Stimulating Effect of an Artificial Electron Mediator, Phenazine Methosulfate, on Rb^+ Transport in HeLa Cells

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ABSTRACT. To obtain evidence of the action of the intracellular redox system as a regulating factor of membrane K^+ transport in HeLa cells, we used an artificial electron mediator, phenazine methosulfate (PMS). An addition of PMS to the incubation medium increased the intracellular accumulation of Rb^+ as an analog of K^+ . This increase, which was ouabain-sensitive, was shown to be due to the stimulation of active Rb^+ transport. The rate of ouabain-sensitive Rb^+ uptake was a linear function of the cellular bulk ATP level. When cells were treated with $20\ \mu\text{M}$ PMS, the linear curve showed a parallel shift upward. The range of the shift, the PMS-stimulated part of the Rb^+ uptake, was independent of the ATP level. The intracellular level of Rb^+ plus K^+ , which was equal to the K^+ level in cells incubated in normal medium, was elevated slightly, but Na^+ level remained unchanged. These results indicated that the stimulation of Rb^+ uptake had no effect on changes in the intracellular levels of monovalent cations. The maximal rate of ouabain-sensitive Rb^+ uptake, J_{max} , was increased, but the apparent K_{m} in relation to extracellular Rb^+ was unaffected. The specific binding of $[^3\text{H}]\text{ouabain}$ was not enhanced. Thus, the total number of Rb^+ binding sites and the affinity of Rb^+ to those sites would not be affected significantly. We concluded that the stimulating action of PMS is due either to the acceleration of Rb^+ pumping at each Na^+ , K^+ -pump or to activation of the resting pumps.

Phenazine methosulfate (PMS) transmits electrons by changing its molecular structure (26). Since Singer and Kearney introduced PMS as an intermediate electron carrier to link succinate dehydrogenase to oxygen (23), this phenazine dye has been used as a practical electron mediator to couple various dehydrogenases to the physiological or artificial electron donors or acceptors. PMS has been reported to affect the membrane transport of solutes and the metabolism of mammalian (2, 3, 8, 11, 25), and bacterial cells (1, 6, 7, 12, 21). With respect to monovalent cation transport, PMS-ascorbate stimulates the valinomycin-induced concentrative Rb^+ uptake into vesicles of the bacterial membrane (10, 13, 14). The active K^+ uptake in mammalian cells has mechanism which differs from the mechanisms of active K^+ or Rb^+ transport in bac-

Abbreviations: PMS, phenazine methosulfate; MEM, minimum essential medium; CCCP, carbonyl cyanide, m-chlorophenylhydrazine.

teria (9). But, the effects of PMS on cellular functions seems to be closely related to the chemical characteristics of PMS, regardless of the cell species.

We previously reported that when the K^+ in the medium was replaced totally with Rb^+ , Rb^+ substituted for K^+ in the membrane transport of HeLa cells (18). Using techniques described in that same report, we recently have found that PMS stimulates active Rb^+ uptake in the same cells. Hence, we have studied the mode of action of PMS, and here discuss the possible mechanisms by which active Rb^+ uptake is accelerated in the presence of PMS.

MATERIALS AND METHODS

Cell Cultures. HeLa cells (strain S3), obtained from Flow Lab., were cultured successively in glass culture flasks ($40 \times 55 \times 150$ mm, Ikemoto Sci. Co.) in modified minimum essential medium (MEM) supplemented with 10% calf serum (17). Cells in the confluent cultures were dispersed with 0.5% trypsin then inoculated at 5×10^4 cells/ml into Roux flasks containing 100 ml of the culture medium. These flasks were kept at 37°C in a CO_2 -incubator in a humid atmosphere of 5% CO_2 and 95% air. After three days, when cultures were in the exponential growth phase, the cells were resuspended by similar procedures then reinoculated at 1.5×10^5 cells/ml into plastic culture dishes (60×15 mm, Falcon) containing 6 ml of the culture medium. The cultures were kept in the CO_2 -incubator for another 24 h after which they were used for the experiments.

Rb^+ Uptake. Cells first were incubated for 2 h in modified MEM containing 20 mM HEPES (pH 7.2) at 37°C . Separate or simultaneous additions of 20 μM phenazine methosulfate (PMS), 10 μM ouabain and 2 μM carbonyl cyanide, m-chlorophenylhydrazone (CCCP) were made when necessary. For Rb^+ uptake, cells were kept for 15 min in medium that contained 5.4 mM $RbCl$ in place of 5.4 mM KCl (Fig. 1). This medium also contained PMS, ouabain or CCCP when required. Procedures for washing cultures, sampling cells, and measuring intracellular alkali metal cations were same as those reported previously (18).

Assays. Cellular protein was assayed by the method of Lowry *et al.* (15). ATP was determined by the luciferin-luciferase system by the modified procedures reported elsewhere (16). Samples used for the protein and ATP assays were obtained from the cultures used for the experiments on Rb^+ uptake. The amount of [^3H]ouabain bound to cells was measured with a liquid scintillation spectrometer (Mark II, Searle Analytic).

Chemicals and Others. Inorganic salts, glucose, glutamine, and miscellaneous materials were purchased from Wako Chem. Indust. Ltd.; $RbCl$ (Suprapur) from Merck; calf serum, LiCl , and quinacrine hydrochloride from Nakarai Chem. Ltd.; serum albumin (bovine, fraction V), phenazine methosulfate, carbonyl cyanide m-chlorophenylhydrazone, ouabain, ATP, triiodothyronine, firefly lantern extract, and HEPES from Sigma Chem. Co.; trypsin (1 : 250) from Difco Lab.; and [^3H]ouabain (19.5 Ci/mmol) was from New England Nuclear. To facilitate the preparation of modified MEM we used concentrated mixtures of amino acids ($50\times$) and vitamins ($100\times$) purchased from Flow Lab.

RESULTS

Time course curves of intracellular Rb^+ contents after replacement of the K^+ in the medium with Rb^+ , showed that PMS increased Rb^+ accumulation (Fig. 1). Rubidium ion accumulation decreased markedly in the presence of ouabain whether or not PMS was added. These contents were also equal to the Rb^+ contents of cells

Fig. 1. Effect of phenazine methosulfate (PMS) on Rb^+ accumulation in HeLa cells. A. Intracellular Rb^+ level as a function of time after replacement of extracellular K^+ with Rb^+ . Symbols: ●, control; ■, 20 μM PMS; ▲, incubation at 0°C; ○, 10 μM ouabain; and □, 20 μM PMS + 10 μM ouabain. B. The ouabain-sensitive Rb^+ accumulation in relation to time after replacement. Data replotted from the related data in A. Symbols: ●, control; and ■, 20 μM PMS. Each point is the mean of four samples; the vertical bar shows the S. D. of the mean.

incubated at 0°C. The ouabain-sensitive Rb^+ accumulation increased linearly for at least 15 min after cation replacement, and PMS stimulated the ouabain-sensitive fraction. We replotted the data in Fig. 1A according to Equation [2] of a previous paper (18) taking C_p , the pool size of K^+ exchangeable with Rb^+ , as 1.039 and 0.881 $\mu\text{mol/mg}$ of protein for cells incubated with and without PMS (Fig. 2). The rubidium free fraction of the K^+ pool, *i.e.* the part of the pool not yet exchanged with Rb^+ , decreased logarithmically with time after cation replacement. The size of this fraction did not change significantly in the presence of PMS. Also, the direct determination of intracellular K^+ indicated that the K^+ efflux was unaffected on incubation with the dye. Fig. 1 and 2 show that the increase in Rb^+ accumulation was caused by the stimulating action of PMS on ouabain-sensitive Rb^+ uptake, not by any effect on the K^+ efflux. These experiments were conducted after 1 h of incubation with PMS; however, a temporary increase in the K^+ or Rb^+ efflux occurred when the preliminary incubation was shorter (data not shown).

The ouabain-sensitive Rb^+ uptake was maximal when cells were treated with 20–25 μM PMS (Fig. 3). The protein contents of attached cells showed that PMS was not toxic to cell adhesion, at least when the concentration was less than 25 μM . The PMS concentration that gave maximal stimulation to the ouabain-sensitive Rb^+ uptake, without interference with cell attachment, was 20–25 μM . The total Rb^+ uptake in relation to the preliminary incubation time with PMS altered slightly within 2 h. There seemed to be an increasing tendency for uptake from 30 to 60 min (Fig. 4); the rubidium uptake in the presence of ouabain was increased, when the preliminary

Fig. 2. Effects of phenazine methosulfate (PMS) on the time courses of the Rb^+ -free fraction in the Rb^+ -exchangeable K^+ pool and on the K^+ efflux. Cells first were incubated with PMS and ouabain for 1 h. Rb^+ -free fraction: $\bullet$, control; and $\circ$, 20 μM PMS. As 0.881 and 1.039 $\mu\text{mol}/\text{mg}$ of protein were assigned for the Rb^+ -exchangeable K^+ pool, C_p , in the control and PMS-treated cells, the half saturation times, $t_{1/2s}$, were calculated as 39.7 and 38.1 min; and the initial rates of total Rb^+ uptake, J_{Rbs} , 15.4 and 18.9 nmol/mg of protein per min. K^+ efflux: $\blacktriangle$, control; and $\triangle$, 20 μM PMS. The initial size of the K^+ pool, C_0 , was 1.097 $\mu\text{mol}/\text{mg}$ of protein. See reference (18) in details.

incubation with PMS was short, although it returned to the control value after 60 min. The increase in ouabain-insensitive Rb^+ uptake, as well as the increase in the Rb^+ efflux, seemed to be due to a temporary change in membrane permeability after exposure to PMS.

A correlation between the ouabain-sensitive Rb^+ uptake and the extracellular Rb^+ concentration was shown as normal and Lineweaver-Burk plots (Fig. 5). The maximal rate of the ouabain-sensitive Rb^+ uptake, J_{max} , was accelerated; 24.7 vs. 17.1 nmol/mg of protein per min for PMS-treated and control cells. The apparent half saturation concentration of Rb^+ , a K_m of 0.95 mM, was not changed in the presence of PMS, nor was the specific binding of [^3H] ouabain increased by an addition of PMS (Table 1).

We previously found that treatment with CCCP greatly reduced the cellular ATP content and Rb^+ uptake when glucose was omitted, and that the saturating concentration of CCCP was 2 μM . The ATP content and Rb^+ uptake reduced by treatment with CCCP increased depending upon the concentration of added glucose in the presence or absence of PMS. Full restoration was observed when 0.5 mM glucose was added. With the ATP level on the abscissa and the Rb^+ uptake on the ordinate, we obtained a linear relationship (Fig. 6). There was a parallel upward shift of the curve on the addition of PMS. The range of the shift was almost constant for every

Fig. 3. Rubidium ion uptake and protein content of HeLa cells attached to the culture dishes, in relation to the concentration of phenazine methosulfate (PMS). Cells first were incubated for 2 h in modified MEM (17) with various concentrations of PMS. Rb^+ uptake took place for 15 min. Rb^+ uptake: ●, PMS; and ○, PMS+10 μM ouabain. Cellular protein content: ▲, PMS; and △, PMS+10 μM ouabain. Each point is the mean of four samples; the vertical bar shows the S.D. of the mean.

Fig. 4. Rubidium ion uptake into HeLa cells in relation to the preliminary incubation period with phenazine methosulfate (PMS). Rb^+ uptake took place for 15 min in medium that was the same as the preliminary incubation medium, but, it contained Rb^+ in place of K^+ . Symbols: ●, 20 μM PMS; ○, 20 μM PMS+10 μM ouabain; and △, 10 μM ouabain alone. Each point is the mean of four samples; the vertical bar shows the S.D. of the mean.

Fig. 5. Effect of phenazine methosulfate (PMS) on ouabain-sensitive Rb⁺ uptake in HeLa cells. A. Ouabain-sensitive Rb⁺ uptake in relation to the Rb⁺ concentration in the medium. B. Lineweaver-Burk plot of the data shown in A. Symbols: ●, control; and ○, 20 μM PMS.

ATP content examined. As Rb⁺ uptake was unaffected by glucose and PMS in the presence of ouabain, the upward shift of the curve was confirmed to be caused by the stimulating action of PMS on ouabain-sensitive Rb⁺ uptake. Simultaneous treatments of cells with PMS and ouabain reduced the ATP contents significantly, whereas treatment with PMS or ouabain alone did not change the level significantly in comparison to the control in the presence of CCCP.

The intracellular contents of Rb⁺ plus K⁺, which are identical with K⁺ contents of cells incubated in normal medium (18), increased slightly in the presence of PMS (Fig. 7); but, Na⁺ was not significantly changed by the same treatment. The bulk ATP content was decreased by an addition of PMS even in the presence of 5.6 mM glucose. This is in sharp contrast to the full recovery of the contents when CCCP was added simultaneously (Fig. 6). We also have found that CCCP restores the glycolytic activity of HeLa cells inhibited by an addition of PMS (unpublished data).

TABLE 1. EFFECT OF PHENAZINE METHOSULFATE (PMS) ON [³H]OUABAIN BINDING IN HELa CELLS

Addition	Ouabain bound	
	10 min	30 min
	(molecules × 10 ¹² /mg protein)	
None	2.71	5.59
10 μM Ouabain	2.11	2.67
20 μM PMS	2.77	4.95
20 μM PMS + 10 μM Ouabain	2.67	2.47

[³H]Ouabain was added as 0.15 μM with 0.85 μM non-radioactive ouabain. Cells first were incubated with 20 μM PMS and 10 μM ouabain, separately or together, for 2 h when necessary.

Fig. 6. Rubidium ion uptake as a function of the cellular ATP content. Cells first were incubated for 2 h with 20 μM phenazine methosulfate (PMS) with various concentrations of glucose and 2 μM CCCP. Symbols: ●, control; ○, 10 μM ouabain; ■, 20 μM PMS; and □, 20 μM PMS+10 μM ouabain. Each point is the mean of four samples; the vertical bar shows the S.D. of the mean.

Fig. 7. Effects of phenazine methosulfate (PMS) and ouabain on Rb^+ uptake and the intracellular contents of ATP, Na^+ , and $\text{Rb}^+ + \text{K}^+$ in HeLa cells. Cells first were incubated with PMS for 2 h in the presence or absence of ouabain. Rb^+ uptake took place in the medium as the preliminary incubation medium, but it contained Rb^+ in place of K^+ . A. Control; B, 10 μM ouabain; C, 20 μM PMS; and D, 20 μM PMS+10 μM ouabain. Each column is the mean of four samples; the vertical bar shows the S.D. of the mean.

DISCUSSION

Effect of PMS on the Concentration of Rb⁺ Accumulated. Rb⁺ uptake in the presence of ouabain, which was not affected by an addition of phenazine methosulfate (PMS), was almost equal to the Rb⁺ uptake at 0°C. The Na⁺, K⁺-pump is not believed to work at low temperatures; for incubations longer than 90 min under these conditions, the intracellular Rb⁺ content remained 0.12–0.15 μ moles/mg of protein, which is the Rb⁺ content produced by passive transport. In contrast, after a 2-hour incubation without ouabain, Rb⁺ accumulated as a substitute for K⁺ to about 5.5 times the value produced by passive transport. The Rb⁺ accumulation increased on treatment with PMS. These results clearly show that PMS stimulates the active and ouabain-sensitive influx of Rb⁺ as a substitute for K⁺.

Possible Action Mechanisms of PMS. The PMS-stimulated fraction remained constant during ouabain-sensitive Rb⁺ uptake as bulk ATP content was varied over a wide range. Also, the ATP content was reduced somewhat when PMS was added, even in the presence of glucose. PMS oxidizes NADH or NADPH directly (19, 20, 24); hence, the flux of reducing equivalents from glucose to the pentose phosphate pathway increases (5, 11, 25). This results in a reduction of glycolytic activity as observed in HeLa cells (unpublished data). The decrease in the NADH level also inhibits ATP synthesis through oxidative phosphorylation. These data and the interpretation of the lowered ATP content lead to the conclusion that the PMS-stimulation of active Rb⁺ uptake is caused by a mechanism other than the action of the dye on the ATP supply to the Na⁺, K⁺-pumps. The possibility that the decrease in the ATP content is the result of increased ATP consumption in the presence of PMS cannot be ruled out.

The intracellular content of Rb⁺ plus K⁺ increased slightly but the amount of Na⁺ did not change significantly during the 2-hour treatment with PMS, evidence that the effect of PMS was not due to activation of the pump by any changes in the intracellular contents of alkali metal cations. On the addition of PMS-ascorbate, bacterial vesicles which contain the respiratory chain have been reported to develop an electrochemical proton gradient across the membrane (21) and active transport of specific solutes is reported to be derived from respiratory energy via the pH gradient (22). We could obtain no evidence of a change in proton exodus across the membrane of HeLa cells in response to ouabain in the presence of PMS.

Ehrlich tumor cells treated with dinitrophenol and iodoacetate have been reported to recover their uptake of amino acid rapidly when PMS-ascorbate is added (8). Quinacrine suppressed this uptake almost completely, and restoration by an addition of PMS-ascorbate also was blocked by quinacrine (2). In contrast, we could not demonstrate that quinacrine (or triiodothyronine), the specific inhibitor of membrane NADH dehydrogenase (4), inhibits the ouabain-sensitive Rb⁺ uptake stimulated by PMS. This clearly indicates that different mechanisms are at work in the PMS-stimulation of the two transport systems.

We have shown that PMS increases the rate of maximal ouabain-sensitive Rb⁺ uptake, the $J_{\max}$, without changing the apparent K_m . The specific binding of [³H]-ouabain was not increased by the presence of the phenazine dye. Thus, PMS seems neither to change the affinity of Rb⁺ for its binding sites, nor to increase the total number of these sites. These results lead us to the conclusion that PMS stimulates the rate of Rb⁺ pumping at each Na⁺, K⁺-pump, or that PMS increases the number of working pumps by activating the resting ones. Whether PMS stimulates the pump directly or indirectly through a redox system is now being studied.

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