Interrelation between Membrane Transport and the Contents of Alkali Metal Cations in HeLa Cells

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Abstract The relation of membrane transport of alkali cations to their external concentrations or to their cellular contents was studied in HeLa cells. Chilling the cells at 0°C reversed cell Na⁺ and K⁺ to a mirror image of the normal pattern. Upon rewarming to 37°C the ouabain-sensitive Rb⁺ uptake became 2-fold faster than the control. A kinetic analysis revealed that the stimulation was due to an increase in the maximal rate of Rb⁺ uptake, J_{max} . The increase in apparent K_m was relatively small. The analysis also showed that the ouabain-sensitive cation transport system seemed to have two binding sites for Rb⁺. The stimulation of Rb⁺ uptake was related to an increase in cell Na⁺, and an addition of ouabain abolished such a relation. Net Na⁺ flux which was in the direction from inside the cells to the medium at hypernormal cell Na⁺ was increased when cell Na⁺ increased. In contrast, net Na⁺ flux which was in the opposite direction in the presence of ouabain was reduced and became almost 0 at cell Na⁺ of 900 nmol/mg of protein. The Na⁺/Rb⁺ coupling ratio in the ouabain-sensitive cation transport was apparently less than 1 at nearly physiological cell Na⁺, but it approached 1.5 when cell Na⁺ was sufficiently high. The sum of cell K⁺ plus Rb⁺ varied inversely with cell Na⁺, and this relation was unaffected upon treatment with ouabain. When Rb⁺ uptake declined below 80% of the control, cell K⁺ plus Rb⁺ was reduced, however, 40% of the sum of cell cations was still preserved even after complete inhibition of the cation pumps by ouabain treatment for 2 hr. Interrelations of these results are discussed.

Key Words: Rb⁺ transport, HeLa cells, ouabain, sodium ion, potassium ion.

Kinetic studies on the membrane cation pumps have repeatedly been made by using specific materials such as red cells (CAVIERES and ELLORY, 1975; GLYNN and KARLISH, 1975; HOBBS and DUNHAM, 1976; GARAY and GARRAHAN, 1979),

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ghosts of red cells (GLYNN, 1962; GARRAHAN and GLYNN, 1967; PRIESTLAND and WHITTAM, 1968), membrane vesicles prepared from red cells (BLOSTEIN *et al.*, 1979), reconstituted vesicles (SWEADNER and GOLDIN, 1975; ANNER, 1980), and squid axons (BRINLEY and MULLINS, 1968; DE WEER *et al.*, 1979). The advantage of using these materials is that their cation contents can be regulated artificially by techniques including reversible hemolysis, replacing external and internal media separately, internal perfusion, or microinjection. In contrast, cultured mammalian cells have not frequently been used for that purpose, although one can easily select pertinent types of cells from different sources and examine the growth phenomena in relation to cation transport. One reason for the obstruction to use of cultured cells is the difficulty of controlling the intracellular concentrations of alkali metal cations, which seems partly to be due to uneven distribution of the cations inside the cells (PALMER and CIVAN, 1977; GUPTA and HALL, 1978).

HeLa cells are known to have much denser [^3H] ouabain-binding sites on the cell membrane than red cells (BAKER and WILLIS, 1969). This seemed partly to overcome the disadvantage of using cultured cells in the study of cation transport. Therefore, we attempted to make a kinetic analysis of cation pump activities in this type of cells. In the present paper, we show data on the dependence of fluxes of alkali metal cations on their external concentrations and their cellular contents. Furthermore, the data concerning a variation in the Na^+/Rb^+ coupling ratio in the active transport as a function of cell Na^+ in HeLa cells are shown. Our results show that these cells are sufficiently useful for the kinetic analysis of cation pump activities if cellular contents of alkali metal cations, which are closely related to the pumping activities, are determined simultaneously.

MATERIALS AND METHODS

Cell cultures. HeLa cells (strain S3) obtained from Flow Lab. were serially cultivated with a modified Eagle's minimum essential medium (mMEM, MIYAMOTO *et al.*, 1976) supplemented with 10% (v/v) calf serum in Roux flasks. The mMEM used in this study contained double amounts of glucose and amino acids, and a triple amount of glutamine as compared with Eagle's original medium; HEPES was omitted. Cells in the growing cultures were detached from the flasks by 0.5% trypsin and reinoculated in plastic culture dishes (52 mm in diameter, Wako Pure Chem. Co.) at a density of $1.5\text{--}2 \times 10^5$ cells/ml. The cultures were placed in a CO_2 -incubator in a humid atmosphere of 5% CO_2 and 95% air at 37°C.

Preincubation and chilling. After 24-hr culture, the culture medium was replaced with mMEM containing 5.6 mM glucose and 20 mM HEPES (pH 7.2). Cells were preincubated for another 20 min in the presence or absence of 10 μM ouabain. The medium was again exchanged with a slightly modified medium,

which contained 5.4 mM RbCl instead of 5.4 mM KCl. Rubidium ion uptake into cells took place at 37°C for various periods as indicated in legends to figures. For chilling cells the culture dishes containing the preincubation medium were placed for 3–4 hr in an ice-bath at 0°C, unless otherwise stated. To determine passive Rb⁺ uptake cells were preincubated with the medium containing 10 μ M ouabain for 10 min before chilling. The activity of Na⁺, K⁺-pump was completely inhibited by such a treatment (MIYAMOTO *et al.*, 1978a).

Assays. The cultures were rinsed six times with ice-cold 0.15 M LiCl in less than 15 sec (MIYAMOTO *et al.*, 1978b). Three to 6 ml of ice-cold redistilled water was put in each culture dish. Cells were detached from the dish and suspended by a silicone-rubber policeman rotating at the rate of 120 rpm. To assay alkali metal cations, 3 ml of 30 mM LiCl was added to the same amount of each cell suspension. The mixture was put in a screw-capped test tube and placed for at least 24 hr at room temperature to lyse cells and extract the cations. The concentrations of K⁺, Rb⁺, and Na⁺ were determined by flamephotometry, for which 15 mM LiCl was used as the internal standard. For protein measurement, 1 ml of each cell suspension was mixed with 1 ml of 1 N NaOH; cell protein was assayed by the method of LOWRY *et al.* (1951).

Reagents and others. The specially pure grade of KCl, LiCl, other inorganic salts, glucose, and Folin-phenol reagent were purchased from Wako Pure Chem. Co.; ouabain, 2-deoxyglucose, HEPES, and carbonyl cyanide *m*-chlorophenylhydrazine (CCCP) from Sigma Chem. Co.; RbCl (Suprapur) from Merck; furosemide from Hoechst Japan, Ltd.; trypsin (1:250) from Difco Lab.; calf serum from Nakarai Chem., Ltd. For convenience, the concentrated solutions of vitamins (50 $\times$) and amino acids (100 $\times$) purchased from Flow Lab. were used to prepare mMEM.

RESULTS

After a 3.5-hr preincubation of HeLa cells at 0°C, measurements of Rb⁺ uptake and the contents of K⁺ plus Rb⁺ and of Na⁺ were started, and the data obtained are shown as a function of incubation time at 37°C (Fig. 1). Rubidium ion uptake into rewarmed cells was about two times higher than that into control cells. Rb⁺ uptake was suppressed in both control and rewarmed cells to a similar extent by ouabain. The ouabain-sensitive Rb⁺ accumulation increased linearly with time up to 20 min in both rewarmed and control cells. Based on this finding, we measured Rb⁺ uptake for 5–15 min, unless otherwise stated, in order to obtain the rate of uptake which was expressed as nmol/mg of protein per min. Cell Na⁺ was increased to about 1 μ mol/mg of protein, *i.e.*, almost equal to cell K⁺ in the control, at the end of the chilling period. Sodium content, however, was decreased rapidly in 20 min after the start of rewarming to 37°C. In contrast, cell K⁺ was decreased to about 0.3 μ mol/mg of protein after chilling. The

Fig. 1. Time courses of Rb^+ accumulation and the contents of alkali metal cations in HeLa cells. For chilling cells, HeLa cells were preincubated with the modified minimum essential medium for 3 hr at 0°C . Rubidium ion uptake took place at 37°C after chilling. A) Time course of Rb^+ accumulation. Symbols: ●, control; □, $10\ \mu\text{M}$ ouabain; ▲, rewarmed after chilling; and ◇, rewarmed after chilling in the presence of $10\ \mu\text{M}$ ouabain. Inset: time course of ouabain-sensitive Rb^+ accumulation calculated from the data in A. Symbols: ●, control; and ▲, rewarmed after chilling. B) Time course of cellular contents of alkali metal cations. Symbols: ●, Na^+ of control cells; ○, K^+ plus Rb^+ of control cells; ▲, Na^+ of rewarmed cells; and △, K^+ plus Rb^+ of rewarmed cells. Each point indicates mean of four samples, and vertical bar the S.D. of the mean.

K^+ content increased rapidly after rewarming and the pattern of change in this cation content was like a mirror image of that of Na^+ content. The rate of ouabain-sensitive Rb^+ uptake into rewarmed cells was kept constant for 20 min, while the intracellular cations changed markedly. Therefore, the cation pumps seemed to be fully activated during the period. The ouabain-sensitive Rb^+ uptake as a function of Rb^+ concentration in the medium, $[\text{Rb}^+]_o$, was compared between control and rewarmed cells (Fig. 2A). The Rb^+ uptake was almost saturated when $[\text{Rb}^+]_o$ was about 3 mM in control cells, but it was not in rewarmed cells even when $[\text{Rb}^+]_o$ was increased to about 6 mM. From these data, the root square of the reciprocal of the rate of ouabain-sensitive Rb^+ uptake (J) was replotted against the reciprocal of $[\text{Rb}^+]_o$ in Fig. 2B. Using such a plot, the curves obtained were straight for both control and rewarmed cells. Therefore, the following equation was derived:

$$1/J = (1/J_{\max})(1 + k/[\text{Rb}^+]_o)^2, \quad (1)$$

where k is a constant. In control cells, this equation was valid in the range of

Fig. 2. The ouabain-sensitive Rb⁺ uptake and the contents of alkali metal cations in relation to external Rb⁺ concentration, [Rb⁺]_o, in HeLa cells. For chilling cells, HeLa cells were preincubated with the modified minimum essential medium for 3 hr at 0°C, then Rb⁺ uptake took place for 15 min at 37°C. A) The ouabain-sensitive Rb⁺ uptake. Symbols: ●, control; and ▲, rewarmed after chilling. For explanation for the dotted curves see RESULTS. The Rb⁺-substituted medium contained a tracer amount of K⁺, i.e., [K⁺]_o = 24 to 32 μM. Therefore, measured Rb⁺ uptake was corrected as: (Rb⁺ uptake) × {([K⁺]_o + [Rb⁺]_o) / [Rb⁺]_o }, since Rb⁺ uptake has been proved to change in proportion to [Rb⁺]_o / ([K⁺]_o + [Rb⁺]_o) provided [K⁺]_o + [Rb⁺]_o was kept constant at both low and high concentrations (MIYAMOTO *et al.*, 1978b). B) Double reciprocal plot of the square root of the ouabain-sensitive Rb⁺ uptake (*J*) against [Rb⁺]_o. Symbols are the same as those in A. C) Cellular contents of alkali metal cations. Cells were sampled at the end of Rb⁺ uptake. Symbols: ●, Na⁺ of control cells; ○, K⁺ plus Rb⁺ of control cells; ▲, Na⁺ of rewarmed cells; and △, K⁺ plus Rb⁺ of rewarmed cells. Each point indicates mean of four samples and vertical bar the S.D. of the mean.

[Rb⁺]_o less than about 1 mM, but a significant disagreement was seen at a higher concentration range; the application of the simple Michaelis-Menten equation was found to be more appropriate in this case. On the other hand, in rewarmed

cells the equation describes fairly precisely the observed rate. From the straight lines in Fig. 2B $J_{\max}$'s were calculated to be 28.3 and 52.5 nmol/mg of protein per min for control and rewarmed cells; and k 's 0.61 and 0.82 mM, respectively. Dotted lines in Fig. 2A were drawn according to Eq. (1) and using these values. The decrease in cell Na^+ in rewarmed cells was more dominant when $[\text{Rb}^+]_o$ was higher (Fig. 2C). This result suggests that the higher active Na^+ efflux occurs in exchange with the higher active Rb^+ uptake. Slight increases in cell Na^+ in control cells at low $[\text{Rb}^+]_o$ seemed to be due either to decreases in Na^+ efflux or to $\text{Na}^+ : \text{Na}^+$ exchange (GARAY and GARRAHAN, 1973), since there was no such

Fig. 3. Rb^+ uptake and Na^+ flux as functions of the Na^+ content in HeLa cells. To vary the Na^+ content the cells were preincubated with the modified minimum essential medium for various periods at 0°C . Cation fluxes were calculated from the differences of cation contents between 5 and 10 or between 5 and 15 min after reincubation at 37°C with the medium containing 5.4 mM RbCl instead of 5.4 mM KCl . Cell Na^+ in abscissa indicates the Na^+ content at 5 min (see RESULTS). A) Rb^+ uptake. B) Na^+ efflux. Symbols: $\bullet$, control; and $\circ$, 10 μM ouabain. Each point indicates mean of four samples.

tendency in ouabain-treated cells (data not shown).

Rb⁺ uptake and Na⁺ efflux in relation to cell Na⁺ are shown in Fig. 3. The cell Na⁺ contents (abscissa) are the values measured 5 min after exposure to Rb⁺, since the rate of Rb⁺ uptake, especially in the presence of ouabain, and Na⁺ efflux became constant after 5 min. Fluxes of Rb⁺ and Na⁺ were calculated from the difference of cellular contents of these cations between 5 and 10 or between 5 and 15 min, during these periods the cell cations changed linearly with time (data on Na⁺ efflux from ouabain-treated cells not shown). Rubidium ion flux was unidirectional and increased as cell Na⁺ became higher in the control, although it remained nearly constant at a low rate in the presence of ouabain (Fig. 3A). When cell Na⁺ was higher, cells lost more Na⁺ (Fig. 3B). At a cell Na⁺ around 200 nmol/mg of protein, which was in the normal range of contents, the steady state of Na⁺ fluxes seemed to be established across the cell membrane. When cell Na⁺ increased to about 900 nmol/mg of protein, passive movement of Na⁺ in the presence of ouabain was shown to be arrested. Cell K⁺ plus Rb⁺, *i.e.*, the sum of cell K⁺ and Rb⁺ contents, showed a reciprocal correlation with cell Na⁺ (Fig. 4). This relationship was not altered significantly in the presence of ouabain.

Cell K⁺ plus Rb⁺ was observed as a function of Rb⁺ uptake which was varied by separate or combined treatments with the inhibitors: ouabain, furose-

Fig. 4. Correlation between the contents of Na⁺ and of K⁺ plus Rb⁺ in HeLa cells. Cells were preincubated for various periods at 0 or 37°C to change the Na⁺ content. Rb⁺ uptake took place for 15 min at 37°C in medium containing 5.4 mM RbCl in place of 5.4 mM KCl. Thick lines indicate the statistic regression lines and thin curves the confidential intervals of the regression lines at $\alpha=0.05$. A) Control. ●, $y = -1.72x + 1.75$, $r = -0.786$. B) Ten μM ouabain. ○, $y = -1.61x + 1.74$, $r = -0.765$.

Fig. 5. Relation between Rb^+ uptake and the content of K^+ plus Rb^+ in HeLa cells. Rb^+ uptake and the cation content were indicated as percent of the control. Cells were preincubated with the inhibitors for 2 hr, then Rb^+ uptake was assayed for 15 min with the same inhibitors. Symbols: ●, 2 μM CCCP or 4 mM 2-deoxyglucose with various concentrations of glucose from 0 to 5.6 mM; ○, 10 μM ouabain; and Δ, 10 μM ouabain + 0.9 mM furosemide. Each point indicates mean of 8–35 samples. Vertical and horizontal bars indicate the S.D. of the mean in the two directions.

mide, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and 2-deoxyglucose (Fig. 5). CCCP and 2-deoxyglucose were used with various concentrations of glucose. As far as Rb^+ uptake was maintained between 120 and 80% of the control, cell K^+ plus Rb^+ was kept at the normal contents, whereas the sum of cell cations was reduced depending on the rate of Rb^+ uptake as the rate was slowed down below 80%. Even after complete inhibition of the cation pumping by 2-hr treatments with ouabain, about 40% of cell K^+ plus Rb^+ was still preserved. Simultaneous treatments with ouabain and furosemide did not reduce the cell cations further than the treatments with ouabain alone.

DISCUSSION

Rubidium ion is accumulated in HeLa cells in preference to K^+ when observed after a long incubation of more than 24 hr. Nevertheless, Rb^+ was shown to behave similarly to K^+ in membrane transport when influx measurements were carried out in short-term experiments lasting a few hour (MIYAMOTO *et al.*, 1978b). Therefore, Rb^+ has been used as an analog for K^+ to investigate cation transport, and cell K^+ plus Rb^+ can be taken as cell K^+ in HeLa cells as observed in the normal medium.

When HeLa cells were incubated at low temperature, cell Na^+ has been reported to increase concomitantly with a decrease in cell K^+ , and the ouabain-

sensitive $^{86}\text{Rb}^+$ uptake is stimulated (Cook *et al.*, 1975). An increase in the Na^+ content in red cells seemed to increase both the apparent K_m for external K^+ and the maximum rate of ouabain-sensitive K^+ influx, $J_{\max}$, when cell K^+ and external Na^+ were extremely low (SACHS, 1977). We showed similar changes in cell Na^+ and K^+ , and the stimulation of ouabain-sensitive Rb^+ uptake after chilling HeLa cells. Our result revealed that the increase in $J_{\max}$ was significant, but the apparent K_m for external Rb^+ was only slightly increased. This suggests three possible actions of chilling to stimulate Rb^+ pumping in HeLa cells: (1) stimulation of the activity of the cation pump; (2) an increase in the number of pump sites; or (3) both. The ouabain-sensitive $^{86}\text{Rb}^+$ uptake was reported to become more than double, whereas $[^3\text{H}]$ ouabain binding remained unchanged after chilling of HeLa cells (Cook *et al.*, 1975). In addition, a reasonable period of time must be required to synthesize the new cation pump sites after rewarming to 37°C , while the active Rb^+ uptake was stimulated immediately after the re-incubation. Hence, the stimulation of pumping activity should be attributed to action (1) involving an activation of resting pump sites. The inhibition of active Na^+ efflux by cell K^+ was also reported in red cells (KNIGHT and WELT, 1974). Therefore, a decrease in K^+ content in chilled cells might have some contribution to the activation of cation pumps.

The relationship between cell Na^+ and cell K^+ plus Rb^+ showed that, if cell Na^+ was the same, cell K^+ plus Rb^+ was in a similar range, regardless of an addition of ouabain. For this reason, the concentration gradients of cations and the difference of potentials across the cell membrane would be similar between control and ouabain-treated cells provided cell Na^+ was the same. This implies that passive cation fluxes are similar in both cases if cellular Na^+ contents are the same. We calculated the ouabain-sensitive cation fluxes by subtracting fluxes in the presence of ouabain from those for control cells with the same Na^+ contents. The ouabain-sensitive Na^+ flux and Rb^+ uptake were replotted as functions of cell Na^+ (Fig. 6). These fluxes had a tendency to increase when cell Na^+ increased from the normal content. The ratio of ouabain-sensitive Na^+ efflux to ouabain-sensitive Rb^+ uptake (the Na^+/Rb^+ coupling ratio) was not always constant. The ratio was apparently less than 1.0 when cell Na^+ was at about physiological level, but the ratio approached 1.5 as cell Na^+ became sufficiently high. This finding was contrary to the constant ratio of 1.5 irrespective of a variation in ionic environments as observed in red cells by POST and JOLLY (1957). LINDENMAYER *et al.* (1974) proposed a model of Na^+ , K^+ -ATPase, which included 3 Na^+ sites and 2 K^+ sites. The reconstituted vesicles containing Na^+ , K^+ -ATPase were shown to transport 1.8 Rb^+ for every 3 Na^+ on average in solutions with different proportions of NaCl and KCl (SWEADNER and GOLDIN, 1975). However, the ratio of binding sites, 1.5, would not always be identical with the Na^+/Rb^+ ratio in the cation transport. For example, the ratio even in the reconstituted vesicles was found to vary with intravesicular Na^+

Fig. 6. The ouabain-sensitive Rb^+ influx and Na^+ efflux, and the ratio of the two fluxes in relation to the Na^+ content in HeLa cells. Solid curve for the Rb^+ influx was obtained from the difference of values represented by the two curves shown in Fig. 3A, and solid curve for the Na^+ efflux from that shown in Fig. 3B. Broken curve indicates the ratio of values represented by the solid curves at each cell Na^+ .

and K^+ concentrations (ANNER, 1980), and also the ratio was changed as a function of cell Na^+ in squid axons (MULLINS and BRINLEY, 1969; BRINLEY and MULLINS, 1974). In cultured heart cells, the coupling ratio was calculated at 1.5 under physiological conditions, whereas it was increased by treatment with low concentrations of ouabain (LAMB and MCCALL, 1972). These results imply that the Na^+/K^+ coupling ratio can vary when cellular ionic conditions are modified. It has been reported that, when external K^+ concentration is in the physiological range, the ouabain-sensitive K^+ or Rb^+ uptake in cardiac muscle may not reflect the rate of Na^+ efflux; but that, when external K^+ concentration is lowered, the ouabain-sensitive uptake of these cations may indicate the rate of active Na^+ efflux (AKERA *et al.*, 1981). We demonstrated that, when external Rb^+ concentration was low, the mode of ouabain-sensitive Rb^+ uptake could be accounted for by a pump model with two equivalent Rb^+ -binding sites; whereas the mode seemed to be incompatible with the model when external Rb^+ concentration was high (Fig. 2). These findings suggest that the Na^+/Rb^+ ratio of 1.5 at high cell Na^+ may represent the coupling ratio in active pumping in HeLa cells. However, the value of less than 1.0 obtained at low cell Na^+ may not represent the coupling ratio. One possible reason for this was that $\text{Rb}^+:\text{K}^+$ exchange which was equivalent to $\text{K}^+:\text{K}^+$ exchange (GLYNN *et al.*, 1970; SIMONS, 1974) might interfere with the coupling ratio in control cells more strongly than the ratio in rewarmed cells, because control cells contained higher K^+ and lower Na^+ than

rewarmed cells.

The rate of Rb^+ uptake was slowed by treatments with the inhibitors (Fig. 5). The inhibitors included carbonyl cyanide *m*-chlorophenylhydrazone and 2-deoxyglucose, which affected the cation pumping by reducing the cellular ATP contents (unpublished data); and ouabain and furosemide, which abolished the cation pumping and the ouabain-insensitive $\text{Rb}^+:\text{K}^+$ exchange diffusion, respectively (TUPPER, 1975; MIYAMOTO *et al.*, 1978b). Since cell cations were not yet in complete equilibrium after 2-hr treatment with ouabain, the preserved contents of K^+ plus Rb^+ (40% in average) would still decrease with time. Passive fluxes of K^+ and probably Na^+ might be restricted by decreases in their concentration gradients across the cell membrane, which could be the reason of the preservation of the 40% level. Ineffectiveness of furosemide indicated that the $\text{Rb}^+:\text{K}^+$ exchange diffusion did not contribute to the cation contents in HeLa cells.

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