c^+ and decrease in K_c^+ were shown only in normal culture media or K^+-free media in HeLa cells [12,18] and Ehrlich ascites tumor cells [19,20]. Our detailed examinations revealed that the cations tended to reach different steady levels in chilling HeLa cells for 3 h in isotonic media of different extracellular cation compositions (Figs. 1 and 2). Thus, the chilling technique is a convenient method for changing $[Na^+]_c$ while keeping the pump activity intact.

Vaughan and Cook [10] reported that the Na^+/K^+-pump in HeLa cells has similar affinities for K^+ and Rb^+. No significant difference was found in the influx and washout kinetics of $^{42}K^+$ and $^{86}Rb^+$, or in the inhibitions of unidirectional $^{42}K^+$ or $^{86}Rb^+$ influx by ouabain or furosemide in 3T3 cells [21]. Influx of $^{86}Rb^+$ did not

deviate significantly from a 1 : 1 ratio with influx of $^{42}K^+$ in dog kidney cells [9]. In primary astroglial cultures $^{86}Rb^+$ influx also showed the 1 : 1 ratio with K^+ influx after the cells, which had been exposed to ouabain, were allowed to reaccumulate K^+ with or without $^{86}Rb^+$ added [22]. Furthermore, equalities of the apparent K_d values for K^+ and Rb^+ for ouabain-sensitive, furosemide-sensitive, and ouabain- and furosemide-insensitive influxes into HeLa cells were demonstrated [7]. Hence, Rb^+ has been found to be useful in investigating functions of the Na^+/K^+-pump in some cultured cells, including HeLa cells. Therefore, in this work we conducted experiments on total replacement of K_e^+ by Rb^+.

Effects of extracellular cations

Extracellular Na^+ is known to cause competitive inhibition of the Na^+/K^+-pump activated by extracellular K^+ in red cells and giant axons [23–26]. We observed similar competitive inhibition of active Rb^+ influx into HeLa cells by Na_e^+. The apparent $K_{1/2}$ for Rb_e^+ was 0.76 mM in the presence of 140 mM Na_e^+. This apparent $K_{1/2}$ is almost the same as that of 0.8 mM reported for the cells by Cook et al. [18], but slightly smaller than values of 1.0–1.5 mM reported for the same cells [10,27] and 3T3 cells [28,29]. Using the value of 0.76 mM, we calculated the $K_{1/2}$ in the absence of Na_e^+ to be 0.16 mM. Values for this $K_{1/2}$ in cultured cells have not been reported previously. The K_i for Na_e^+ is 36.8 mM, which is much lower than the Na^+ concentration in normal culture medium. The $K_{1/2}$ and K_i values seem similar to those obtained in red cells, giant axons and frog epithelial cells [23–26,30–33]. The $K_{1/2}$ values are similar to the K_m values of $(Na^+ + K^+)$-ATPase stimulated by K^+ [25,34,35] and of Rb^+ binding to the enzyme [36]. Allosteric inhibition of ouabain-sensitive K^+ influx by Na_e^+ in red cells [26,37] and negative cooperative effects of Na^+ on K^+ activation of $(Na^+ + K^+)$-ATPase [34] have been reported. However, the problem of whether Na_e^+ acts in HeLa cells only as dead-end inhibitor or as an inhibitor with both competitive and allosteric effects remains unsolved.

Effects of intracellular cations

In a review of the physiological role of K_c^+ it was pointed out that the cation behaves as an inhibitor of the Na^+/K^+-pump [38]. A competitive or heterotropic influence of K_c^+ on the pump stimulated by Na_c^+ has been observed in red cells or their ghosts [39,40] and in squid axons [32]. Similar inhibitory effects of K_c^+ on $(Na^+ + K^+)$-ATPase [34,41] are well known. In cultured plasmocytoma cells, active K^+ influx became greater when K_c^+ was lowered, suggesting regulation of K^+ influx by K_c^+ [42]. However, the results did not exclude a possible role of Na_c^+, because Na_c^+ was not determined. In contrast, no stimulatory effect on Na^+ efflux was observed in leucocytes when K_c^+ was reduced [43]. We showed that the effect of K_c^+ on active Rb^+ influx could be ignored (Table III). Furthermore, our observation of a parallel relation of a family of lines with different fixed $[Na^+]_c$ (Fig. 5A) provides further evidence for the insensitivity of the pump to K_c^+, because, similar parallelism was observed between results with two series of red cells with different fixed $[Na^+]_c$ when K_c^+ was omitted [31]. The apparent K_m for K_c^+ at internal K^+ discharge sites is far higher than the K_i for K_c^+ as a competitor of Na_c^+ [44]. This implies that in HeLa cells the affinity for Rb_c^+ is extremely low at the internal Na^+ loading sites and especially at the K^+ discharge sites, and hence that Rb^+ influx through the pump is almost irreversible. The pumps of mature high-K^+ sheep red cells and reticulocytes, which have high densities of pump sites, were relatively insensitive to increase in $[K^+]_c$, whereas K_c^+ markedly inhibited the pump in mature low-K^+ sheep red cells [45]. This indicates that the insensitivity of the pump to K_c^+ in HeLa cells may be related to the large number of pumps and high K^+ concentration in the growing phase. The $K_{1/2}$ for Na_c^+ was 20 mM, which was in the range of Na^+ concentrations in control cells. Therefore, even a small increase in $[Na^+]_c$ for some reason should raise the pump activity significantly, as suggested by Pollack et al. [46], whereas a change in $[K^+]_c$ should have relatively little effect. The $K_{1/2}$ obtained was slightly higher than the apparent $K_{1/2}$ of Na^+ efflux (about 10–15 mM) reported for HeLa cells [46], and also higher than the values obtained in red cells [31,39].

Mechanisms of cation transport

Eqn. 6 corresponding to the families of parallel lines at various fixed $[Na^+]_c$ and $[Rb^+]_e$ in Figs. 5A and 6A, seems to describe a ping-pong bi-bi mechanism [47]. However, for the classical ping-pong mechanism to operate the products of the reaction, i.e. Na_e^+ and K_c^+, must theoretically be zero in the initial step of the reaction. Since no technique has yet been established to eliminate the cations in HeLa cells completely, active Rb^+ influx must be assayed in the presence of these ions. Nevertheless, as we have shown that the influence of K_c^+ on the pump can be ignored and that Na_e^+ apparently acts as a competitive inhibitor of K_e^+, the influx kinetics of Rb^+ may be described by Eqn. B4 (Appendix 2) and understood to follow a consecutive sequence explained by a model of the primary active transport (Fig. 9).

We demonstrated the existence of two external K^+ loading sites with almost the same affinities (Appendix 1). The presence of two sites with different affinities in CHO cells was also suggested recently by Graves and Wheeler [2]. These findings are inconsistent with results on other cells, including HeLa cells [6,10,28]. Although the main purposes of the latter studies were not to elucidate the mechanism of active transport, they demonstrated a Michaelis-Menten relation between the active or total K^+ or Rb^+ influx and its extracellular concentration. Such a relation would imply the presence of only one site. There have been many studies on the number of K^+ loading sites per pump in red cells [26,31,39,41] and giant axons [30] and of K^+ binding sites of $(Na^+ + K^+)$-ATPase in preparations from various sources [48–50]. Most results have indicated the existence of two sites with similar or equal affinities or with different affinities. Therefore, it seems reasonable to consider that there are two external K^+ loading sites per pump unit in cultured cells. Recently, protein assays by direct quantitation of amino acids have revealed that each α-subunit of the $(Na^+ + K^+)$-ATPase molecule involves a functioning phosphorylation site [51–53]. This rules out the 'half-of-the-site' mechanism of the pump, which has been proposed as essential for simultaneous transport of Na^+ and K^+ by unitary mechanism.

Thus a kinetic model of the pump with the characteristics of a consecutive sequence and binary mechanism in HeLa cells seems consistent with results. According to Eqn. 6, two sodium ions and two potassium ions are considered to be exchanged. If 3 Na^+/2 K^+ coupling occurs, it is unknown how the remaining one sodium ions moves outwards based on this kinetic model.

Appendix 1

The number of external K^+ loading sites in a pump unit can be estimated from a Hill plot (Fig. 8A). For this purpose we used the results obtained for cells with a high fixed $[Na^+]_c$ reported in a previous paper (Fig. 2B of Ref. 12), since they covered a sufficiently wide range of $[Rb^+]_e$. The Hill coefficient (n) was found to be 1.5, suggesting the existence of at least two sites. When K''_{Rb1} and K''_{Rb2} are the apparent $K_{1/2}$ values for the first and second sites, we obtain the following equation:

$$1/J = (1/\mathrm{app}J_{max})(1 + K''_{Rb1}/[Rb^+]_e)(1 + K''_{Rb2}/[Rb^+]_e) \quad \text{(A1)}$$

where J is the ouabain-sensitive Rb^+ influx, and $\mathrm{app}J_{max}$ is the apparent maximum ouabain-sensitive Rb^+ influx. The equation best describes results when $K''_{Rb1} \cdot K''_{Rb2} = 0.686$ and $K''_{Rb1} + K''_{Rb2} = 1.657$. From this, the parameters were calculated as 0.85 and 0.81 (Fig. 8B). Namely, the two sites have almost the same affinities. Thus Eqn. A1

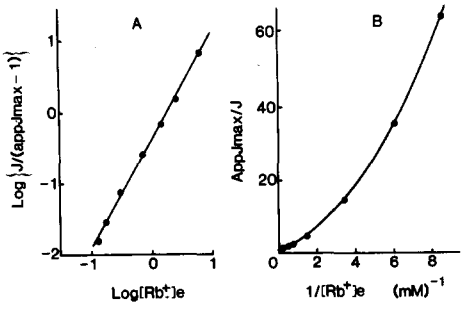

Fig. 8 Estimation of the number of external K^+-loading sites in HeLa cells. (A) Hill plot. (B). Double-reciprocal plot of a relative ouabain-sensitive Rb^+ influx vs. extracellular Rb^+ concentration. The apparent J_{max} is 51.8 nmol/mg protein per min (10 mM/min).

can be simplified to

$$1/J = (1/\mathrm{app}J_{max})\left(1 + K''_{Rbe}/[Rb^+]_c\right)^2 \quad (A2)$$

where K''_{Rbe} is the apparent $K_{1/2}$ for Rb_e^+ of the sites under the conditions described. As suggested in the previous paper this equation may hold, since all points for a double-reciprocal plot of $\sqrt{J}$ vs. $[Rb^+]_e$ fell on a straight line. However, the equation has not yet been analyzed precisely.

Appendix 2

The transport mechanism indicated by the equations in Results seems to be explained by a model, which involves eight enzyme-containing species and eight interconversion steps (Fig. 9). For simplicity, a sequence of reactions occurring in a single channel is treated in the model. Subscript e and c indicate the solutes (A and B) located in the extracellular and intracellular compartments. Also, ′ and ″ refer to external and internal faces of the membrane. k_i and k_{-i} are the rate constants for forward and backward reactions at the slow translocation steps, while the K values are the dissociation or equilibrium constants at the rapidly equilibrating steps. According to the proposal of Heinz [54], we assume that conformation

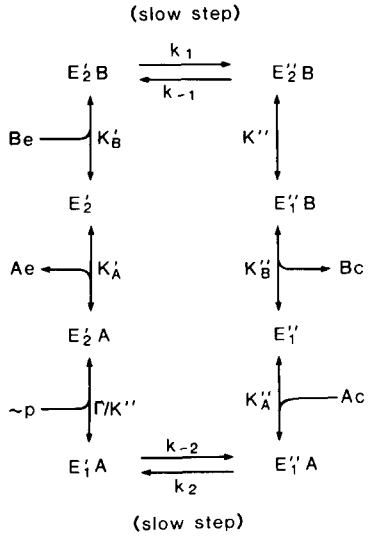

Fig. 9. Schematic presentation of a model system transporting two different solutes in consecutive sequence.

change from E_1 to E_2 is symmetric on both sides of the membrane and spontaneous, whereas that to the reverse direction requires energy supplied from coupled hydrolysis of a energy-rich substrate s to p. Then, we obtain

$$[E'_2A]/[E'_1A] = \Gamma/K'' \quad (B1)$$

where $\Gamma = (s/p)K_{ps}$, $[E''_1B]/[E''_2B] = K''$, and K_{ps} is the dissociation constant of the hydrolytic reaction.

Influx of B, J_B, is equal to the rate of reaction at the rate-limiting step between E'_2B and E''_2B:

$$J_B = k_1 \cdot [E'_2B] - k_{-1} \cdot [E''_2B] \quad (B2)$$

An application of the method of Cha [55] to the model, which has incorporated the relation indicated by Eqn. B1, leads to a general expression as

$$J_B = [E_t]\left\{k_1 \cdot k_2 \cdot \frac{[B_c]}{K'_B}\frac{[A_c]}{K''_A} - k_{-1} \cdot k_{-2} \cdot \frac{1}{\Gamma}\frac{[A_e]}{K'_A}\frac{[B_c]}{K''_B}\right\}$$
$$/\left[\left\{k_{-2} \cdot \frac{K''}{\Gamma}\frac{[A_e]}{K'_A} + k_1 \cdot \frac{[B_e]}{K'_B}\right\}\right.$$
$$\times\left\{1 + \frac{[A_c]}{K''_A} + \left(1 + \frac{1}{K''}\right)\frac{[B_c]}{K''_B}\right\}$$
$$+\left\{1 + \left(1 + \frac{K''}{\Gamma}\right)\frac{[A_e]}{K'_A} + \frac{[B_e]}{K'_B}\right\}$$
$$\left.\times\left\{k_2 \cdot \frac{[A_c]}{K''_A} + k_{-1} \cdot \frac{1}{K''}\frac{[B_c]}{K''_B}\right\}\right] \quad (B3)$$

where $[E_t]$ is the total concentration of the enzyme-containing species. Eqn. B3 can be converted to a simple form, provided that $K'_A \gg [A_e]$, $K''_B \gg [B_c]$, $K'' > 1$, $\Gamma \gg K''$, and k_{-1} and k_{-2} are small:

$$J_B = J_{max}/\{1 + K'_{Be}/[B_e] + K''_{Ac}/[A_c]\} \quad (B4)$$

where $J_{max} = [E_t] \cdot k_1k_2/(k_1 + k_2)$, $K'_{Be} = K'_B \cdot k_2/(k_1 + k_2)$, and $K''_{Ac} = K''_A \cdot k_1/(k_1 + k_2)$. Eqn B4 corresponds to Eqn. 6 in Results and describes active transport of the solutes through the channel, since the equation holds when Γ is much larger than K''. The proposed model interpretes that the

solute A and B are bound to the site and moved through the channel in a consecutive sequence, but not simultaneously.

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