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Kinetic Study on the Effects of Intracellular K^+ and Na^+ on Na^+,K^+,Cl^- Cotransport of HeLa Cells by Rb^+ Influx Determination

Toshitaka Ikehara, Hisao Yamaguchi, Keiko Hosokawa, Akira Takahashi, and Hiroshi Miyamoto
Department of Physiology, School of Medicine, The University of Tokushima, Tokushima 770, Japan

Summary. The effects of intracellular K^+ and Na^+ (K^+c, Na^+c) on the Na^+,K^+,Cl^- cotransport pathway of HeLa cells were studied by measuring ouabain-insensitive, furosemide-sensitive Rb^+ influx (JRb) at various intracellular concentrations of K^+ and Na^+ ($[K^+]c$, $[Na^+]c$). When $[K^+]c$ was increased and $[Na^+]c$ was decreased, keeping the sums of their concentrations almost constant, JRb as a function of the extracellular Rb^+ or Na^+ concentration ($[Rb^+]e$, $[Na^+]e$) was stimulated. However, the apparent $K_{0.5}$ for Rb^+e or Na^+e remained unchanged and the ratio of the apparent $K_{0.5}$ for K^+c and the apparent K_i for Na^+c was larger than 1. When JRb was increased by hypertonicity by addition of 200 mM mannitol, the apparent maximum JRb increased without change in the apparent $K_{0.5}$ for Rb^+e. These results show that K^+c stimulates and Na^+c inhibits JRb, without change in the affinities of the pathway for Rb^+e and Na^+e. The affinity for K^+c is slightly lower than that for Na^+c. Hypertonicity enhances JRb without any change in the affinity for Rb^+e. We derived a kinetic equation for JRb with respect to K^+c and Na^+c and proposed a general and a special model of the pathway. The special model suggests that, in HeLa cells, JRb takes place when Rb^+e binds to the external K^+ binding site of the pathway after the binding of K^+c to the internal regulatory site.

Key Words furosemide · HeLa cells · Na^+,K^+,Cl^- cotransport · potassium · rubidium influx · sodium

Introduction

The transmembrane chemical gradients of K^+ and Na^+ are produced by the Na^+ pump in most cells. The Na^+ gradient generates the driving force for symports and antiports of many essential substances. One Na^+-dependent symport is electrically silent Na^+,K^+,Cl^- cotransport, which is known to be important for Na^+ reabsorption in Henle's loops of the renal tubules. This cotransport pathway is specifically sensitive to loop diuretics such as furosemide, bumetanide and pyretanide. The pathway has been demonstrated to work in the plasma membranes of many types of animal cells such as squid axons [3, 30], fish cells [29], mammalian cells including tumor and cultured cells [1, 2, 12, 14, 21, 34] and different species of erythrocytes [4, 7, 8, 10, 32]. $K^+(Rb^+)$ influx mediated by the cotransport pathway in HeLa cells constitutes a significant part of the K^+ influx comparable with that mediated by the Na^+ pump [17].

Studies on the kinetic mechanisms of $K^+(Rb^+)$ influx into mammalian cells through the cotransport pathway have revealed the requirements for Na^+ and Cl^- in the medium. Na^+, K^+ and Cl^- are moved across the cell membrane at a stoichiometry of 1:1:2 in different types of cells [20, 24, 33] and some kinetic parameters for the diuretic-sensitive cotransport in relation to extracellular ion have been estimated [5, 34]. Moreover, a few investigators have proposed models of the cotransport pathway with special reference to the sequence of bindings of the related ions on the internal and external faces of the cell membrane in human and duck erythrocytes [5, 9, 23]. We have also determined some kinetic parameters for the furosemide cotransport pathway of HeLa cells confirming the 1:1:2 stoichiometry of Na^+, Rb^+ and Cl^-, and proposed models of the sequence of ion bindings to the external face and the reaction of ATP on the internal face of the cell membrane [18, 25].

As an extension of our preceding studies, in this work we investigated the effects of intracellular K^+ and Na^+ on ouabain-insensitive, furosemide-sensitive Rb^+ influx into HeLa cells. We estimated the ratio of the $K_{0.5}$ and the K_i for intracellular K^+ and Na^+, respectively, with respect to the Rb^+ influx. We proposed kinetic equations and transport models for the effects of intracellular K^+ and Na^+ on the furosemide-sensitive cotransport pathway.

Materials and Methods

Cell Culture

HeLa cells (strain S3) purchased from Flow Laboratories (McLean, VA) were serially cultured in glass flasks before use.

The cells in growing cultures were detached from the flasks with 0.5% trypsin (1:250, Difco Laboratories, Detroit, MI) and inoculated at a cell density of 5×10^4 cells/ml into plastic culture dishes (60 mm diameter, No. 25010, Corning Glass Works, Corning, NY) containing 5 ml of a culture medium. The medium used was a modified Eagle's minimum essential medium (mMEM) based on Earle's saline solution [26], enriched with amino acids and vitamins and supplemented with 10% (vol/vol) calf serum. The cultures were kept at 37°C for 48 hr in a CO_2 incubator in a humid atmosphere of 5% CO_2 in air.

Assays

Cells growing exponentially in culture dishes were used for assay of Rb^+ influx. As reported elsewhere [16], the intracellular concentrations of K^+ and Na^+ were changed gradually with time by adding 5 μl of 10 mM ouabain to dishes containing 5 ml of conditioned culture medium (final ouabain concentration, 10 μM). The cells were preincubated in this medium for different periods 5 of 15 min to 5 hr at 37°C in the CO_2 incubator.

The culture medium was removed and the cells were washed twice within a few seconds with a medium containing 5 mM glucose and various inorganic salts. The medium was similar to Earle's solution but contained 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) to maintain the pH at 7.2. As HeLa cells do not distinguish Rb^+ from K^+ in ouabain-sensitive uptake and ouabain-insensitive, furosemide-sensitive influx [18, 25, 27], KCl in the medium was totally replaced by 5 mM RbCl and other potassium salts were replaced by sodium salts. Thus, the medium was isotonic but finally contained 5 mM Rb^+ and 145 mM Na^+. It was named Rb^+ medium. After the washings, the cells were incubated in Rb^+ medium with or without 0.2 mM furosemide in the presence of 10 μM ouabain and allowed to take up Rb^+ for the various periods described in Results. Then they were washed six times with cold 150 mM $LiNO_3$ in 15 sec by the technique described elsewhere [15]. A volume of 3.5 ml of chilled, deionized and distilled water was introduced into each culture dish and the attached cells were scraped off with a silicon-rubber policeman. A 1-ml aliquot of the resulting cell suspension was mixed with an equal amount of 1 N NaOH for protein assay. Another 2-ml aliquot was kept overnight at room temperature to lyze the cells completely and be used for cation assay. Rb^+, K^+ and Na^+ were measured by flamephotometry as described previously [15], except that another type of flamephotometer (type 170-30, Hitachi, Tokyo, Japan) was used. First, Rb^+ influx was expressed in nmol/mg of protein · min and the intracellular contents of K^+ and Na^+ were expressed in nmol/mg of protein, and then the values were converted to mmol/liter of cell water · min and mmol/liter of cell water, respectively.

Protein was assayed with Folin-phenol reagent by the method of Lowry et al. [22], with bovine serum albumin (fraction V) as a standard.

The osmotic pressure of the incubation medium was increased by addition of various concentrations of mannitol up to 300 mM to the medium. Osmotic pressure was determined with an osmometer (Osmostat OM-6020, Kyoto Daiichi Kagaku, Kyoto, Japan), with sucrose as a standard.

The cellular water content was determined in replicate cultures with [^{14}C]-urea (specific activity 2.1 GBq/mmol, Amersham International, Buckinghamshire, UK) as described in details elsewhere [16]. For equilibrium of the intra- and extracellular concentrations of radioactive urea, the agent was added to the conditioned culture medium at 37 kBq/ml and ouabain was added for the last 15 to 30 min of the preincubation period. Then the labeled cells were washed with cold 150 mM $LiNO_3$ and sampled. Radioactivity was measured with a liquid scintillation spectrometer (LSC-62, Aloka, Tokyo, Japan). The cellular water content was expressed as ml/mg of protein.

Reagents and Other Substances

Super-pure grade RbCl was purchased from Merck; guaranteed grade NaCl, KCl, $LiNO_3$, other inorganic salts and mannitol were from Wako Pure Chemical (Osaka, Japan); ouabain, furosemide, HEPES and bovine serum albumin (fraction V) were from Sigma Chemical (St. Louis, MO); glucose and calf serum from Nacalai Tesque (Kyoto, Japan). Concentrated solutions of vitamins (100×) and amino acids (50×) purchased from GIBCO Laboratories (Grand Island, NY) were used to prepare mMEM.

Symbols and Definitions

e and *c*:	Subscripts referring to external and internal faces of the cell membrane
[ ]:	Concentration of an ion
*J*Rb:	Ouabain-insensitive, furosemide-sensitive Rb^+ influx
*app.J*Rb*max*:	Apparent maximum *J*Rb
*J*Rb*max*:	Apparent maximum *J*Rb at $[Na^+]c = 0$
*K*K*e*:	Apparent $K_{0.5}$ for K^+e
*K*Rb*e*:	Apparent $K_{0.5}$ for Rb^+e in relation to *J*Rb
*K*Na*e*:	Apparent $K_{0.5}$ for Na^+e in relation to *J*Rb
*K*K*c*:	Apparent $K_{0.5}$ for K^+c in relation to *J*Rb
*K*Na*c*:	Apparent K_i for Na^+c in relation to *J*Rb

Results

Basic Experiments

To change $[K^+]c$ and $[Na^+]c$ without significant change in the sum of the two cation concentrations, we incubated HeLa cells for various periods in the medium containing 10 μM ouabain as described in Materials and Methods.

The time courses of Rb^+ accumulation in HeLa cells after change to Rb^+ medium were investigated using the cells with two different combinations of $[K^+]c$ and $[Na^+]c$ (Fig. 1). Irrespective of whether $[K^+]c$ was more or less than $[Na^+]c$, ouabain-insensitive Rb^+ accumulation occurred linearly with time for at least 30 min both in the absence and presence of 0.2 mM furosemide (Fig. 1*A* and *B*). *J*Rb was obtained by subtracting Rb^+ influx in the presence of furosemide from that in its absence. The influx took place at the initial rates for 30 min, though $[K^+]c$ and $[Na^+]c$ changed somewhat during the incubation. Based on these results, we assayed Rb^+ influx in 15 min, unless otherwise stated. From the

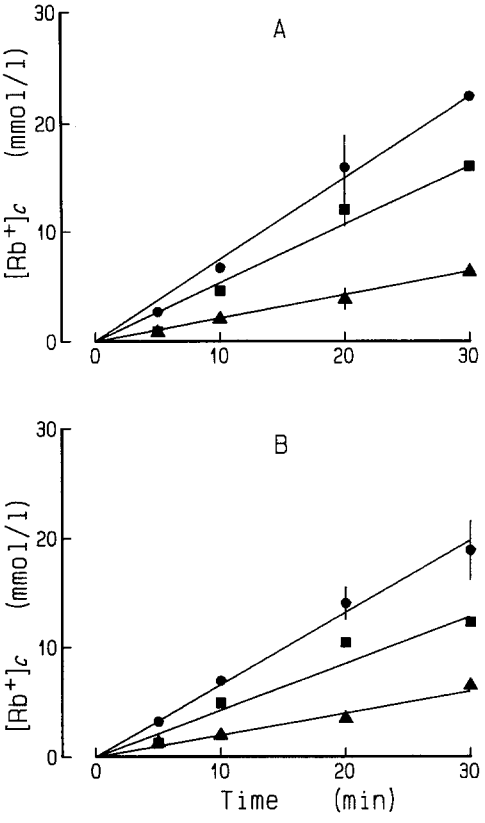

Fig. 1. Time course of changes in $[Rb^+]c$ of HeLa cells after medium change to the Rb^+ medium. The cells initially contained different combinations of $[K^+]c$ and $[Na^+]c$ in the presence of ouabain. (*A*) Changes in $[Rb^+]c$ of the cells initially with a $[K^+]c$ of 94.8 and $[Na^+]c$ of 50.4 mmol/liter of cell water. (*B*) Changes in $[Rb^+]c$ of the cells initially with a $[K^+]c$ of 61.7 and $[Na^+]c$ of 71.4 mmol/liter of cell water. ●, $[Rb^+]c$ in the presence of 10 μM ouabain; ▲, $[Rb^+]c$ in the presence of 10 μM ouabain and 0.2 mM furosemide; ■, ouabain-insensitive, furosemide-sensitive Rb^+ accumulation. Points and bars are means ± SD for four samples.

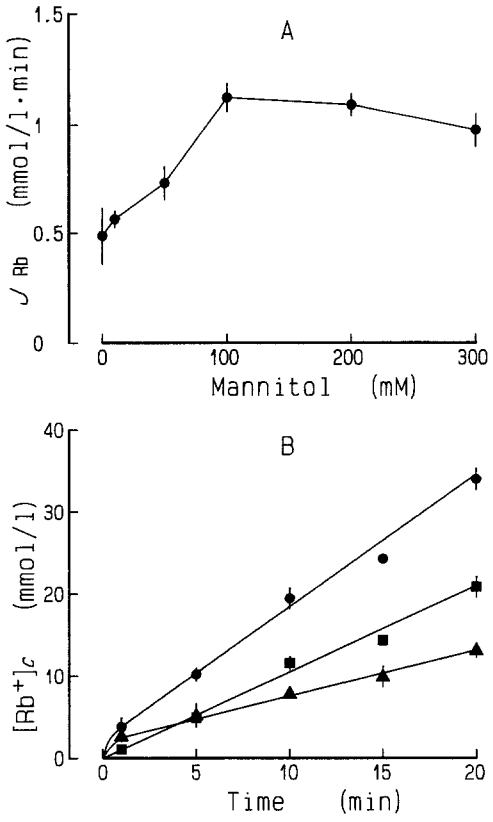

Fig. 2. Effects on *J*Rb into HeLa cells of hypertonicity produced by addition of mannitol to the medium. (*A*) Effects of various concentrations of mannitol on *J*Rb. The osmolalities were determined to be 292, 301, 336, 386, 485 and 586 mOsM upon additions of 0, 10, 50, 100, 200 and 300 mM mannitol, respectively. (*B*) Time-dependent Rb^+ accumulation in the cells in the presence of 200 mM mannitol. ●, 10 μM ouabain; ▲, 10 μM ouabain and 0.2 mM furosemide; ■, ouabain-insensitive, furosemide-sensitive Rb^+ accumulation. Initially $[K^+]c$ and $[Na^+]c$ were 117 and 22 mmol/liter of cell water, respectively. Points and bars are means ± SD for four samples.

results in Fig. 1*A* and *B*, the values of *J*Rb were 0.54 and 0.43 mmol/liter of cell water · min.

As we needed to stimulate *J*Rb to avoid saturation of the flux at relatively low $[Rb^+]e$, at time zero we place the cells in hyperosmotic Rb^+ media containing various concentrations of mannitol. As shown in Fig. 2*A*, *J*Rb increased with increase in the mannitol concentration to a plateau at 100 mM that was nearly double the rate without mannitol. The time course of hypertonicity-stimulated Rb^+ accumulation in the cells during incubation in medium containing 200 mM mannitol in the presence and absence of furosemide was also examined (Fig. 2*B*). Although the cell water content presumably decreased from the initial value of 7.78 μl and so $[K^+]c$ and $[Na^+]c$ changed from the normal values with time during the incubation, ouabain-insensitive, furosemide-sensitive Rb^+ accumulation proceeded linearly with time for at least 20 min.

*J*Rb stimulated by the addition of 200 mM mannitol was tested as a function of $[Rb^+]e$ (Fig. 3). The Rb^+ influx into the cells with normal cation concentrations seemed to be saturated at a $[Rb^+]e$ of more than 3 mM in the isotonic medium, whereas the influx was enhanced and not saturated even at 5 mM in the hypertonic medium. The regression curves in Fig. 3*A* as well as those in other similar figures shown below were based on Eq. (A3) in the Appendix and drawn as best-fit curves by application of the nonlinear least-squares method. Double reciprocal plots of *J*Rb *vs.* $[Rb^+]e$ show that points stand in two lines, except those corresponding to saturated *J*Rb in the isotonic medium. The intercepts of the lines with the horizontal axis are not signifi-

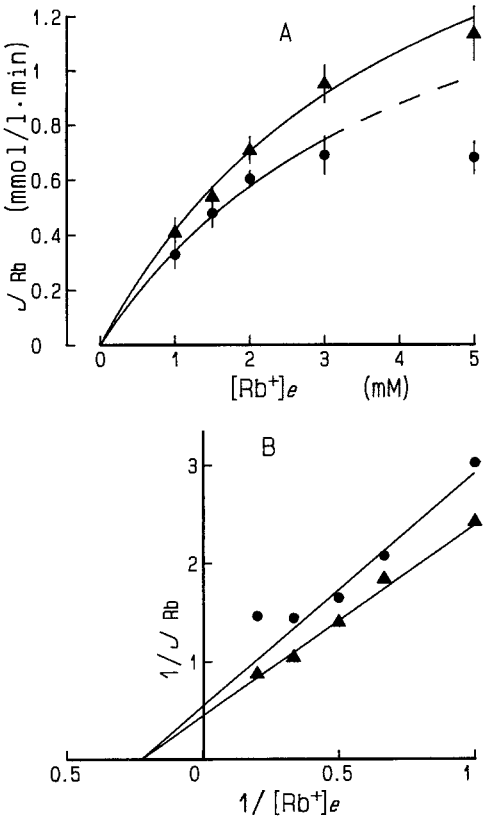

Fig. 3. Effect on *J*Rb into HeLa cells of hypertonicity (485 mOsM) produced by addition of 200 mM mannitol. (*A*) *J*Rb as a function of $[Rb^+]e$. Curves were drawn by the nonlinear least-squares method. (*B*) Double-reciprocal plots of the data in *A*. Lines are the regression lines. Experimental points are proved to significantly regress to the lines by the F-test ($P < 0.005$). ●, control; ▲, 200 mM mannitol. Initially the cells contained the same $[K^+]c$ and $[Na^+]c$ as the cells used in Fig. 2. Points and bars are means ± SD for four samples.

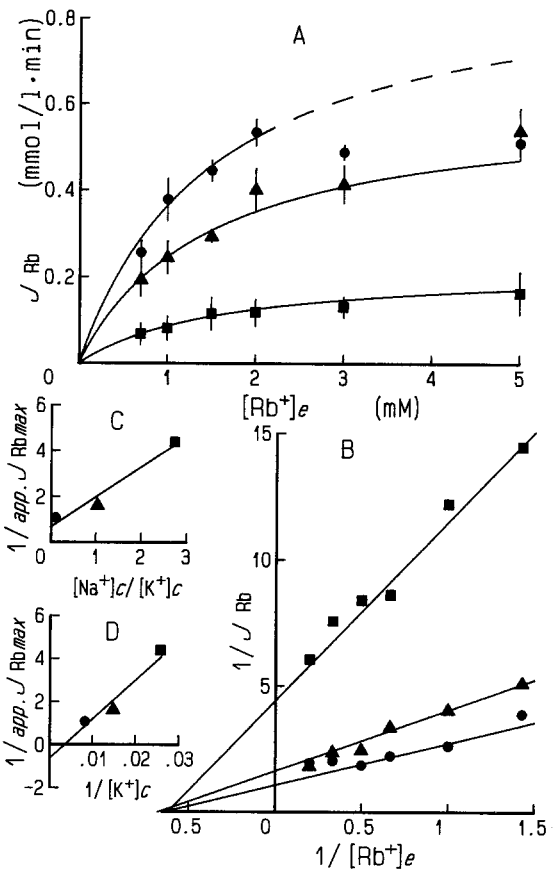

Fig. 4. *J*Rb into HeLa cells with different combinations of $[K^+]c$ and $[Na^+]c$. (*A*) *J*Rb in relation to $[Rb^+]e$. Curves are regression curves obtained by the nonlinear least-squares method. (*B*) Double-reciprocal plots of data in *A*. Lines are the regression lines. (*C*) Intercepts of the regression lines with the vertical axis in *B* in relation to $[Na^+]c/[K^+]c$. (*D*) Similar plots to those in *D* but in relation to $1/[K^+]c$. The line is the regression line. Points in *B*, *C* and *D* are proved significantly to regress to the lines, respectively, by the F-test ($P < 0.005$). Initially $[K^+]c$ and $[Na^+]_c$ were: ●, 120 and 14; ▲, 67 and 70; ■, 39 and 106 mmol/liter of cell water, respectively. Points and bars are means ± SD for four samples.

cantly different by the *t*-test ($P > 0.05$), indicating that the lines converge to approximately the same point on the axis. Therefore, *J*Rb*max* increases but *K*Rb*e* is not altered in hypertonic conditions.

Effects of the Intracellular Cation Concentrations

The effects of $[K^+]c$ and $[Na^+]c$ on *J*Rb were investigated as a function of $[Rb^+]e$ in three groups consisting of cells with three different combinations of $[K^+]c$ and $[Na^+]c$ (Fig. 4*A*). The results clearly indicate that *J*Rb tends to become larger when $[K^+]c$ is larger and $[Na^+]c$ is smaller. *J*Rb was saturated at a $[Rb^+]e$ of only 2 mM in the group with normal cation concentrations in this case, but the values of *J*Rb for the other two groups were not saturated up to 5 mM. Curves were drawn by the nonlinear least-squares method. Double reciprocal plots of the data on *J*Rb *vs.* $[Rb^+]e$ indicate that the points for the three groups give three regression lines, except for two points of the saturated *J*Rb (Fig. 4*B*). The lines show three, closely adjacent intercepts on the horizontal axis, no two of which were significantly different by the *t*-test ($P > 0.1$). These results reveal that values of the apparent *J*Rb*max* are different, but those of *K*Rb*e* are almost equal in the three groups. The mean *K*Rb*e* was 1.55 mM in this case. Replots of $1/app.J\text{Rb}max$ as a function of $[Na^+]c/[K^+]c$ suggest a linear arrangement of the three points corresponding to these three groups (Fig. 4*C*). The regression line passed a point on the vertical axis that represents *J*Rb*max* attainable when

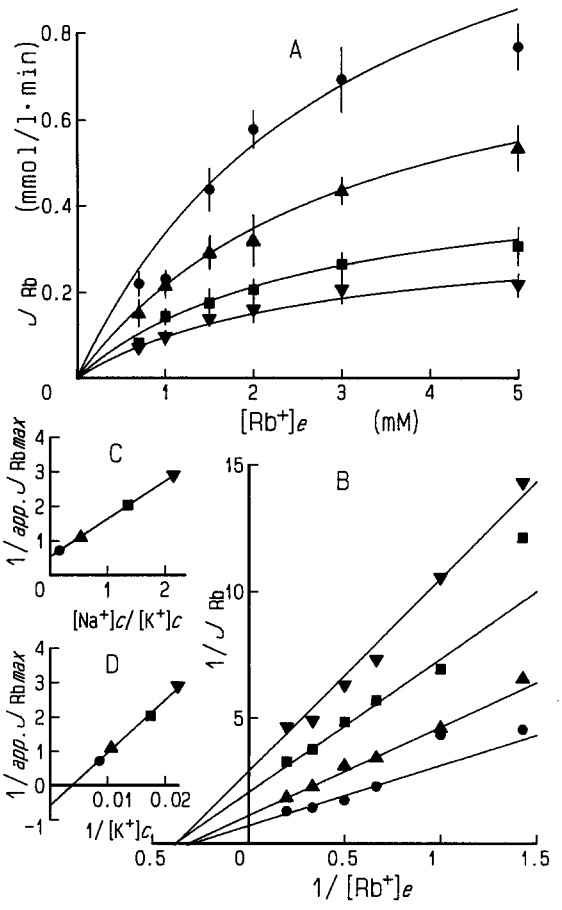

Fig. 5. Similar results to those shown in Fig. 4 except that the cells with four different combinations of $[K^+]c$ and $[Na^+]c$ were used and 200 mM mannitol was added to the medium. *See* Fig. 4 for details of plots of data and statistical analysis. Initially $[K^+]c$ and $[Na^+]c$ were ●, 115 and 20; ▲, 93 and 51; ■, 57 and 78; ▼, 45 and 96 mmol/liter of cell water, respectively.

$[Na^+]c$ is zero, i.e., 1.49 mmol/liter of cell water · min. The slope of the regression line relates to the ratio of the apparent $K_{0.5}$ for K^+c and the apparent K_i for Na^+c, i.e., $K = KKc/KNac = 1.95$, as understood from Eqs. (A1) and (A3) in the Appendix. The value of K suggests lower affinity of the pathway for K^+c than for Na^+c with respect to *J*Rb. The apparent *J*Rb*max* also shows a linear relation with $1/[K^+]c$ (Fig. 4*D*). This is reasonable as explained by Eq. (A2), because the means of the sums of $[K^+]c$ plus $[Na^+]c$ of the three groups are regarded as practically equal, i.e., 138.7 ± 5.7 (mean ± SD) mmol/liter of cell water. The intercept of the regression line with the vertical axis shows a negative value. This is also reasonable, because the value of 1-*K* is negative.

The values of *J*Rb of four groups of the cells with different $[K^+]c$ and $[Na^+]c$ upon addition of 200 mM mannitol to the Rb^+ medium were determined (Fig. 5). Again, stimulation of *J*Rb by hypertonicity seemed to be marked when $[K^+]c$ was high and $[Na^+]c$ was low (Fig. 5*A*). Figure 5*B* shows double-reciprocal plots of the data in Fig. 5*A*. The points for the four groups gave four separate regression lines, which showed closely adjacent intercepts with the horizontal axis. These intercepts were not significantly different according to the *t*-test ($P > 0.1$). The mean *K*Rb*e* was estimated to be 2.84 mM, which was somewhat larger than the value shown in Fig. 4. The four values of $1/app.JRbmax$ obtained in Fig. 5*B* are replotted as a function of $[Na^+]c/[K^+]c$ in Fig. 5*C*. This plot shows a linear arrangement of four points and *J*Rb*max* was estimated as 1.88 mmol/liter of cell water · min from its intercept with the vertical axis. From the slope of the regression line, $KKc/KNac = 2.07$. A replot of the four values of $app.JRbmax$ in relation to $1/[K^+]c$ also gave a regression line (Fig. 5*D*), in common with the results in Fig. 4.

Next, we tested the effect of $[Na^+]e$ on *J*Rb in three groups of the cells with different $[K^+]c$ and $[Na^+]c$ (Fig. 6). These cells were incubated in the media containing 5 mM Rb^+ and various $[Na^+]e$. The points are fitted to regression curves drawn by the nonlinear least-squares method and indicate the stimulating effect of Na^+e on *J*Rb. Double-reciprocal plots of *J*Rb *vs.* $[Na^+]e$ demonstrated that the points for the three groups gave three different regression lines, with closely adjacent intercepts with the horizontal axis (Fig. 6*B*). These intercepts were not significantly different as judged by the *t*-test ($P > 0.1$). From the mean of these intercepts, we calculated a value of *K*Na*e* of 15.3 mM at 5 mM Rb^+e. Replots of the $1/app.JRbmax$ *vs.* $[Na^+]c/[K^+]c$ showed a linear relation of the two parameters (Fig. 6*C*). The regression line shows a *J*Rb*max* value of 1.11 mmol/liter of cell water · min and *KKc*/*KNac* of 1.39.

We collected data for *J*Rb measured at various $[K^+]c$ and $[Na^+]c$ from four experiments conducted independently and showed them as a function of $[K^+]c/[Na^+]c$ (Fig. 7). Values of *J*Rb of control cells containing initially normal $[K^+]c$ and $[Na^+]c$ slightly differed in different experiments. Therefore, values of the influx at various intracellular cation concentrations were represented relatively as percentages of that of control cells in each experiment. The relation of *J*Rb *vs.* $[K^+]c/[Na^+]c$ seemed to be described by a regression curve drawn by the nonlinear least-squares method. When the ratio $[K^+]c/[Na^+]c$ was extremely low, *J*Rb was strongly suppressed. With increase in the concentration ratio, *J*Rb increased to a plateau. The regression curve was not consistent with the broken curve rep-

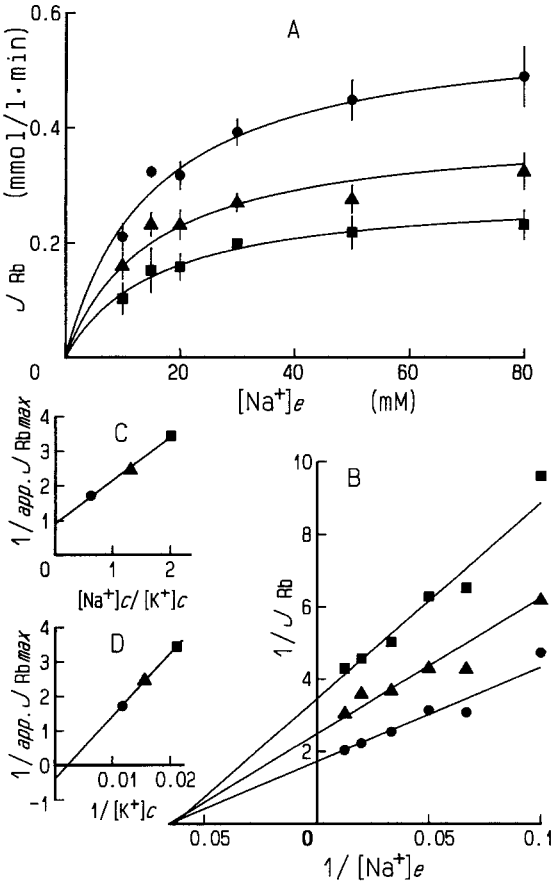

Fig. 6. Effect of various $[Na^+]e$ in the presence of 5 mM Rb^+e on *J*Rb of HeLa cells with different combinations of $[K^+]c$ and $[Na^+]c$. (*A*) *J*Rb as functions of $[Na^+]e$. Curves are drawn by the nonlinear least-squares method. (*B*) Double-reciprocal plots of data shown in *A*. The three lines are the regression lines corresponding to the three curves in *A*. (*C*) Relation between intercepts of the regression lines on the vertical axis in *B* and $[Na^+]c/[K^+]c$. The line is the regression line. Points in *B*, *C* and *D* are proved to significantly regress to the lines, respectively, by the F-test ($P < 0.005$). Initially $[K^+]c$ and $[Na^+]c$ were: ●, 85 and 54; ▲, 64 and 84; ■, 47 and 95 mmol/liter of cell water, respectively. Points and bars are means ± SD for four samples.

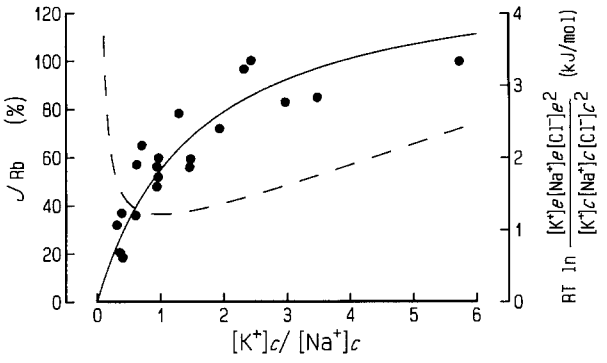

Fig. 7. *J*Rb and the transmembrane gradient of the sums of chemical potentials of K^+, Na^+ and Cl^- in HeLa cells as functions of $[K^+]c/[Na^+]c$. The solid curve is the regression curve obtained by the nonlinear least-squares method and the broken curve represents the transmembrane gradient of the sum of chemical potentials of the three ions. *J*Rb is expressed as a percentage of that for control cells initially containing a normal $[K^+]c$ and $[Na^+]c$. Data were collected from results of four separate experiments. From the regression curve, the apparent *J*Rb *max* was estimated as 140% and *K*K*c*/*K*Na*c* as 1.54.

resenting the transmembrane gradient of the sum of the chemical potentials of Na^+, K^+ and $2Cl^-$.

Kinetic parameters such as *K*Rb*e*, *K*Na*e* and the ratio of the $K_{0.5}$ for K^+c and the K_i for Na^+c, i.e., *K*K*c*/*K*Na*c* are summarized in the Table. The value of *K*K*c* was more than that of *K*Na*c* in all experiments.

Discussion

In the present study, we used Rb^+ as an analogue of K^+ and assayed Rb^+ influx by totally replacing K^+ in the medium by Rb^+. This was possible because Rb^+ is not distinguished from K^+ significantly in ouabain-insensitive, furosemide-sensitive uptake by HeLa cells [25].

It has been demonstrated that ouabain-insensitive, furosemide-sensitive Rb^+, $^{22}Na^+$, or $^{36}Cl^-$ influx is extremely inhibited by omission of any one of the three ions from the medium and that furosemide-sensitive cotransport takes place with a 1:1:2 stoichiometry for Na^+, Rb^+ and Cl^- in HeLa cells [25] as in other types of cells [20, 24, 34]. ATP is not used as an energy source for the cotransport pathway, but is required as a regulator or a modifier [12, 17]. Instead, the transmembrane gradient of the sum of the potentials of Na^+, K^+ and $2Cl^-$ has been suggested to drive the diuretic-sensitive K^+ influx in Ehrlich ascites tumor cells [14]. However, the regression curve of *J*Rb as a function of the ratio $[K^+]c/[Na^+]c$ in HeLa cells did not simply coincide with the curve representing the transmembrane gradient of the sum of chemical potentials of these ions (Fig. 7). The disagreement does not mean that the potential gradient does not drive *J*Rb, but suggests certain modification of the gradient-driven Rb^+ influx by specific actions of K^+c and Na^+c. The best method for testing the effects of K^+c and Na^+c on the cotransport pathway is to change their concentrations independently. However, so far there is no such ideal method that gives no inhibitory influence to the pathway of HeLa cells. Hence, we incubated the cells for various periods in the presence of ouabain as the second best technique. This technique changed $[K^+]c$ and $[Na^+]c$ in association without

Table. Kinetic parameters for the affinities of the Na^+,K^+,Cl^--cotransport pathway of HeLa cells to extracellular and intracellular cations with respect to ouabain-insensitive, furosemide-sensitive Rb^+ influx

Case	Kinetic parameters			Remarks
	*K*Rb*e*	*K*Na*e*	*K*Kc/*K*Nac	
A	4.40			Fig. 3
B	1.55		1.95	Fig. 4
C	2.84		2.07	Fig. 5
D		15.3	1.39	Fig. 6
E			1.54	not shown
F		23.8	1.18	not shown
G	2.26		1.02	not shown
H	1.89		1.05	not shown
I	3.57			not shown
Mean ± SD	2.57 ± 1.08	19.6 ± 6.0	1.46 ± 0.42	

The experiments indicated as "not shown" in the Remarks are similar to those for Figs. 7, 6, 5, 4 and 3, respectively.

any significant influence to the pathway. Since the sum of the cation concentrations was kept constant, cellular osmotic pressure was not significantly altered. *J*Rb was stimulated when $[K^+]c$ increased and $[Na^+]c$ decreased, whereas *K*Rb*e* seemed to remain unchanged. This implies that an increase in $[K^+]c$ or a decrease in $[Na^+]c$ stimulates *J*Rb by increasing the number of active cotransport pathways without changing the affinity of the pathway for Rb^+e. Studies on the effects of the intracellular cations in erythrocytes have been reported. In contrast to HeLa cells, in human erythrocytes an increase in Na^+c or a decrease in K^+c stimulates *J*K in the absence of Na^+e [5]. Furosemide-sensitive $^{86}Rb^+$ influx into human erythrocytes in vivo is negatively correlated with $[K^+]c$, but not significantly correlated with $[Na^+]c$ [10]. These results seem to conflict with another report that Na^+e-dependent, furosemide-sensitive Rb^+ influx into erythrocytes does not require K^+c and is almost independent of Na^+c, provided $[Na^+]c$ is not very high [9]. The discrepancy of the results for erythrocytes might be partly due to difference in the pathways of K^+ influx, because the former results would include the Na^+e-independent K^+ exchange pathway. As the component of Na^+e- or Cl^-e-independent $Rb^+(K^+)$ influx involved in *J*Rb is very small in HeLa cells [25], the different properties of *J*Rb as a function $[K^+]c$ or $[Na^+]c$ in HeLa cells and human erythrocytes may reflect different effects of the intracellular cations on the cotransport pathway in these cells.

We showed that the $K_{0.5}$ for K^+c, *K*Kc, is somewhat larger than the K_i for Na^+c, *K*Nac (Table). This suggests that the affinity of Na^+c to the cotransport pathway in relation to *J*Rb is slightly higher than the affinity of K^+c. As it was technically difficult to change the intracellular concentration of K^+ or Na^+ independently without influencing the cotransport function, we could not succeed in obtaining the values of *K*Kc and *K*Nac separately.

Hypertonicity produced by addition of mannitol stimulated *J*Rb and prevented its saturation at a $[Rb^+]e$ of more than 2–3 mM (*compare* Figs. 4 and 5). Enhancement of *J*K(*J*Rb) in hypertonic media has been reported for duck red cells [32], HeLa cells [35] and chicken enterocytes [28]. Although hypertonicity increased *J*Rb*max*, it did not affect the affinity of the cotransport pathway represented by *K*Rb*e* (Fig. 2), suggesting that the number of active pathways increases in these conditions without change in the transport activity of each single pathway. This pattern of stimulation seems to be somewhat different from that reported for HeLa cells also incubated in hypertonic conditions produced by addition of mannitol in which both *J*Rb and the $K_{0.5}$ for K^+e were shown to increase [35]. Unlike sucrose, mannitol is known to permeate the cell membrane slightly [13]. Though the permeable nature of mannitol might reduce water leakage from the cells when incubated in hypertonic medium, cell shrinkage is not avoided. Hence, $[K^+]c$ and $[Na^+]c$ would change and influence *J*Rb. However, our results showed linear ouabain-insensitive, furosemide-sensitive accumulation of Rb^+ in HeLa cells for at least 20 min, indicating that *J*Rb was maintained at the rate corresponding to the initial intracellular ionic conditions. The hypertonicity-induced shrinkage of the cells would strongly stimulate the cotransport pathway as explained by the model of feedback control of cell volume [11].

The mechanisms of the effects of important intracellular ions on *J*Rb of HeLa cells cannot be fully understood until the role of Cl^-c has been clarified. However, our results reveal that K^+c stimulates and Na^+c inhibits *J*Rb, and that the binding affinity of K^+c is slightly smaller than that of Na^+c. The binding of any of the intracellular cations to the internal site does not affect the binding affinity of the external site for $K^+e(Rb^+e)$. Instead, it influences the number of active cotransport pathways. Based on these results, a general and a special transport model were proposed to elucidate the mechanism of the Rb^+ influx (Fig. 8). The special model suggests the presence of one regulatory cation binding site on the internal face of the pathway in HeLa cells. When K^+c binds to the internal site and Rb^+e to the external site, Rb^+ is moved into the cells; but when Na^+c or none of the intracellular cations binds to the internal site, Rb^+ is not transported.

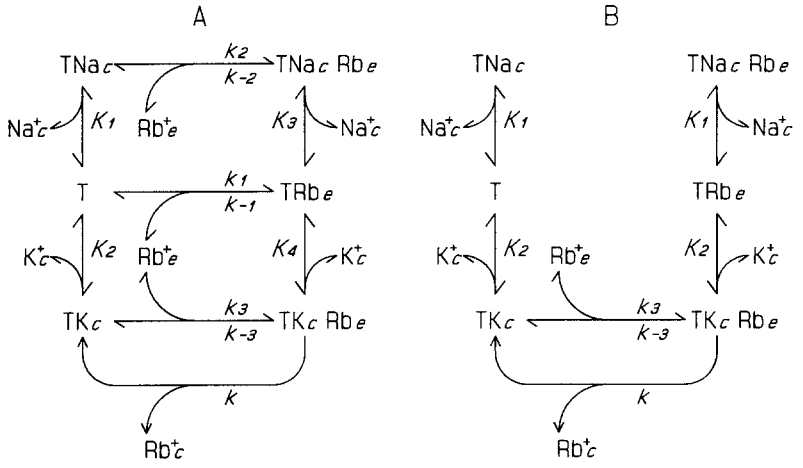

Fig. 8. Schematic presentation of kinetic models for the effects of K^+c and Na^+c on *J*Rb. (*A*) General model under certain physical restrictions. (*B*) Special model suitable for HeLa cells. T, the Na^+,K^+,Cl^--cotransport pathway. *See* the Appendix for the definitions of *k*, *kn*, *k-n* and *Kn*.

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Appendix

Kinetic Equations

The result shown in Fig. 5*C* indicates a linear relation of values for $1/app.JRbmax$ and $[Na^+]c/[K^+]c$ and can be expressed as

$$1/app.JRbmax = (1/JRbmax)(1 + K[Na^+]c/[K^+]c), \quad (A1)$$

where $1/JRbmax$ is the reciprocal of the apparent maximum value of JRb at $[Na^+]c = 0$ and corresponds to the intercept of the regression line on the vertical axis. K is a constant related to the slope of the line. The value of $1/JRbmax$ and the slope of the line are estimated to be 0.532 and 1.10 liter of cell water · min/mmol, respectively. Based on these values, K is calculated to be 2.07.

Though $[Na^+]c$ and $[K^+]c$ were changed experimentally by incubating the cells for various periods in the presence of ouabain, the sum of $[Na^+]c$ and $[K^+]c$ did not change significantly as shown in Results. Therefore, a constant M may be assigned to the sum, i.e., $M = [K^+]c + [Na^+]c$. Therefore,

$$1/app.JRbmax = (1/JRbmax)(1 - K + KM/[K^+]c). \quad (A2)$$

This equation suggests a linear relation between $1/app.JRbmax$ and $1/[K^+]c$. In fact, on plotting $1/app.JRbmax$ against $1/[K^+]c$, the points fell on a regression line (Fig. 5*D*). The intercept of the line on the vertical axis, −0.552 liter of cell water · min/mmol, approximately equals the value of $(1/JRbmax)(1 - K) = 0.532(1-2.07) = -0.569$ liter of cell water · min/mmol. As the slope of the line was 153 min, the value of M could be estimated by the expression (the slope)/$\{K(1/JRbmax)\}$ as $153/(2.07 \times 0.532) =$ 138.9 mmol/liter of cell water. This value is well consistent with the mean of the sum of $[K^+]c$ and $[Na^+]c$, 138.0 ± 5.2 mmol/liter cell water, obtained experimentally in Fig. 5. Similar consistency was observed when the results in Figs. 4 and 6 were used, suggesting that the estimations are reasonable and reproducible.

We have reported kinetic equations that explain the roles of Na^+e and Rb^+e [17, 25]. From these equations and the expressions given above, we finally derive the equation

$$1/JRb = (1/JRbmax)\{1 + ([Na^+]c/KNac)(KKc/[K^+]c)\}$$
$$\{1 + (1 + KNae/[Na^+]e)KRbe/[Rb^+]e\}, \quad (A3)$$

where $KKc/KNac = K$.

This equation indicates the roles of K^+c and Na^+c in Rb^+ influx mediated by the ouabain-insensitive, furosemide-sensitive cotransport pathway, and the contributions of external cations.

Transport Models

We propose a kinetic model of the Na^+,K^+,Cl^--cotransport pathway to explain the stimulating effect of K^+c and the inhibitory effect of Na^+c on JRb (Fig. 8*A*). We assume the presence of one regulatory cation binding site on the internal face of the pathway, to which K^+c or Na^+c binds rapidly. For simplicity, only the Rb^+ influx is considered in the model. Rb^+ is moved into the cells mediated by the pathway after the bindings of Rb^+e and K^+c to the external and the internal sites, respectively ($T \cdot Kc \cdot Rbe \rightarrow Rb^+c + T \cdot Kc$), whereas Rb^+ is not transported when the pathway is bound with Na^+c or none of the intracellular cations ($T \cdot Nac \cdot Rbe$ or $T \cdot Rbe$). By application of the method of Cha [6] to the transport model, we can derive the kinetic equation

$$JRb = J'Rbmax/\langle\{1 + K_4/[K^+]c + K_4[Na^+]c/(K_3[K^+]c)\} + \{1 + K_2/[K^+]c + K_2[Na^+]c/(K_1[K^+]c)\}\{k + k_{-3} + k_{-2}K_4/[K^+]c + k_{-1}K_4[Na^+]c/(K_3[K^+]c)\}(1/[Rb^+]e)/\{k_3 + k_2K_2/[K^+]c + k_1K_2[Na^+]c/(K_1[K^+]c)\}\rangle, \quad (A4)$$

where k is the rate constant for the Rb^+ influx, and kn and $k-n$ are those for the association and dissociation reactions of Rb^+e and the pathway, respectively (n = 1, 2, or 3). Kn is the dissociation constant with respect to the binding reaction of K^+c or Na^+c with the pathway (n = 1, 2, 3 or 4).

If Rb^+e binds to the external site only after the binding of K^+c to the internal site but not when Na^+c or none of the intracellular cations binds, then $k_1 = k_{-1} = k_2 = k_{-2} = 0$. In addition, if the dissociation constants are not changed by the binding of Rb^+e, then $K_1 = K_3$ and $K_2 = K_4$. Therefore, Eq. (A4) is simplified to the expression

$$JRb = J'Rbmax/\langle\{1 + K_2/[K^+]c + ([Na^+]c/K_1)(K_2/[K^+]c)\}\{1 + (k + k_{-3})/(k_3[Rb^+]e)\}\rangle. \quad (A5)$$

Eq. (A5) is further simplified to the final equation

$$JRb = JRbmax/\langle\{1 + ([Na^+]c/KNac)(KKc/[K^+]c)\}\{1 + KRbe/[Rb^+]e\}\rangle, \quad (A6)$$

where $JRbmax = J'Rbmax/(1 + K_2/M)$, $KKc = K_2(K_1 + M)$, $KNac = K_1(K_2 + M)$ and $KRbe = (k + k_{-3})/k_3$.

Eq. (A6) composes a major part of Eq. (A3) and leads us to propose a special model shown in Fig. 8*B*. This model is consistent with our experimental results and explains the mechanism for the effects of K^+c and Na^+c on the activity of the pathway in HeLa cells. It also clarifies the order of the bindings of these cations to the internal regulatory site in relation to the binding of Rb^+e to the external K^+ site.

We assumed rapid equilibrium bindings of the cations to the cotransport pathway in this study. The assumption has been criticized, because such a physical restriction requires complex adjustments of the kinetic models [19, 31]. In addition, the effects of the intracellular cations on the bindings of the other essential, extracellular ions such as Na^+e and Cl^-e are not sufficiently understood at present. Therefore, further studies are needed to construct the complete models for explaining the effects of all of the related ions based on solid mathematical grounds.