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Kinetic Mechanism of Na^+, K^+, Cl^--Cotransport as Studied by Rb^+ Influx into HeLa Cells: Effects of Extracellular Monovalent Ions

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Summary. Ouabain-insensitive, furosemide-sensitive Rb^+ influx (J_{Rb}) into HeLa cells was examined as functions of the extracellular Rb^+, Na^+ and Cl^- concentrations. Rate equations and kinetic parameters, including the apparent maximum J_{Rb}, the apparent values of K_m for the three ions and the apparent K_i for K^+, were derived. Results suggested that one unit molecule of this transport system has one Na^+, one K^+ and two Cl^- sites with different affinities, one of the Cl^- sites related with binding of Na^+, and the other with binding of $K^+(Rb^+)$. A 1 : 1 stoichiometry was demonstrated between ouabain-insensitive, furosemide-sensitive influxes of $^{22}Na^+$ and Rb^+, and a 1 : 2 stoichiometry between those of Rb^+ and $^{36}Cl^-$. The influx of either one of these ions was inhibited in the absence of any one of the other two ions. Monovalent anions such as nitrate, acetate, thiocyanate and lactate as substitutes for Cl^- inhibited ouabain-insensitive Rb^+ influx, whereas sulfamate and probably also gluconate did not inhibit J_{Rb}. From the present results, a general model and a specialized cotransport model were proposed: 1) In HeLa cells, one Na^+ and one Cl^- bind concurrently to their sites and then one $K^+(Rb^+)$ and another Cl^- bind concurrently. 2) After completion of ion bindings Na^+, $K^+(Rb^+)$ and Cl^- in a ratio of 1 : 1 : 2 show synchronous transmembrane movements.

Key Words chloride · cotransport · HeLa cells · rubidium influx · potassium · sodium

Introduction

Evidence has been reported for the presence of cotransport mechanisms of Na^+, K^+ and Cl^-, Na^+ and Cl^-, and K^+ and Cl^- in the plasma membranes of a wide variety of animal cells, such as the intestine of a marine teleost (Musch et al., 1982), squid axon (Russell, 1983), chicken heart cells (Aiton et al., 1981), avian red blood cells (Haas & McManus, 1985) and mammalian cells or tissues (Frizzell et al., 1979; Aull, 1981; Ifshin et al., 1983; Wiater & Dunham, 1983; Lauf, 1984). These cotransport systems are all sensitive to inhibitors of Na^+ absorption in the thick ascending limb of Henle's loop, such as furosemide, bumetanide and piretanide. An appreciable part of Na^+ or K^+ transport depends on extracellular Cl^- and is also linked to Cl^- transport. Substitution of another monovalent anion for Cl^- has been reported to reduce Na^+ or K^+ flux in mammalian cells (Geck et al., 1980; Aiton et al., 1981; Chipperfield, 1981; Jayme et al., 1984). However, the loop-diuretics do not have synergistic effects with Cl^- deficiency in reducing cation transport (Aiton et al., 1981; Chipperfield, 1981). These results imply that Cl^--dependent cation fluxes are identical with diuretic-sensitive cotransport of the cations.

There are many reports on the kinetic characteristics and physiological roles of the diuretic-sensitive systems of mammalian cells including cultured cells (Gargus & Slayman, 1980; Koenig et al., 1983; Duhm & Göbel, 1984*a*,*b*; Jayme et al., 1984; Owen, 1984; Tivey et al., 1985). As the total net charge of the ions moved by the diuretic-sensitive systems is usually zero and the transport activity of the systems is not influenced by changes in membrane potential (Geck et al., 1980; McRoberts et al., 1982), the systems appear to be electrically silent.

The ratio of ions moved through the cotransport system has usually been demonstrated to be close to unity or to certain integral values in these cells under different conditions. Although, this ratio was found to vary considerably depending on the ion concentrations on the two sides of the cell membrane (Brugnara et al., 1983) and the combinations of ions appears to vary under different conditions, e.g. a change in the cell volume induces different ion combinations (Hoffman et al., 1983; Haas & McManus, 1985). One-for-one coupling of diuretic-sensitive K^+/K^+ or Na^+/Na^+ exchange has been shown to take place in Ehrlich ascites tumor cells (Tupper, 1975) and human red blood cells (Duhm & Göbel, 1984*a*; Brand & Whittam, 1985), whereas diuretic-sensitive $K^+(Rb^+)$ efflux is two or three

times higher than $K^+(Rb^+)$ influx in 3T3 cells (Atlan et al., 1984). It is unknown whether the cotransport and the K^+/K^+ or Na^+/Na^+ exchange are carried out by the same mechanism (Chipperfield, 1981) or by separate mechanisms (Duhm & Becker, 1979); many reported results are contradictory and different in different types of cells, and different groups have obtained discrepant results even for a single type of cells.

In an attempt to explain some of the reasons for this diversity of findings, in this work we studied the mechanism of furosemide-sensitive Rb^+ influx associated with the effects of extracellular Na^+, $K^+(Rb^+)$ and Cl^-. The stoichiometry of influxes of these ions and the inhibitory effects of other monovalent anions on the diuretic-sensitive cotransport system also are described. Finally, a general and a specialized model are proposed for the kinetic mechanism of ion fluxes through the cotransport system.

Materials and Methods

Cell Culture

HeLa S3 cells purchased from Flow Laboratories were maintained by serial cultivation in glass flasks. Each culture flask contained 10 ml of culture medium consisting of a modified minimum essential medium (mMEM, Miyamoto et al., 1976) supplemented with 10% (vol/vol) calf serum. After about a week, when the cultures became confluent, the cells were dispersed with 0.5% trypsin and suspended in the same culture medium, inoculated at a density of 6×10^4 cells/ml into Roux flasks containing 100 ml of culture medium. The cultures were incubated for 72 hr at 37°C in a CO_2 incubator in a humid atmosphere of 5% CO_2 in air. Then, the cells were again detached by trypsin treatment and reinoculated into plastic culture dishes (60 mm diameter, Corning Glass Works) at 4×10^4 cells/ml with 5 ml of culture medium. The dishes were placed for another 48 hr in the CO_2 incubator. HEPES buffer was omitted from the culture medium.

Assay of Ion Influxes

Unless otherwise stated, 50 μl of 10 mM ouabain were added to each culture dish and the cultures were incubated for 30 min to allow complete inhibition of the Na^+,K^+-pump. Then the culture medium was replaced by a Rb^+-substituted medium containing 5.6 mM glucose and 20 mM HEPES (pH 7.2) without serum for assay of Rb^+ influx. In addition to ouabain (10 μM), furosemide (0.1 mM) was also added to this Rb^+-substituted medium, when required. The Rb^+-substituted mdium contained Rb^+ instead of K^+ and the concentrations of monovalent ions were adjusted as described in the Results. Rb^+ influx was assayed for 15 min, since furosemide-sensitive and -insensitive Rb^+ influxes took place at constant rates at least 20 min (*see* Fig. 2). Rates were expressed in nmol/mg protein per min or mmol/liter cell water per min. After assay of Rb^+ influx, the medium was discarded. Procedures for washing cells and determining alkali cations by flame photometry were as reported in our previous papers (Ikehara et al., 1984*a*,*b*). $^{22}Na^+$ influx was assayed in parallel with Rb^+ influx after preincubation of the cells with ouabain, and measurements were made on four replicate cultures in each experimental group. For assay of $^{22}Na^+$ influx, $^{22}NaCl$ (847.42 mCi/mg, New England Nuclear) at 1 μCi/ml was added in Rb^+-substituted medium. The cells were incubated in this radioactive medium for 15 min at 37°C, and then washed four times with ice-cold 0.15 M $LiNO_3$. They were then lysed in 2.5 ml of 3 N NaOH per culture dish and 1 ml of lysate was used for assay of radioactivity in an Autogamma Scintillation Spectrometer (Packard). $^{22}Na^+$ influx was also expressed in mmol/liter cell water per min. Influx of $^{36}Cl^-$ was assayed in replicate cultures in parallel with that of Rb^+ influx after preincubation with ouabain. Usually, samples were taken from four culture dishes in each group. For the assay, $Na^{36}Cl$ solution (0.1 mCi/ml, Amersham International Plc) was diluted with Rb^+ medium to a radioactivity of 0.5 μCi/ml. The cells were incubated for 15 min in this radioactive medium at 37°C and then washed four times with ice-cold 0.15 M $LiNO_3$. They were then lysed in 1 ml of 2 N NaOH per culture dish and 0.7 ml of the lysate was transferred to vials containing 10 ml of liquid scintillation cocktail. Radioactivities were determined in a liquid scintillation spectrometer (LSC-602, Aloka). Influxes of $^{22}Na^+$ and $^{36}Cl^-$ were expressed in mmol/liter cell water per min.

Other Assays

The cell diameter was measured by microscopy and the cell volume was calculated from the diameter, assuming that the cells are spheroid or ellipsoid, because for measurement by micrometry the cells were detached from the culture dishes with 0.5% trypsin and suspended in the preincubation medium. It has been reported that the cell volume is not altered by trypsinization (Lamb & MacKinnon, 1971). The mean cell volume determined in the present study was 3.03 pl. The number of cells was assayed with an electronic cell counter (Sysmex CC-110, TOA Medical Electronics Co.). For protein assay, the cells were solubilized in 0.5 N NaOH and protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. The mean protein content was 0.416 ng/cell. The water content of the cells was determined by use of ^3H_2O (5 mCi/ml, Amersham International Plc) and inulin-[^{14}C]-carboxylic acid (2.4 mCi/g, New England Nuclear). The cells from replicate cultures were placed for 30 min in preincubation medium containing the radioactive substances at 1 and 0.5 μCi/ml, respectively in addition to ouabain. Their mean water content was 83.6%. Radioactivities were measured in a liquid scintillation spectrometer (LSC-700, Aloka). Determinations of the cellular contents of protein and water, and the cell volume were necessary for calculation of the intracellular cation concentrations and ion influxes.

Reagents and Miscellaneous Substances

RbCl (super-pure) was purchased from Merck; specially pure grade NaCl, LiCl, $LiNO_3$, glucose and calf serum were from Nakarai Chem. Ltd.; KCl, choline chloride, choline (50% solution) and various inorganic salts were from Wako Pure Chemical Co.; ouabain, furosemide, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), HEPES buffer and bovine serum albumin (fraction V) were from Sigma Chemical Co.; trypsin (1 : 250) was from Difco Laboratories; concentrated mixtures of amino acids (50×) and vitamins (100×) used to prepare the modified mini-

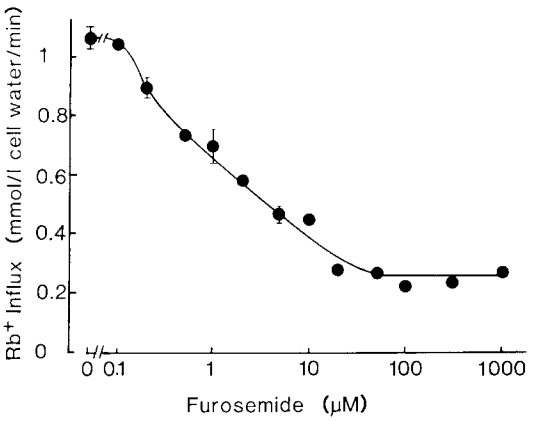

Fig. 1. Dose-response curve for the effect of furosemide on ouabain-insensitive Rb^+ influx into HeLa cells. The initial mean intracellular concentrations of Na^+ and K^+ were 44 and 123 mmol/liter cell water, since the cells were preincubated with 100 μM ouabain for 30 min

mum essential medium were from Gibco Laboratories. Amiloride hydrochloride dihydrate was donated from Merck Sharp and Dohme Co., Inc.

Symbols and Definitions

Symbols and their definitions used in the present paper are listed below:

e and c	subscripts referring to external and internal faces of the cell membrane
[ ]	the concentration of an ion
J_{Rb}	unidirectional ouabain-insensitive, furosemide-sensitive Rb^+ influx
app.$J'_{Rb\,max}$	the apparent maximum value of J_{Rb}
$J'_{Rb\,max}$	app.$J'_{Rb\,max}$ at infinite $[Rb^+]e$
app.K'_{Na}	the apparent K_m for Na^+e in relation to J_{Rb}
K'_{Na}	app.K'_{Na} in the absence of Rb^+e
K''_{Rb}	the apparent K_m for Rb^+e in relation to J_{Rb} in the presence of K^+
app.K'_{Rb}	K''_{Rb} in the absence of K^+e
K'_{Rb}	app.K'_{Rb} at infinite $[Na^+]e$
K'_{Cl1}	the apparent K_m for Cl^-e in relation to J_{Rb} and relevant to one of the Cl^- binding site
K'_{Cl2}	the same parameter relevant to another Cl^- binding site
K'_K	the apparent K_i for K^+ in relation to J_{Rb}

Results

Dose-Response to Furosemide

The dose-response curve for the effect of furosemide on ouabain-insensitive Rb^+ influx into HeLa cells showed that the drug inhibited the cation influx at concentrations of above 0.1 μM and that the inhibition reached a maximim at about 50 μM (Fig. 1). At this concentration, Rb^+ influx was decreased to about 25% of the control, and represented ouabain- and furosemide-insensitive passive transport. The concentration of furosemide for half-maximal inhibition, IC_{50}, was about 1 μM. Judging from the rapid onset of inhibition after administration of the drug (Brazy & Gunn, 1976), furosemide acted on the outer surface of the cell membrane, and so preincubation with this drug was not necessary. However, when the cells were preincubated with furosemide for 30 min, the concentration required for maximal inhibition was decreased to about 10 μM (*data not shown*).

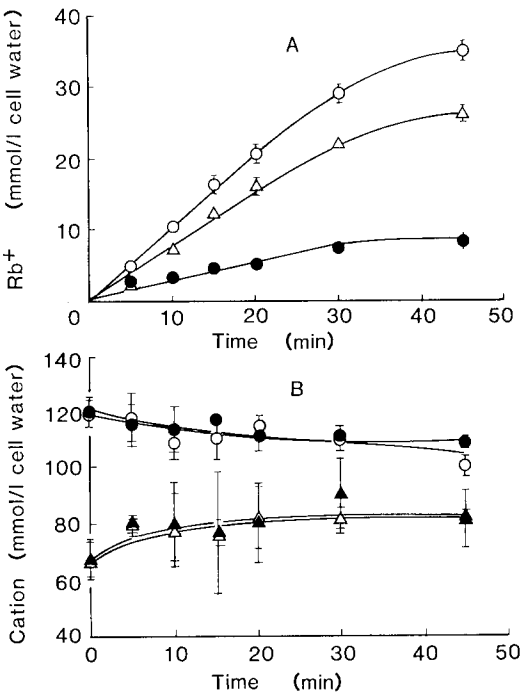

Fig. 2. Time-dependent changes in the intracellular cation concentrations in HeLa cells after replacement of 5 mM K^+ in the medium by 5 mM Rb^+. (*A*) Accumulation of Rb^+. ○, 10 μM ouabain; ●, 10 μM ouabain + 0.1 mM furosemide; △, furosemide-sensitive. Circles and bars are means ± SD and triangles and bars are means ± SE for four samples. (*B*) Changes in the intracellular concentrations of Na^+ and K^+ plus Rb^+. △, Na^+ with 10 μM ouabain; ▲, Na^+ with 10 μM ouabain and 0.1 mM furosemide; ○, K^+ + Rb^+ with 10 μM ouabain; ●, K^+ + Rb^+ with 10 μM ouabain and 0.1 mM furosemide. Points and bars are means ± SD for four samples

Time-Dependent Change in the Cation Content

The effect of 0.1 mM furosemide on the time course of ouabain-insensitive Rb^+ accumulation in cells after replacement of K^+ in the medium by Rb^+ is shown in Fig. 2*A*. Both in the presence and absence of furosemide, the intracellular Rb^+ content in-

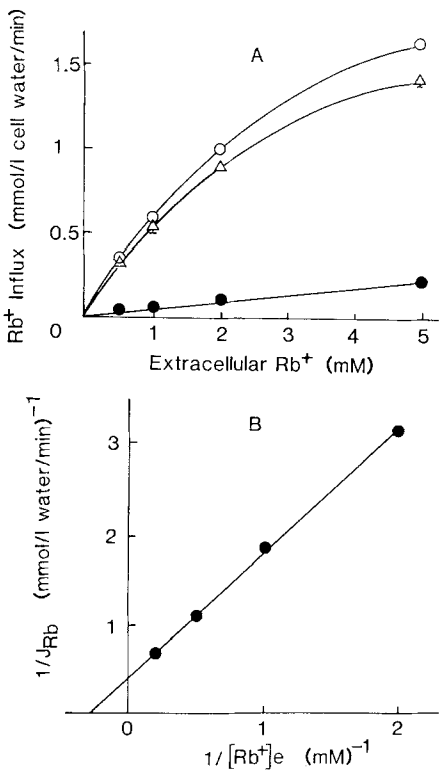

Fig. 3. Ouabain-insensitive Rb^+ influxes into HeLa cells as functions of the extracellular Rb^+ concentration. Media contained NaCl (140 mM) and RbCl plus choline chloride, keeping the sum of the concentrations of the two at 5 mM. (*A*) Effect of furosemide on ouabain-insensitive Rb^+ influx. ○, 10 μM ouabain; ●, 10 μM ouabain plus 0.1 mM furosemide; △, furosemide-sensitive. (*B*) Double-reciprocal plot of J_{Rb} against $[Rb^+]e$. The app.K'_{Rb} is 3.33 mM and $J'_{Rb\,max}$ is 1.39 mmol/liter of cell water per min. The initial mean intracellular concentrations of Na^+ and K^+ were 48 and 113 mmol/liter cell water, respectively. The linear curve in *B* is the regression line obtained by the least-squares method

creased linearly with the incubation time for about 20 min after which the rate of increase decreased and, in particular, the increase almost stopped in the presence of furosemide. Furosemide-sensitive Rb^+ accumulation, calculated by subtracting the Rb^+ content with furosemide from that without furosemide, also increased linearly for about 20 min. Figure 2*A* shows that the rate of increase in the furosemide-sensitive component of Rb^+ influx was 0.77 mmol/liter cell water per min (4.7 nmol per mg protein per min), which represented 75% of the ouabain-insensitive Rb^+ influx in the present experiment. The change in $[Na^+]c$ was a mirror image of $[K^+]c + [Rb^+]c$ (Fig. 2*B*). There were no significant differences between either $[Na^+]c$ or $[K^+]c + [Rb^+]c$ in cells incubated in the presence of 10 μM ouabain alone or with 0.1 mM furosemide. Therefore, addition of furosemide did not influence $[Na^+]c$ and $[K^+]c$, when K^+e was not replaced by Rb^+e.

Effects of Transport Inhibitors

The ouabain-sensitive component of Rb^+ influx, i.e., active transport driven by the Na^+,K^+-pump, was about 51% of the total Rb^+ uptake. Of the remaining ouabain-insensitive Rb^+ influx, the furosemide-sensitive and -insensitive components constituted about 35 and 14%, respectively, indicating that furosemide-sensitive Rb^+ influx constituted the major part (71%) of ouabain-insensitive Rb^+ influx. A specific inhibitor of anion exchange (DIDS) and another inhibitor of Na^+/H^+ exchange (amiloride) had no significant effects on ouabain-insensitive or ouabain-, furosemide-insensitive Rb^+ influxes and hence, on furosemide-sensitive and -insensitive Rb^+ influxes (*data not shown*).

Effect of Rb^+ Concentration

Rb^+ influx into HeLa cells in the presence of 10 μM ouabain was determined at various values of $[Rb^+]e$ (Fig. 3). Figure 3*A* shows that ouabain-insensitive Rb^+ influx was due to two different components: one was furosemide-insensitive Rb^+ influx, which was a minor component and changed in proportion to $[Rb^+]e$, and furosemide-sensitive Rb^+ influx, which was the main component. This latter component increased along a saturable curve with increase in $[Rb^+]e$. In a double-recripocal plot of the furosemide-sensitive Rb^+influx (J_{Rb}) *vs.* $[Rb^+]e$, all the points fell on a line (Fig. 3*B*). The points of intersection of this line with the abscissa and the ordinate represent the reciprocal of app.K'_{Rb} and $J'_{Rb\,max}$ in relation to ouabain-insensitive, furosemide-sensitive Rb^+ influx. This relation between J_{Rb} and $[Rb^+]e$ can be described by simple Michaelis-Menten kinetics:

$$1/J_{Rb} = (1/J'_{Rb\,max})(1 + \text{app.}K'_{Rb}/[Rb^+]e), \qquad (1)$$

and suggests the presence of one K^+ site per unit molecule of the furosemide-sensitive cotransport system.

Effect of Extracellular K^+

The relation between J_{Rb} and $[Rb^+]e$ in the presence of three different values of $[K^+]e$ was examined (Fig. 4). Figure 4*A* shows that inhibition of J_{Rb} was stronger when $[K^+]e$ was higher. Double-reciprocal plots of J_{Rb} and $[Rb^+]e$ gave three different lines for

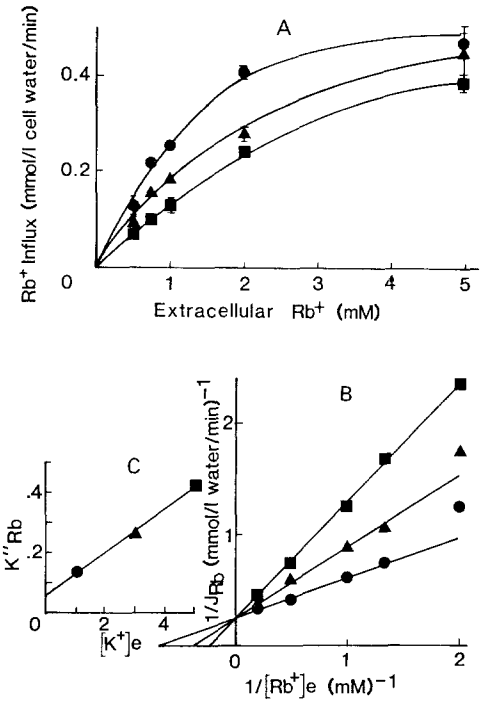

Fig. 4. Effect of the extracellular K^+ concentration on ouabain-insensitive, furosemide-sensitive Rb^+ influxes into HeLa cells. Medium contained 140 mM Na^+, 140 mM Cl^- and Rb^+ + K^+ + choline ion, keeping the sum of the concentrations of three cations at 5 mM. (*A*) J_{Rb} as functions of $[Rb^+]e$. Points and bars are means ± SE for differences between ouabain-insensitive Rb^+ influxes and ouabain-, furosemide-insensitive Rb^+ influxes in the four conditions. (*B*) Double-reciprocal plot of J_{Rb} *vs.* $[Rb^+]e$. (*C*) The apparent K_m for Rb^+e (K''_{Rb}) in relation to $[K^+]e$. The relation is shown by the expression K''_{Rb} = app. $K'_{Rb}(1 + [K^+]e/K'_K)$. ●, 1 mM K^+e; ▲, 3 mM K^+e; ■, 5 mM K^+e. The initial mean intracellular concentrations of Na^+ and K^+ were 65 and 116 mmol/liter cell water. The lines in *B* and *C* are the regression lines drawn by the least-squares method

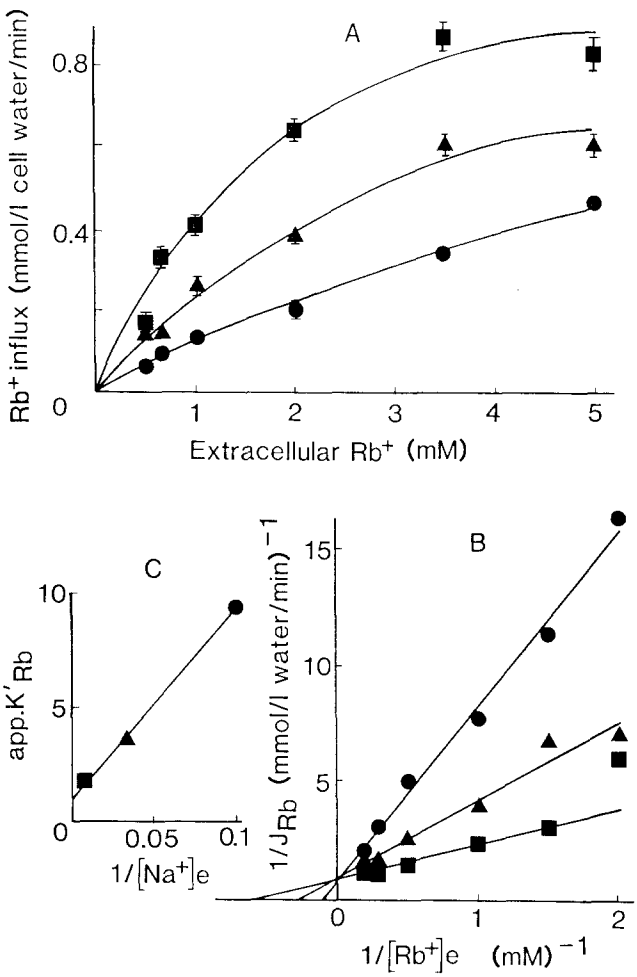

Fig. 5. Effect of the extracellular concentration of Na^+ on ouabain-insensitive, furosemide-sensitive Rb^+ influx into HeLa cells. Medium contained Na^+, Rb^+, choline ion and 140 mM Cl^-, keeping the sum of the concentrations of the cations at 145 mM. (*A*) J_{Rb} as functions of $[Rb^+]e$. *See* legend to Fig. 4 for explanation of points and bars. (*B*) Double-reciprocal plots of J_{Rb} *vs.* $[Rb^+]e$. (*C*) The app.K'_{Rb} in relation to the reciprocal of $[Na^+]e$. The relation is expressed by the equation, app.K'_{Rb} = $K'_{Rb}(1 + K'_{Na}/[Na^+]e)$. Symbols: ●, 10 mM Na^+e; ▲, 30 mM Na^+e; ■, 140 mM Na^+e. The linear curves in *B* and *C* are the regression lines obtained by the least-squares method. The mean initial intracellular concentrations of Na^+ and K^+ were 60 and 100 mmol/liter cell water

results at the three values of $[K^+]e$ (Fig. 4*B*). Two points at the lowest $[Rb^+]e$ (0.5 mM) deviated from the straight lines, probably because of the large values of the statistical coefficients of variation, but other points fell on these three lines. The straight lines converged to a certain point on the ordinate, which indicated competitive interaction between Rb^+e and K^+e. Values for the apparent K_m for Rb^+e corresponding to the three different $[K^+]e$ values were plotted as a function of $[K^+]e$, and also gave a linear curve (Fig. 4*C*).The functional relation illustrated in Fig. 4*B* and *C* are well described by the expression as

$$1/J_{Rb} = (1/J'_{Rb\,max})\{1 + (1 + [K^+]e/K'_K)\text{app.}K'_{Rb}/[Rb^+]e\}. \quad (2)$$

The slope of the linear curve in Fig. 4*C* represents the ratio of app.K'_{Rb}/K'_K and equals 0.71 in the present case.

Effect of Extracellular Na^+

The effects on J_{Rb} of three different values for $[Na^+]e$ at various $[Rb^+]e$ values are shown in Fig. 5. Na^+e stimulated J_{Rb} (Fig. 5*A*). In double-reciprocal plots of J_{Rb} against $[Rb^+]e$ the points fell on three straight lines for the three different values of $[Na^+]e$ (Fig. 5*B*). The lines converged to the same point on the ordinate, which indicates that app.K'_{Rb} changes depending on $[Na^+]e$, whereas $J'_{Rb\,max}$ is not affected by Na^+e. These results suggest that change in $[Na^+]e$ affects the affinity of Rb^+e to the furo-

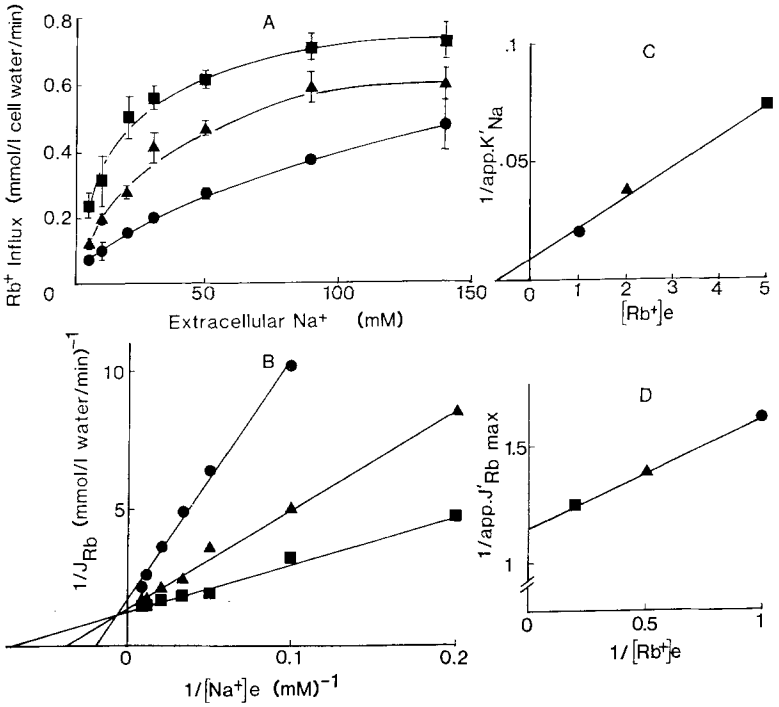

Fig. 6. Effect of the extracellular Rb^+ concentration on ouabain-insensitive, furosemide-sensitive Rb^+ influx. Medium contained 140 mM Cl^-, keeping the sum of the concentrations of Na^+, Rb^+ and choline at 145 mM. (*A*) J_{Rb} as functions of $[Na^+]e$. *See* legend to Fig. 4 for explanation of points and bars. (*B*) Double-reciprocal plots of J_{Rb} *vs.* $[Na^+]e$. (*C*) The intersections on the abscissa, i.e. 1/app. K'_{Na}, as a function of $[Rb^+]e$. The relation is expressed as $1/\text{app}.K'_{Na} = 1/K'_{Na}(1 + [Rb^+]e/K'_{Rb})$. (*D*) Double-reciprocal plot of app. $J'_{Rb\,max}$ against $[Rb^+]e$. The relation is described by the equation $1/\text{app}.J'_{Rb\,max} = (1/J'_{Rb\,max})(1 + K'_{Rb}/[Rb^+]e)$. Symbols: ●, 1 mM Rb^+e; ▲, 2 mM Rb^+e; ■, 5 mM Rb^+e. The lines in *B*, *C* and *D* are regression lines obtained by the least-squares method. The mean initial intracellular concentrations of Na^+, K^+, and Cl^- were 75, 109 and 65 mmol/liter cell water, respectively

semide-sensitive transport system, but does not affect the number of active molecules of the transport system. A plot of the values of app.K'_{Rb} obtained from the points of intersection of the three linear curves on the abscissa as a function of the reciprocal of $[Na^+]e$ again demonstrated a linear relation (Fig. 5*C*). The functional relations shown in Fig. 5*B* and *C* are exactly explained by the equation as

$$1/J_{Rb} = (1/J'_{Rb\,max})\{1 + (1 + K'_{Na}/[Na^+]e)K'_{Rb}/[Rb^+]e\}. \quad (3)$$

The points of intersection of the linear curve in Fig. 5*C* with the abscissa and the ordinate represent K'_{Rb} and $1/K'_{Na}$, respectively. Hence, K'_{Rb} and K'_{Na} were calculated to be 0.99 and 83.0 mM in the present case.

Effect of Extracellular Rb^+ at Different Na^+ Concentrations

J_{Rb} was determined as a function of $[Na^+]e$ in the presence of three different $[Rb^+]e$ (Fig. 6) and was confirmed to increase when $[Na^+]e$ or $[Rb^+]e$ increased (Fig. 6*A*). Double-reciprocal plots of J_{Rb} *vs.* $[Na^+]e$ also showed linear relations. The lines obtained by this procedure did not converge at any point on either the abscissa or the ordinate, but at a point on the left of the ordinate and above the abscissa (Fig. 6*B*). The reciprocal of the value of this point projected onto the horizontal axis is equal to K'_{Na} as can be understood by the following explanation. By plotting the values of the three points at which the straight lines intersected the abscissa as a function of $[Rb^+]e$, we obtained a linear relation (Fig. 6*C*). There was also a linear relation between values of the points of intersections on the ordinate in Fig. 6*B* and the reciporcal of $[Rb^+]e$ (Fig. 6*D*). These functional relations can be satisfactorily described by Eq. (3). The points of intersection of the linear curve with the vertical and horizontal axes in Fig. 6*C* represent the reciprocal of K'_{Na} and K'_{Rb}, respectively, and thus these two parameters were calculated to be 105 and 0.76 mM in this experiment. From the crossing point on the ordinate in Fig. 6*D*, $J'_{Rb\,max}$ was calculated to be 0.87 mmol/liter cell water per min in the present case. The results shown in Fig. 6 imply the presence of one Na^+ site per unit molecule of furosemide-sensitive cotransport system.

Effects of Extracellular Cl^-

J_{Rb} as a function of $[Rb^+]e$ was determined at three different values of $[Cl^-]e$ in the presence of a sufficiently high $[Na^+]e$ (140 mM) (Fig. 7*A*). Figure 7*A* shows that with change in $[Rb^+]e$ at a constant $[Cl^-]e$, J_{Rb} is represented by a saturable curve and that the curve was shifted upward with increase in $[Cl^-]e$. Double-reciprocal plots of J_{Rb} *vs.* $[Rb^+]e$ gave three separate linear curves at the three values of $[Cl^-]e$ (Fig. 7*B*). The lines seemed to converge to

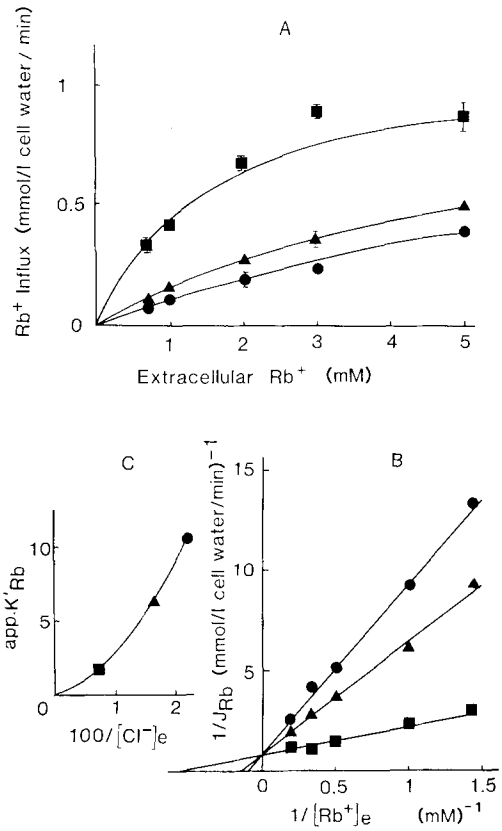

Fig. 7. Effect of extracellular Cl^- concentration on ouabain-insensitive, furosemide-sensitive Rb^+ influx into HeLa cells. (*A*) Relation of J_{Rb} to $[Rb^+]e$. ●, 45 mM Cl^-e; ▲, 60 mM Cl^-e; ■, 140 mM Cl^-e. Isotonicity of the medium, $[Na^+]e$ (140 mM), $[Rb^+]e$ (0.7 to 5 mM) and $[Cl^-]e$ (45, 60 and 140 mM) were adjusted by addition of NaCl, sodium sulfamate, RbCl and choline chloride. Points are differences between means of Rb^+ influxes in the presence of 10 μM ouabain without and with 0.1 mM furosemide. Points and bars are means ± SE of differences for four samples. (*B*) Double-reciprocal plots of the data in *A*. (*C*) Plot of app.K'_{Rb} against the reciprocal of $[Cl^-]e$. The regression curve is expressed by the quadratic equation $y = 15919x^2 + 117.61x + 0.0176$, which should be theoretically app.$K'_{Rb} = (1 + K'_{Cl1}/[Cl^-]e)K'_{RbCl}/[Cl^-]e$. Therefore, $K'_{Cl1} = 15919/118 = 135$ mM. Also, $K'_{Cl2} = K'_{RbCl}/[Rb^+]e = 23.5$ mM in the presence of 5 mM Rb^+e. The regression lines in *B* and the regression curve in *C* were obtained by the least-squares method. The mean initial intracellular Na^+ and K^+ concentrations were 44 and 154 mmol/liter cell water

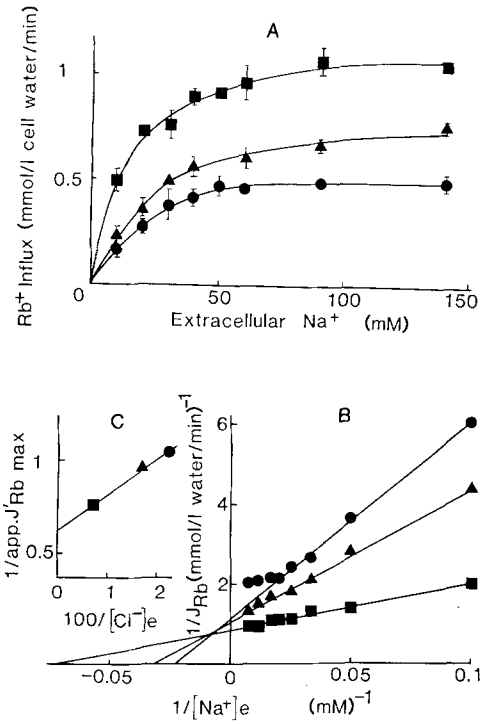

Fig. 8. Effect of Cl^- concentration in the medium on ouabain-insensitive, furosemide-sensitive Rb^+ influx into HeLa cells. (*A*) J_{Rb} as functions of $[Na^+]e$. ●, 45 mM Cl^-e; ▲, 60 mM Cl^-e; ■, 140 mM Cl^-e. *See* legend to Fig. 7 for explanation of points and bars. $[Na^+]e$ (10 to 140 mM), $[Rb^+]e$ (5 mM) and $[Cl^-]e$ (45, 60 and 140 mM) were adjusted by addition of NaCl, sodium sulfamate, RbCl and choline chloride. Isotonicity of the medium was maintained with sucrose. (*B*) Double-reciprocal plots of J_{Rb} against $[Na^+]e$. (*C*) Relation between the reciprocals of app. $J'_{Rb\,max}$ and $[Cl^-]e$. The regression line is expressed as 1/app. $J'_{Rb\,max} = (1/_2J'_{Rb\,max})(1 + K'_{Cl2}/[Cl^-]e)$. The regression lines in *B* and *C* were drawn by the least-squares method. The mean initial intracellular Na^+ and K^+ concentrations were 42 and 149 mmol/liter cell water

a point on the ordinate, and this point, which corresponded to $J'_{Rb\,max}$ and was independent of $[Cl^-]e$. The plots were very similar to those shown in Fig. 5 for J_{Rb} *vs.* $[Rb^+]e$ in the presence of different fixed values of $[Na^+]e$ and 140 mM Cl^-e. Values for app.K'_{Rb} obtained from the three points of intersection of the three lines with the abscissa were replotted against the reciprocal of $[Cl^-]e$ (Fig. 7*C*), and gave points located on a quadratic regression curve that passed a point on the ordinate close to the origin of the coordinates. The functional relations of J_{Rb}, $[Rb^+]e$ and $[Cl^-]e$ can be explained by the expression

$$1/J_{Rb} = (1/{}_1J'_{Rb\,max})\{1 + (1 + K'_{Cl1}/[Cl^-]e)K'_{RbCl}/([Rb^+]e[Cl^-]e)\}, \quad (4)$$

where ${}_1J'_{Rb\,max}$ is the apparent maximum J_{Rb} at infinite $[Rb^+]e$, which is independent of the value of $[Cl^-]e$. K'_{Cl1} would be related to one of the Cl^- sites closely relevant to Na^+ binding (*see* Appendix). K'_{RbCl} is a constant especially related with the simultaneous bindings of Rb^+e and Cl^-e to their sites, and is understood from Eqs. (A.8) and (A.12) in the Appendix. The values of K'_{Cl1} and K'_{Cl2} were calculated from the data in Fig. 7*C* to be 135 and 23.5 mM (*see* legend to Fig. 7 and Eq. 5) in the present conditions.

Next, we examined J_{Rb} as a function of $[Na^+]e$ in the presence of three different values of $[Cl^-]e$ at a sufficiently high $[Rb^+]e$ (5 mM) (Fig. 8). Figure 8*A*

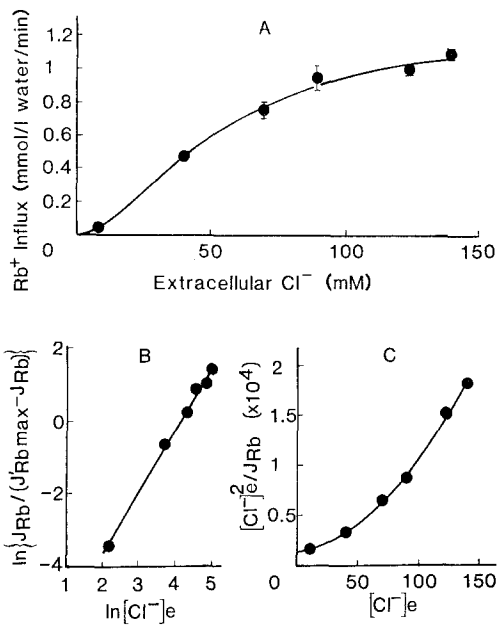

Fig. 9. (*A*) Ouabain-insensitive, furosemide-sensitive Rb^+ influx into HeLa cells as a function of the extracellular Cl^- concentration. $[Cl^-]e$ was adjusted by addition of various concentrations of sodium sulfamate, keeping the sum of the concentrations of Cl^- and sulfamate ion at 140 mM. $[Na^+]e$ and $[Rb^+]e$ were kept at 140 and 5 mM, respectively. *See* legend to Fig. 7 for explanation of points and bars. (*B*) Hill plot of the data shown in *A*. The regression line was drawn by the least-squares method. The Hill coefficient is 1.72 in the present case. (*C*) Plot of $[Cl^-]e^2/J_{Rb}$ *vs.* $[Cl^-]e$. Points correspond to those in *A*. The mean initial intracellular concentrations of Na^+ and K^+ were 69 and 114 mmol/liter cell water

shows that J_{Rb} gave a saturable curve at a constant value of $[Cl^-]e$, and that the curve was shifted upward on increasing $[Cl^-]e$ from 45 to 140 mM. Double-reciprocal plots of J_{Rb} *vs.* $[Na^+]e$ at the three values of $[Cl^-]e$ gave three separate lines (Fig. 8*B*). All pairs of these three straight lines appeared to intersect at different points located to the left of the ordinate and above the abscissa. This result is different from that reported for double-reciprocal plots of J_{Rb} *vs.* $[Na^+]e$ at different values of $[Rb^+]e$, which indicated convergence of the regression lines to about the same point (*see* Fig. 6*B*). The three points of intersection of the lines with the ordinate in Fig. 8*B*, i.e. $1/\text{app.}J'_{Rb\,max}$, were plotted against $1/[Cl^-]e$. As shown in Fig. 8*C*, the three points also fell on a line. From the intersection of this line with the ordinate, we obtained the apparent maximum J_{Rb} at infinite $[Cl^-]e$ in the presence of sufficiently high $[Rb^+]e$ ($_2J'_{Rb\,max}$). The relations shown in Fig. 8 can be expressed by the equation

$$1/J_{Rb} = (1/{}_2J'_{Rb\,max})[1 + \{1 + K'_{NaCl}/([Na^+]e[Cl^-]e)\} \cdot K'_{Cl2}/[Cl^-]e], \quad (5)$$

where K'_{NaCl} is a constant especially connected with the simultaneous bindings of Na^+e and Cl^-e to their binding sites and can be understood from Eqs. (A.6) and (A.10) in the Appendix. K'_{Cl2} is considered to be relevant to Rb^+ binding (*see* Appendix). The slope of the line in Fig. 8*C* is equal to $K'_{Cl2}/{}_2J'_{Rb\,max}$. The values of $_2J'_{Rb\,max}$ and K'_{Cl2} were calculated to be 1.42 mmol/liter cell water per min and 28.3 mM, respectively, in the present conditions.

A plot of J_{Rb} in relation to $[Cl^-]e$ in the presence of sufficiently high $[Na^+]e$ (140 mM) and $[Rb^+]e$ (5 mM) gave a sigmoidal curve (Fig. 9*A*), which seemed to reach almost a plateau at 140 mM Cl^-e. From a double-reciprocal plot of J_{Rb} and $[Cl^-]e$ (*data not shown*), the apparent maximum J_{Rb} at infinite $[Cl^-]e$ ($_3J'_{Rb\,max}$) was found to be 1.33 mmol/liter cell water per min by extrapolation of the regression curve. A Hill plot was made using this value of $_3J'_{Rb\,max}$ (Fig. 9*B*). The Hill coefficient was calculated to be 1.72 in this experiment and its mean value was 1.69 (Table 2), which suggests the presence of at least two Cl^- sites per unit molecule of the cotransport system. We next examined the relation of $[Cl^-]e^2/J_{Rb}$ with $[Cl^-]e$. Figure 9*C* shows the arrangement of points of a quadratic regression curve obtained by the least-squares method. The regression equation was expressed as $[Cl^-]e^2/J_{Rb} = a[Cl^-]e^2 + b[Cl^-]e + c$, where $a = 0.7501$, $b = 15.525$ and $c = 1621.3$. Coefficient a can be shown to be equal to the reciprocal of the value of $_3J'_{Rb\,max}$ described above. Assuming that the ratios c/b and b/a of the coefficients are identical with K'_{Cl1} and K'_{Cl2}, respectively, we calculated them to be 104.4 and 20.7 mM. These relations lead to the expression

$$1/J_{Rb} = (1/{}_3J'_{Rb\,max})\{1 + (1 + K'_{Cl1}/[Cl^-]e)K'_{Cl2}/[Cl^-]e\}, \quad (6)$$

The curve in Fig. 9*A* was drawn according to this equation, and shows good conformity to the experimental results, thus indicating the presence of two Cl^- sites with different affinities.

Kinetic Parameters

Values for the apparent maximum J_{Rb} determined by the different procedures described above are summarized in Table 1. There was no significant difference between either two of the four mean values of the apparent maximum J_{Rb}. This demonstrates equality of the values, regardless of the difference in the procedures used for their calculation, and supports the conclusion that $_jJ'_{Rb\,max}$ is compatible with $J'_{n\,max}$ represented by Eq. (A.5) in the Appendix.

The kinetic parameters for Na^+e and Rb^+e in relation to J_{Rb} and the ratio of $K'_K/\text{app.}K'_{Rb}$ are listed

Table 1. Comparison of the apparent maximal values of ouabain-insensitive, furosemide-sensitive Rb^+ influx into HeLa cells under various ion conditions of the medium[a]

Case	Mean ± SD (mmol/liter cell water/min)	n	Extracellular ion		
			$[Na^+]$	$[Rb^+]$	$[Cl^-]$
$J'_{Rb\,max}$	1.18 ± 0.50	7	irrelevant	infinite	high
${}_1J'_{Rb\,max}$	1.20 ± 0.13	3	high	infinite	irrelevant
${}_2J'_{Rb\,max}$	1.26 ± 0.21	3	irrelevant	high	infinite
${}_3J'_{Rb\,max}$	1.17 ± 0.23	4	high	high	infinite

[a] There is no significant difference between the two values for the mean in either pair of values for the maximal Rb^+ influx ($P > 0.1$). The initial intracellular concentrations (mean ± SD) of Na^+ and K^+ were 55 ± 12 and 128 ± 26 mmol/liter cell water.

Table 2. Kinetic parameters in relation to furosemide-sensitive Rb^+ influx into HeLa cells[a]

Case	Mean ± SD	n[b]
K'_{Na}	105 ± 22 mM	3
K'_{Rb}	0.83 ± 0.11 mM	4
K'_K/app. K'_{Rb}	1.47 ± 0.41	5
K'_{Cl1}	103 ± 22 mM	8
K'_{Cl2}	23.9 ± 4.4 mM	9
Hill coefficient	1.69 ± 0.03	3

[a] The mean initial intracellular concentrations of Na^+ and K^+ were 62 ± 3 and 120 ± 6 mmol/liter cell water (377 ± 18 and 731 ± 158 nmol/mg protein), respectively. The values were obtained directly or by calculation by the procedures described in the Results. The value of K'_{Cl1} was obtained with 140 mM Na^+e and that of K'_{Cl2} with 5 mM Rb^+e.

[b] n, number of samples. *See* Results for details.

in Table 2. These parameters were obtained by the procedures used in the experiments shown in Figs. 3 to 6, and the mean values for K'_{Na} and K'_{Rb} are 105.0 and 0.83 mM. Also, K'_{Cl1}, K'_{Cl2} and Hill coefficient for Cl^-e are shown, which were obtained by the procedures described for the experiments shown in Figs. 7 to 9. The mean values of the apparent K_m for Cl^-e are 103 and 23.9 mM. Since the ratio of K'_{Na}/K'_{Cl1} is 105/103 and the ratio of $[Na^+]e/[Cl^-]e$ is 140/140 under normal conditions, $K'_{Na}/K'_{Cl1} = [Na^+]e/[Cl^-]e$, which confirms the validity of Eq. (A.13). Similarly, $K'_{Rb}/K'_{Cl2} = [Rb^+]e/[Cl^-]e$, which supports Eq. (A.14). Using these relations, we can rewrite Eqs. (4) to (6) more simply as

$$1/J_{Rb} = (1/J'_{Rb\,max})\{1 + (1 + K'_{Na}/[Na^+]e)K'_{Rb}/[Rb^+]e\}, \quad (7)$$

in the presence of a constant $[Cl^-]e$, and

$$1/J_{Rb} = (1/J'_{Rb\,max})\{1 + (1 + K'_{Cl1}/[Cl^-]e)K'_{Cl2}/[Cl^-]e\}, \quad (8)$$

in the presence of constant $[Na^+]e$ and $[Rb^+]e$. These equations correspond to each other with different expressions and equal Eqs. (A.4) and (A.9) in the absence of $Ne(K^+e)$.

Rb^+ influx is significantly influenced by $[Na^+]c$, $[K^+]c$ and $[Cl^-]c$ (*unpublished data*), suggesting that the kinetic parameters must change depending on the intracellular concentrations of these ions, as can be explained by the cotransport models shown in the Appendix.

Stoichiometry of Cotransport

The effect of addition of Rb^+e or Cl^-e on ouabain-insensitive $^{22}Na^+$ influx and that of Cl^-e on Rb^+ influx were examined in the presence of sufficient $[Na^+]e$ (Table 3). Omission of Rb^+e reduced ouabain-insensitive $^{22}Na^+$ influx to the same level as that attained by further addition of 0.1 mM furosemide, indicating inhibition of furosemide-sensi-

Table 3. Effects of Rb^+ and Cl^- in the medium on ouabain-insensitive $^{22}Na^+$ and Rb^+ influxes into HeLa cells in the presence or absence of 0.1 mM furosemide

Extracellular ion	Flux component	Influx (mmol/liter cell water/min)	
		$^{22}Na^+$	Rb^+
5 mM Rb^+, 140 mM Cl^-			
	Total	2.00 ± 0.12	1.27 ± 0.04
	Furosemide-insensitive	0.98 ± 0.11	0.24 ± 0.01
	Furosemide-sensitive	1.02 ± 0.20	1.03 ± 0.07
5 mM Choline, 140 mM Cl^-			
	Total	0.90 ± 0.04[a]	
	Furosemide-insensitive	0.85 ± 0.06[a]	
	Furosemide-sensitive	0.05 ± 0.07	
5 mM Rb^+, 5 mM Cl^- 135 mM sulfamate			
	Total	0.87 ± 0.05[b]	0.30 ± 0.02[c]
	Furosemide-insensitive	0.79 ± 0.09[b]	0.21 ± 0.01[c]
	Furosemide-sensitive	0.07 ± 0.10	0.09 ± 0.04

[a,b,c] No significant difference between the two values in the respective pairs ($P > 0.1$, $P > 0.1$ and $0.05 < P < 0.1$, respectively). The medium contained 140 mM Na^+. The fluxes were assayed in the presence of 10 μM ouabain only or 10 μM ouabain plus 0.1 mM furosemide.

Table 4. Effect of omission of Na^+ or Rb^+ from the medium on ouabain-insensitive influxes of Rb^+ and $^{36}Cl^-$ into HeLa cells

Extracellular cations	Flux component	Influx (mmol/liter cell water/min)	
		Rb^+	$^{36}Cl^-$
140 mM Na^+, 5 mM Rb^+			
	Total	1.13 ± 0.03	2.97 ± 0.46[a]
	Furosemide-insensitive	0.25 ± 0.01	1.16 ± 0.34[a]
	Furosemide-sensitive	0.88 ± 0.05	1.81 ± 0.33
140 mM Na^+, 5 mM Choline			
	Total		1.43 ± 1.36[b]
	Furosemide-insensitive		1.29 ± 0.39[b]
	Furosemide-sensitive		0.31 ± 0.20
5 mM Rb^+, 140 mM Choline			
	Total	0.36 ± 0.02	0.44 ± 0.38[c]
	Furosemide-insensitive	0.26 ± 0.02	0.37 ± 0.40[c]
	Furosemide-sensitive	0.10 ± 0.03	0.07 ± 0.26

[a] Significant difference between the two values ($P < 0.01$).
[b,c] No significant difference between the two values in the respective pairs ($P > 0.1$). The medium contained 140 mM Cl^-. The fluxes were assayed in the presence of 10 μM ouabain only or 10 μM ouabain plus 0.1 mM furosemide. The mean values for the initial intracellular concentrations of Na^+ and K^+ were 52 and 128 mmol/liter cell water.

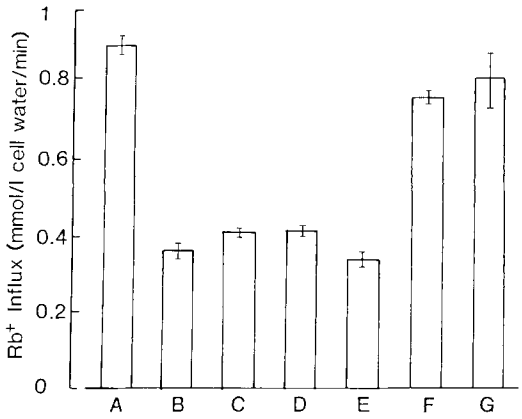

Fig. 10. Effect of various monovalent anions added to the medium on ouabain-insensitive Rb^+ influx into HeLa cells. *A*, 140 mM Cl^-; *B*, 70 mM Cl^- + 70 mM nitrate; *C*, 70 mM Cl^- + 70 mM thiocyanate; *D*, 70 mM Cl^- + 70 mM lactate; *E*, 70 mM Cl^- + 70 mM acetate; *F*, 70 mM Cl^- + 70 mM sulfamate; *G*, 70 mM Cl^- + 70 mM gluconate. The mean initial intracellular concentrations of Na^+ and K^+ were 62 and 93 mmol/liter cell water

tive Na^+ influx, but it did not affect furosemide-insensitive $^{22}Na^+$ influx. Decrease of $[Cl^-]e$ to 5 mM resulted in similar inhibition of furosemide-sensitive $^{22}Na^+$ influx. Ouabain-insensitive Rb^+ influx was also strongly suppressed by the same treatment mainly due to inhibition of furosemide-sensitive influx. This demonstrates a coupling of furosemide-sensitive Na^+ and Rb^+ influxes dependent on Cl^-e. As furosemide-sensitive $^{22}Na^+$ influx and Rb^+ influx were 1.02 and 1.03 mmol/liter cell water per min, there was a 1 : 1 stoichiometry between them under nearly normal conditions.

The effects of omission of Na^+ or $K^+(Rb^+)$ from the medium on ouabain-insensitive Rb^+ and $^{36}Cl^-$ influxes were examined (Table 4). Marked suppression of ouabain-insensitive Rb^+ and $^{36}Cl^-$ influxes were observed in the absence of Na^+e. The suppression was mainly due to inhibition of furosemide-sensitive components. On omission of Rb^+, ouabain-insensitive $^{36}Cl^-$ influx decreased to less than half that in the complete medium, also due to inhibition of the furosemide-sensitive components. Conversely, furosemide-insensitive Rb^+ influx remained unchanged in Na^+-deficient medium, and furosemide-insensitive $^{36}Cl^-$ influx was also not affected by the absence of Rb^+e. But, in the absence of Na^+e, the furosemide-insensitive $^{36}Cl^-$ influx was markedly reduced, an effect that is probably related to inhibition of the anion exchange system. Ouabain-insensitive, furosemide-sensitive Rb^+ and $^{36}Cl^-$ cotransport takes place in a stoichiometry of about 1 : 2 in the complete medium.

Effects of Other Anions

The effects of other monovalent anions in place of extracellular Cl^- on ouabain-insensitive Rb^+ influx into HeLa cells were investigated (Fig. 10). Half the normal Cl^- concentration, i.e. 70 mM, was replaced by the same concentration of these anions since at this concentration the anions had distinct inhibitory effects on the Rb^+ influx. Addition of either nitrate, acetate, thiocyanate or lactate strongly inhibited Rb^+ influx, whereas addition of sulfamate or gluconate was less inhibitory. Taking the results in Fig. 9 also into consideration, we conclude that the slight reduction in Rb^+ influx by partial replacement of Cl^-e by sulfamate, and probably also by gluconate, is simply due to decrease in $[Cl^-]e$ rather than to inhibition by these anions. For this reason we used sulfamate to alter the Cl^- concentration, keeping the total concentration of monovalent anions unchanged, unless otherwise mentioned.

In contrast to the noninhibitory effect of partial replacement with sulfamate, partial replacement of

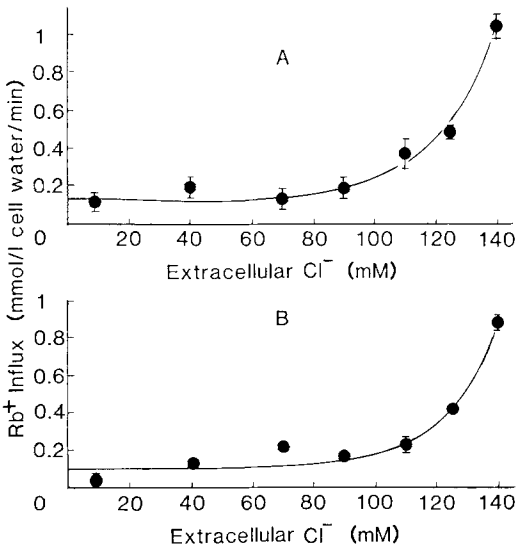

Fig. 11. Effects of nitrate and thiocyanate in place of Cl^- on ouabain-insensitive, furosemide-sensitive Rb^+ influx into HeLa cells as functions of the extracellular Cl^- concentration. (*A*) Effect of nitrate. (*B*) Effect of thiocyanate. The sum of the concentrations of extracellular Cl^- plus any one of the substituted anions was held at 140 mM. The Na^+ and Rb^+ concentrations in the medium were 140 and 5 mM. *See* legend to Fig. 7 for explanation of points and bars. The mean initial intracellular concentrations of Na^+ and K^+ were 69 and 114 mmol/liter cell water

Cl^-e by nitrate or thiocyanate keeping the sum of the extracellular concentrations of Cl^- plus the anion at 140 mM caused significant inhibition of J_{Rb} (Fig. 11). The cation flux was suppressed to a very low level not only when $[Cl^-]e$ was 50 mM, but also even when it was 70 mM. When $[Cl^-]e$ was increased to above 90 mM, J_{Rb} increased abruptly. Therefore, the points seemed to be located on an upward concave curve. When Cl^-e was replaced by nitrate, slight J_{Rb} remained even at extremely low $[Cl^-]e$ (Fig. 11*A*). The inhibition of J_{Rb} by nitrate is well explained by assuming a competitive inhibition of Rb^+ transport coupled with Cl^- by nitrate and that coupled with nitrate by Cl^-. Experimental results seemed to be best described by use of values of 90, 20, 30 and 5 mM for $K'_{Cl1}K'_{Cl2}$, the apparent K_m for nitrate relevant to one of the Cl^- binding sites and the similar parameter relevant to another Cl^- site, respectively, and 1.29 and 0.13 mmol/liter cell water per min for $J'_{Rb\,max}$ coupled with Cl^- and nitrate, respectively, as shown by the curve in Fig. 11*A*. This obviously shows that nitrate has rather higher affinities than Cl^- to Cl^- sites and that a minute part of J_{Rb} is coupled with nitrate transport. A similar result was obtained for thiocyanate, but there was no evidence for cotransport of Rb^+ and thiocyanate (Fig. 11*B*).

Discussion

There are three main components of K^+ influx into cultured mammalian cells in the steady state. These are a ouabain-sensitive Na^+,K^+-pump, a Cl^--dependent cotransport system and a passive process. The Cl^--dependent component of cation fluxes seems to be identical with that mediated via the diuretic-sensitive cotransport system. The concentrations of one of the loop-diuretics, furosemide, for half-maximal inhibition of ouabain-insensitive Rb^+ influx into HeLa cells, IC_{50}, was 1 μM and inhibition was complete with 50 μM furosemide (Fig. 1). This IC_{50} value is lower than other values reported for inhibition of $^{86}Rb^+$ influx such as 3 to 5 μM for MDCK cells (Aiton et al., 1981), HeLa cells (Aiton et al., 1981), 3T3 cells (Atlan et al., 1984) and human red blood cells (Chipperfield, 1980). The reason why our value is lower than reported values for cells including HeLa cells is not yet known. The sensitivity to diuretics seems to vary depending on the anion composition of the medium, since an increase in Cl^-e is reported to shift the dose-response curve for the effect of bumetanide toward higher concentration (Haas & McManus, 1983). With respect to the ratio of diuretic-sensitive Rb^+ influx to total ouabain-insensitive Rb^+ influx, we found that about 71 to 77% of the passive Rb^+ influx into HeLa cells was inhibited by furosemide (*see* Results), which is comparable to the value of 76% for Ehrlich ascites tumor cells (Tupper, 1975), but higher than that of 57% for 3T3 cells (Atlan et al., 1984). Therefore, the inhibition by furosemide and the sensitivity of cells to this drug may vary depending on the cell type and the conditions.

Rb^+ as a Substitute for K^+

K^+ in the medium was totally replaced by Rb^+ in the present study, because previously we found that K^+ and Rb^+ seems to have similar binding constants to the furosemide-sensitive transport system (Miyamoto et al., 1978). The influxes of K^+ and Rb^+ into MDCK cells do not deviate from a 1 : 1 ratio in the presence of both ouabain and furosemide or of either one of these inhibitors (Aiton et al., 1982). Similarly, Ehrlich ascites tumor cells do not distinguish Rb^+ and K^+ in furosemide inhibition (Geck et al., 1980). We demonstrated competitive inhibition of furosemide-sensitive Rb^+ influx by K^+ (Fig. 4). K'_K is not necessarily equal to the apparent K_m for K^+e in diuretic-sensitive K^+ influx. However, the two parameters may be regarded as having closely similar values in the present case. As the mean ratio of $K'_K/\text{app}.K'_{Rb}$ was 1.47, Rb^+ and K^+ seem to have similar affinities to the furosemide-sensitive trans-

port system, although the affinity of Rb^+ is slightly higher.

Effects of Other Anions

Other anions have been reported to be unable to replace Cl^- in supporting diuretic-sensitive or Cl^--dependent cation fluxes (Chipperfield, 1980; Aiton et al., 1981; Aiton & Simmons, 1983; Jayme et al., 1984), except Br^-, which is very slightly effective (Geck et al., 1980). Replacement of Cl^- in the medium by other anions changes the apparent K_m for Cl^- in relation to diuretic-sensitive $^{86}Rb^+$ influx into human red blood cells (Chipperfield, 1984) and HeLa cells (Tivey et al., 1985); nitrate and thiocyanate or isethionate in particular shift the K_m toward higher concentrations than gluconate. Our results showed stronger suppression of ouabain-insensitive Rb^+ influx by nitrate, thiocyanate or acetate than by sulfamate or gluconate (Fig. 10). Nitrate may bind to Cl^--transport sites in the membranes of dog kidney with somewhat lower affinities than Cl^-, and may not itself be transported (Forbush & Palfrey, 1983). Similar results in the present study suggested that nitrate is transported very slightly and thiocyanate scarcely at all in place of Cl^- (Fig. 11*B* and *C*). Unexpectedly, both anions apparently had relatively high binding affinities to the Cl^- sites, competing with Cl^-. However, another possibility that these two ions inhibit Rb^+ influx by binding to a separate modifier site cannot be excluded, as has been pointed out from the competitive effect of acetate in human red blood cells (Dunham et al., 1980). In contrast to nitrate and thiocyanate, sulfamate apparently did not bind to Cl^- sites, i.e. the apparent K_m and K_i values for sulfamate were much higher than the apparent K_m for Cl^-e (Fig. 3*A*). In the presence of sulfamate Rb^+ influx changed only dependent on the concentration of Cl^- in the medium. Gluconate is expected to have a similar effect to sulfamate, although this possibility has not yet been investigated.

Kinetic Parameters

Ouabain-insensitive, furosemide-sensitive $^{22}Na^+$ and $^{36}Cl^-$ influxes into HeLa cells showed a stoichiometry of 1 : 1 and 2 : 1, respectively, with Rb^+ influx. From these results we are convinced that Na^+, Rb^+ and Cl^- influxes into HeLa cells are tightly coupled and mediated by the diuretic-sensitive cotransport system with a stoichiometry of 1 : 1 : 2, which suggest the presence of one Na^+, one K^+ and two Cl^- sites on the molecule of the cotransport system.

The mean app.K'_{Rb} at 140 mM Na^+e was 1.9 mM, as calculated from the data shown in Figs. 3, 4 and 5. The value was compared with the corresponding values for $^{86}Rb^+$ or $^{42}K^+$ influx into various types of cells. The value is approximately twice that of 1 mM for HeLa and MDCK cells (Aiton et al., 1981; 1982), but lower than those of 3.5 mM for 3T3 cells (Atlan et al., 1984), 6 mM for L cells (Gargus & Slayman 1980), and 2.6 to 10 mM for various kinds of red blood cells (Dunham et al., 1980; Chipperfield, 1981; Duhm & Göbel, 1984*b*; Hall & Ellory, 1985). Most of these values were determined under almost normal conditions. The mean value was reduced to 0.83 mM on extrapolation of Na^+e to infinity (K'_{Rb}, *see* Table 2). The only reported value corresponding to this is 9 mM for MDCK cells (Rindler et al., 1982).

On the other hand, the apparent K'_{Na} as 13.6 mM at 5 mM Rb^+e (Fig. 6*B*). This value is similar to that of 10 mM reported for MDCK cells (Aiton et al., 1981), but much lower than other reported values, such as 25 mM for HeLa cells (Aiton et al., 1981), 68.9 mM for 3T3 cells (Atlan et al., 1984), and 35 to 65 mM for red blood cells (Aiton et al., 1981; Duhm & Göbel, 1984*b*). Extrapolation of Rb^+e to zero gave a value of 105 mM (Table 2), which is similar to that of 85 mM for MDCK cells in the absence of K^+e and Cl^-e (McRoberts et al., 1982). Again, corresponding values for other types of cells have not been reported.

Values for the apparent K_m for Cl^-e have also been determined in various cell types. For example, they are reported to be 100 mM for MDCK and HeLa cells (Aiton et al., 1981), 59 to 96 mM for MDCK cells (McRoberts et al., 1982), 70 to 80 mM for human red blood cells (Aiton et al., 1981), and 62.4 mM for duck red cells (Haas & McManus, 1983). Our value obtained with sulfamate as a substitute for Cl^- was about 50 mM (Fig. 9). However, from our results on the kinetic properties of Rb^+ influx as functions of $[Na^+]e$, $[Rb^+]e$ and $[Cl^-]e$, we calculated two different values for the apparent K_m for Cl^-e, i.e., 103 and 24 mM. This finding agrees with the evidence for two cooperative Cl^--binding sites with different values of the apparent K_m, 55.2 and 5.14 mM, in renal epithelial cells (Brown & Murer, 1985) but contrasts with that for two independent and identical Cl^- sites in mouse 3T3 cells (Atlan et al., 1984). Our larger value (K'_{Cl1}, 103 mM) is apparently correlated with Na^+ binding, while our smaller value (K'_{Cl2}, 23.9 mM) is correlated with $Rb^+(K^+)$ binding. As can be understood from Eqs. (A.13) and (A.14) in the Appendix, K'_{Cl1} and K'_{Cl2} should be equal to $K'_{Na}[Cl^-]e/[Na^+]e$ and $K'_{Rb}[Cl^-]e/[Rb^+]e$. Introducing values of 105 mM for K'_{Na}, 0.83 mM for K'_{Rb} (*see* Table 2), and 140, 5 and 140 mM for

$[Na^+]e$, $[Rb^+]e$ and $[Cl^-]e$ into the equations, we demonstrated good agreements of the calculated values with the above ones obtained independently. Therefore, there must be two unequal Cl^- sites on the molecule of the diuretic-sensitive cotransport systems in HeLa cells.

Mechanism of Cotransport

Double-reciprocal plots showed linear relations between J_{Rb} and $[Rb^+]e$ and between J_{Rb} and $[Na^+]e$. In consideration of the 1 : 1 stoichiometry of $^{22}Na^+$ and Rb^+ influxes, these relations support that one Rb^+ ion is moved via the cotransport system coupled with a Na^+ ion. Similar simple Michaelis-Menten kinetics has been observed between $^{22}Na^+$ influx and the Na^+ concentration in membrane vesicles of rabbit kidney, the Hill coefficient being 1 (Koenig et al., 1983). Four possible mechanisms of Na^+ and $K^+(Rb^+)$ bindings to their sites should be considered in relation to our experimental results. The first one is random binding of the cations to the cotransport system. An example of this has been reported for furosemide-sensitive Rb^+ influx into 3T3 cells (Atlan et al., 1984) and the assumptions of random binding of Na^+, K^+ and Cl^- and of the effect of binding of any one ion on the bindings of the other ions have been used to set up flux equations for MDCK cells (McRoberts et al., 1982). The second case is the binding of K^+ to its site before the binding of Na^+. The third case is simultaneous binding of the two cations. However, these three cases are all inconsistent with our experimental results and, therefore, these possibilities are excluded. Fourth, if Na^+ binds to the site before the binding of Rb^+, the flux equation derived theoretically agrees well with Eq. (3) in the Results which suggests that this sequence of cation bindings actually occurs in HeLa cells. This equation implies that Na^+ binding is a trigger for Rb^+ binding, i.e. that there is cooperative interaction of the sites of the two cations, and that $[Na^+]e$ influences the affinity for Rb^+ but not the apparent maximum Rb^+ influx, and that the two cations are moved concomitantly into the cells, i.e. by cotransport. Evidence in the present paper, including the $Na^+/K^+/2Cl^-$ stoichiometry, a quadratic relation of $[Cl^-]e^2/J_{Rb}$ *vs.* $[Cl^-]e$ and the Hill coefficient (1.7), confirmed the participation of two Cl^- ions. In addition, our other results on the effect of Cl^-e on J_{Rb}, which can be described by Eqs. (4) and (5) in the Results, are well understood by assuming a sequence of Cl^- ion bindings to the cotransport system, as explained by a working model (*see* Fig. 12*B*). The sequence is that one Cl^- ion binds to a lower affinity site in response to the binding of one Na^+ ion to its site and then another Cl^- ion binds to the other site with higher affinity in response to the binding of one Rb^+ ion to the K^+ site. Therefore, the cotransport system remains electroneutral throughout the reaction process. There must be cooperative interaction between the two cation binding sites and between the cation sites and Cl^- sites. Cooperativity has also been proposed for the ion binding sites of diuretic-sensitive cotransport systems in MDCK cells (Rindler et al., 1982) duck red blood cells (Haas & McManus, 1982) and renal epithelial cells (Brown & Murer, 1985). In our transport models we assume that the monovalent ions associate with and dissociate from the cotransport system in a symmetric sequence on both sides of the cell membrane. This does not necessarily mean symmetry of transmembrane ion fluxes, since at corresponding reaction steps the rate and dissociation constants on the two sides are assumed generally to be unequal, and this assumption seems to be valid judging from reported results. For example, while furosemide-sensitive Na^+-for-Na^+ exchange in human red blood cells (Brand & Whittam, 1985) and Na^+-for-Na^+ and K^+-for-K^+ exchanges in Ehrlich ascites tumor cells (Tupper, 1975) occur symmetrically, furosemide-sensitive Rb^+ flux in mouse 3T3 cells (Atlan et al., 1984) and Na^+/K^+ cotransport in human red cells (Benjamin & Dunham, 1983) occur asymmetrically. Furosemide-sensitive Na^+ flux is symmetric in Cl^- medium, whereas it becomes asymmetric in nitrate medium (Brand & Whittam, 1985).

Our transport models imply significant influences of intracellular ions on kinetic parameters for extracellular ions. While no obvious influence on furosemide-sensitive Na^+ influx has been found, even on threefold increase in $[Na^+]c$ in human red blood cells (Brand & Whittam, 1984), significant effects of intracellular monovalent ions on ouabain-insensitive or furosemide-sensitive cation fluxes in human red blood cells and squid axons have frequently been reported (Lubowitz & Whittam, 1969; Russell, 1979; 1983; Garay et al., 1981). When $\ln([K^+]c/[K^+]e)$ increases, furosemide-sensitive net K^+ efflux is increased in HeLa cells (Aiton & Simmons, 1983). We also obtained results indicating correlations of transmembrane gradients of Na^+, $K^+(Rb^+)$ and Cl^- with furosemide-sensitive Rb^+ influx (*data not shown*). The sum of the transmembrane gradients of the chemical potentials of Na^+, K^+ and Cl^- is generally accepted to be important in the cotransport system (Schmidt & McManus, 1977; Duhm & Göbel, 1984*a*; Jayme et al., 1984). Therefore, the implication of the effects of intracellular ions seems reasonable and it is premature to complete this transport model until the roles of in-

tracellular ions in the cotransport system have been elucidated.

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Appendix

Having regard to the models proposed for ion-driven cotransport system (Sanders et al., 1984) and for Na^+ and Ca^{++} exchange (Johnson & Kootsey, 1985), we developed a model which explains the mechanism of a cotransport system that synchronously moves ions in a stoichiometry of $Na^+/K^+/2Cl^-$ (Fig. 12*A*). This model includes 64 steps of random binding reactions between ions and the cotransport system and two steps of conformational changes of the system involving ion movements across the cell membrane. One unit molecule of the cotransport system has one binding site of a cation M (Na^+) and another site of a cation N (K^+) and two sites (sites I and II) of an anion A (Cl^-) with different affinities. The association and dissociation of the ions occur rapidly and the sequence of the reactions is symmetric on the two sides of the membrane. After these bindings are complete, the ions are moved slowly across the membrane keeping electroneutrality. The driving forces for inward and outward movements of the ions would be mainly derived from gradients of the chemical potentials of Na^+ and K^+, respectively. K_j and K'_j represent the dissociation constants on the two faces and k_j the rate constant. "T" represents one unit molecule of the cotransport system. By application of the method of Cha (1968) to the model the following equations are obtained.

Let

$$\alpha = k_{-1} + k_2 K'_1 K'_5 K'_{17} K'_{29}/([M]c[N]c[A]c^2), \quad \text{(A.1)}$$

$$\begin{aligned}\beta = {} & 1 + K'_{32}/[M]c + K'_{31}/[N]c + (K'_{29} + K'_{30})/[A]c \\ & + K'_{27}K'_{31}/([M]c[N]c) + (K'_{23}K'_{29} + K'_{25}K'_{30})/([M]c[A]c) \\ & + (K'_{19}K'_{29} + K'_{21}K'_{30}/([N]c[A]c) + K'_{17}K'_{29}/[A]c^2 \\ & + (K'_{11}K'_{19}K'_{29} + K'_{14}K'_{21}K'_{30})/([M]c[N]c[A]c) \\ & + K'_8K'_{17}K'_{29}/([M]c[A]c^2) + K'_5K'_{17}K'_{29}/([N]c[A]c^2) \\ & + K'_1K'_5K'_{17}K'_{29}/([M]c[N]c[A]c^2). \quad \text{(A.2)}\end{aligned}$$

Then, the rate of unidirectional N influx (J_N) can be expressed by the equation

$$\begin{aligned}1/J_N = {} & \{(\alpha + k_1\beta)/(k_1\alpha[Tt])\}[1 + \{\alpha/(\alpha + k_1\beta)\}\{K_{32}/[M]e \\ & + K_{31}/[N]e + (K_{29} + K_{30})/[A]e + K_{27}K_{31}/([M]e[N]e) + (K_{23}K_{29} \\ & + K_{25}K_{30})/([M]e[A]e) + (K_{19}K_{29} + K_{21}K_{30})/([M]e[A]e) \\ & + K_{17}K_{29}/[A]e^2 + (K_{11}K_{19}K_{29} + K_{14}K_{21}K_{30})/[M]e[N]e[A]e) \\ & + K_8K_{17}K_{29}/([M]e[A]e^2) + K_5K_{17}K_{29}/[N]e[A]e^2\} \\ & + \{(\alpha + k_{-2}\beta)/(\alpha + k_1\beta)\}K_1K_5K_{17}K_{29}/([M]e[N]e[A]e^2)], \quad \text{(A.3)}\end{aligned}$$

where $[Tt]$ indicates the total concentration of molecules of the cotransport system. As a special case, we assume that another cation n (Rb^+) is also added to medium and that the complexes of

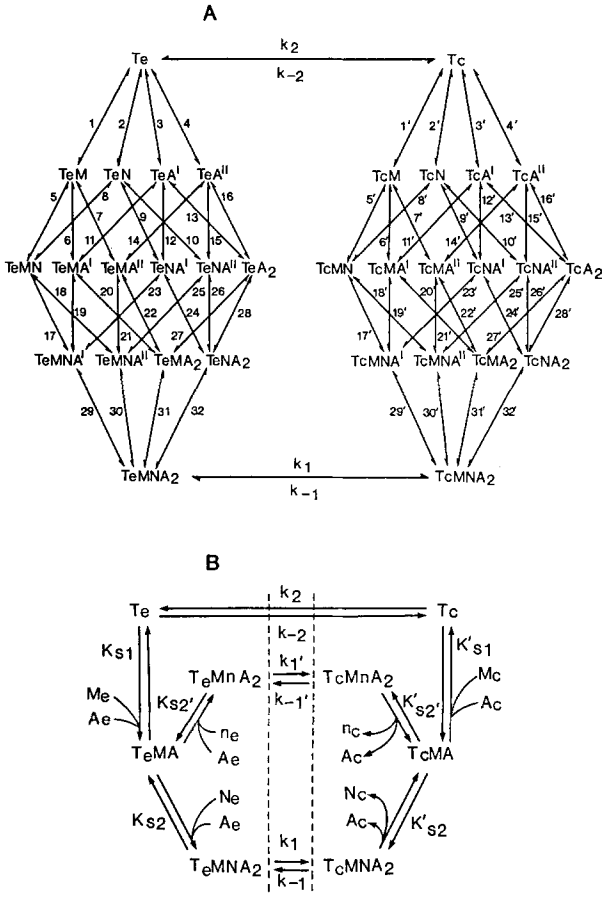

Fig. 12. Schematic presentation of kinetic models of the ouabain-insensitive, diuretic-sensitive Na^+, K^+, Cl^--cotransport system in the cell membrane. (*A*) Generalized model for simultaneous ion movements. (*B*) Specialized model applicable to HeLa cells

the ions and the transport system other than *Te*, $TeMA^1$, $TeMNA_2$, $TeMnA_2$, *Tc*, $McMA^1$, $TcMNA_2$ are not present (Fig. 12*B*). This implies simultaneous binding of one cation *M* and one anion *A* to the system before the simultaneous binding of the second cation *N* or *n* and *A*. Under initial conditions when $[n]c$ is zero, immediately after replacement of medium containing *N* by that containing *N* and *n*, the following rate equation for unidirectional *n* influx is derived by modifying Eq. (A.3).
Let

$$\alpha_s = \{k_2K'_{s1}/([M]c[A]c) + k_{-1}[N]c[A]c/K'_{s2}\}K'_{s2'}/[A]c,$$
$$\beta_s = \{1 + K'_{s1}/([M]c[A]c) + [N]c[A]c/K'_{s2}\}K'_{s2'}/[A]c,$$

then

$$1/J_n = (1/J'_{n\max})\{1 + (1 + K'_{Me}/[M]e + [N]e/K'_{Nei})K'_n/[n]e\}, \tag{A.4}$$

where

$$J'_{n\max} = k_{1'}\alpha_s[Tt]/(\alpha_s + k_{1'}\beta_s), \tag{A.5}$$
$$K'_{Me} = \{(\alpha_s + k_{-2}\beta_s)/\alpha_s\}K_{s1}/[A]e, \tag{A.6}$$
$$K'_{Nei} = \{\alpha_s/(\alpha_s + k_1\beta_s)\}K_{s2}/[A]e, \tag{A.7}$$
$$K'_{ne} = \{\alpha_s/(\alpha_s + k_{1'}\beta_s)\}K_{s2'}/[A]e. \tag{A.8}$$

$J'_{n\max}$ is the apparent maximum rate of unidirectional *n* influx and K'_{Me}, K'_{ne} and K'_{Nei} are the apparent K_m's for *Me* and *ne* and the apparent K_i for *Ne*, respectively. Equation (A.4) becomes identical with Eq. (3) in the Results provided that $[N]e$ is zero. Based on Eqs. (A.6) to (A.8), Eq. (A.4) can be converted to the following Eq. (A.9), which indicates the rate of unidirectional *n* influx as a function of $[A]e$ and is identical with Eq. (6) in the Results in the absence of *Ne*.

$$1/J_n = (1/J'_{n\max})\{1 + (1 + K'_{A1}/[A]e + [A]e/K'_{Ai})K'_{A2}/[A]e\}, \tag{A.9}$$

where

$$K'_{A1} = \{(\alpha_s + k_{-2}\beta_s)/\alpha_s\}K_{s1}/[M]e, \tag{A.10}$$
$$K'_{Ai} = \{\alpha_s/(\alpha_s + k_1\beta_s)\}K_{s2}/[N]e, \tag{A.11}$$
$$K'_{A2} = \{\alpha_s/(\alpha_s + k_{1'}\beta_s)\}K_{s2'}/[n]e. \tag{A.12}$$

K'_{A1} and K'_{A2} are the apparent K_m's for *Ae* at *A* sites relevant to *M* and *n*, respectively, and K'_{Ai} is the apparent K_i. Eqs. (A.6), (A.8), (A.10) and (A.12) lead to the expressions

$$K'_{Me}/K'_{A1} = [M]e/[A]e, \tag{A.13}$$
$$K'_{ne}/K'_{A2} = [n]e/[A]e. \tag{A.14}$$