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Phenazine Methosulfate Stimulation of Ouabain-Sensitive Rb^+ Uptake by HeLa Cells: Effects of Respiratory Inhibitors, Anaerobiosis, and Ascorbate

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Phenazine methosulfate (PMS) stimulates ouabain-sensitive Rb^+ uptake by HeLa cells. This stimulation cannot be attributed to the effect of the dye on the intracellular Na^+ or ATP content. Respiratory inhibitors, such as 5 mM NaCN and 5 μM rotenone, and anaerobic conditions enhance the stimulation of Rb^+ uptake by PMS. Cellular respiration is stimulated, but lactate production is reduced in the presence of PMS, irrespective of the presence of respiratory inhibitors. Cellular NADH is oxidized markedly on addition of PMS plus inhibitors, but it is not affected by addition of the inhibitors only. In the presence of a high concentration of PMS, PMS-stimulated ouabain-sensitive Rb^+ uptake is inhibited by addition of ascorbate. From these results it is concluded that Na/K-pump activity is closely related to the cellular redox state.

Key words: rubidium transport, ouabain, phenazine methosulfate, anaerobiosis, respiratory inhibitors, ascorbate, HeLa cells

Phenazine methosulfate (PMS), which transmits electrons by interchange of molecular structure [1], has been used as an artificial electron mediator to couple various dehydrogenases to their proper electron acceptors [2-5]. Phenazine methosulfate is also known to stimulate Na^+-dependent active transport of amino acids in the presence of NADH or ascorbate in Ehrlich ascites tumor cells [6-9]. We have reported a stimulative effect of the phenazine dye on ouabain-sensitive Rb^+ uptake by HeLa cells [10]. Since the inward transport systems of the cell membranes cannot distinguish between K^+ and Rb^+ [11,12], PMS is considered to stimulate active K^+ transport into cells. We showed that, on addition of PMS, the maximum rate of ouabain-sensitive Rb^+ uptake is increased, whereas the apparent Km for Rb^+ and the number of ouabain-binding sites are not changed [10]. These results suggest that PMS accelerates the turnover of the cation pump and that the acceleration is due to an effect of PMS on some unknown mechanism(s) related to the cation pump activity.

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Recently, we obtained evidence indicating that (1) on incubation of the cells in medium containing PMS either with respiratory inhibitors, such as NaCN and rotenone, or under anaerobic conditions, ouabain-sensitive Rb^+ uptake stimulated by PMS is further enhanced; and (2) addition of ascorbate reduces the PMS-stimulated Rb^+ uptake. From these findings we conclude that PMS-mediated flux of reducing equivalents participates in the regulation of K^+ transport into HeLa cells.

MATERIALS AND METHODS

Cell Cultures

HeLa S3 cells purchased from Flow Laboratories (McLean, VA) were cultured serially in glass culture flasks in a modified Eagle's minimum essential medium (mMEM) supplemented with 10% (v/v) calf serum [13]. Growing cells were harvested with 0.5% trypsin, and the cell suspension was inoculated into plastic culture dishes (60-mm diameter, Wako Pure Chemical Industries, Ltd., Osaka, Japan) at a density of 1.5×10^5 cells/ml. The cultures were incubated for 24 hr in a CO_2 incubator in a humid atmosphere of 5% CO_2 in air at 37°C.

Rb^+ Uptake

The cells were preincubated for 2 hr in mMEM containing 5.6 mM glucose and 25 mM Hepes buffer (pH 7.2) with or without 10 μM ouabain and various concentrations of PMS at 37°C. For assay of Rb^+ uptake, the cells were incubated for 15 min in the same medium but with 5.4 mM RbCl in place of 5.4 mM KCl. The rate of Rb^+ uptake was expressed per mg protein per min, because ouabain-sensitive Rb^+ accumulation, ie, Rb^+ accumulation in the presence of ouabain subtracted from that in the absence of ouabain, was found to be proportional to time, at least for 15 min, regardless of the presence of PMS [10]. The procedures used for washing and sampling the cells and for assaying alkali cations were as described elsewhere [11]. Respiratory inhibitors were added to both preincubation and incubation media when indicated. When 5 mM NaCN was used, the medium was adjusted to pH 7.2 with 1 N HCl. When 10 μM rotenone was added, a final concentration of 2.2% ethanol was also present and ethanol was added to the control medium. For Rb^+ uptake under anaerobic conditions, the cells were placed in a glass dessicator in which 95% of the air was replaced by N_2 gas (99.9%) at 37°C, and the incubation medium used was previously equilibrated with this gas mixture. The control experiment was carried out in the same way but with air instead of N_2 gas.

Other Assays

Cellular protein was assayed by the method of Lowry et al [14] with bovine serum albumin as a standard. ATP was determined with the luciferin-luciferase system by the modified method reported previously [15]. NAD and NADH were determined by the method of Lowry and Passonneau [16] involving glutamate measurement. For assay of dinucleotides, cells collected from 10 culture dishes were washed and solubilized in 3 ml of a cold solution of 0.0 4 N NaOH and 0.5 mM cysteine. The solution was then treated by a modification of the procedure of Schwartz et al [17]. Lactate was assayed with a Lactate UV Test Kit (Boehringer, Mannheim, GmbH), and the rate of oxygen consumption (Q_{O_2}) was measured with an oxygen electrode. Oxygen consumption and lactate production were assayed using cells scraped off the dish with a rubber policeman and suspended in mMEM containing Hepes buffer.

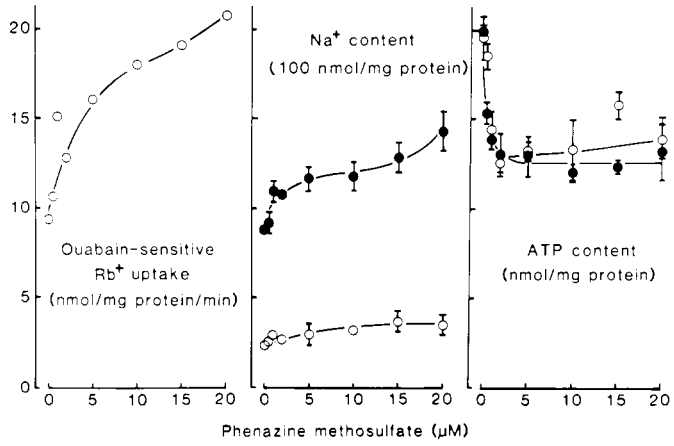

Fig. 1. Effects of various concentrations of phenazine methosulfate (PMS) on ouabain-sensitive Rb^+ uptake and the intracellular contents of Na^+ and ATP in HeLa cells. (○), PMS; (●), PMS plus 10 μM ouabain. Points and bars are means ± SD for four samples.

Reagents

Inorganic salts and NaCN were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), RbCl (superpure) was from Merck (Darmstadt, GmbH), calf serum, LiCl, and rotenone (extra pure) were from Nakarai Chemicals Ltd. (Kyoto, Japan), serum albumin (bovine, fraction V), phenazine methosulfate, ouabain, firefly lantern extract, ATP, NAD, NADH, sodium ascorbate, and Hepes were from Sigma Chemical Co. (St. Louis, MO); trypsin (1:250) was from Difco Laboratories (Detroit, MI), and lactate dehydrogenase (glycerol solution) and glutamate dehydrogenase (glycerol solution) were from Boehringer (Mannheim, GmbH). Concentrated solutions of amino acids (×50) and vitamins (×100) from Flow Laboratories were used to prepare the mMEM.

RESULTS

Intracellular Na^+ and ATP

The effects of various concentrations of PMS on intracellular Na^+ and ATP content and ouabain-sensitive Rb^+ uptake were investigated (Fig. 1). Ouabain-sensitive Rb^+ uptake increases with an increase in the PMS concentration. The increase is especially great in the concentration range below 5 μM PMS. On addition of PMS, the intracellular Na^+ content changes only slightly in the absence of ouabain, whereas the ATP content decreases sharply in the concentration range below 5 μM PMS. This drop in the ATP content is in contrast to the increase in active Rb^+ uptake. Thus, it is unlikely that the stimulatory influence of PMS on ouabain-sensitive Rb^+ uptake is due to any effect of the dye on the intracellular Na^+ or ATP content.

Effects of Respiratory Inhibitors and Anaerobiosis

Ouabain-sensitive Rb^+ uptake is stimulated by treatment with 20 μM PMS. The addition of respiratory inhibitors, such as 5 mM NaCN or 10 μM rotenone, which strongly inhibit respiration and stimulate glycolytic activity (Table I), does not signif-

TABLE I. Effects of Phenazine Methosulfate (PMS) and Respiratory Inhibitors on the Rates of Oxygen Consumption (Q_{O_2}) and Lactate Production (Q_L) by HeLa Cells*

Addition	Rate (nmol/mg protein per min)	
	Q_{O_2}	Q_L
None	4.61	6.43
PMS, 20 μM	30.90	1.15
NaCN, 5 mM	0.97	8.90
PMS, 20 μM + NaCN, 5 mM	30.50	1.74
Rotenone, 10 μM	1.66	9.10
PMS, 20 μM + Rotenone, 10 μM	30.37	0.55

*The cells were suspended in the modified Eagle's minimum essential medium containing 25 mM Hepes (pH 7.2) and 5.6 mM glucose for assays.

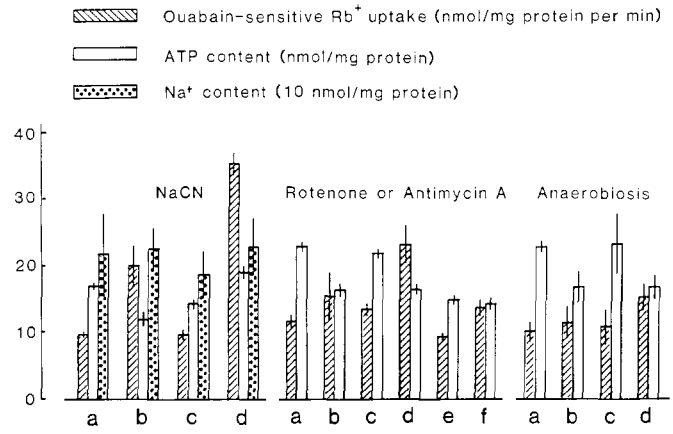

Fig. 2. Effects of 20 μM phenazine methosulfate (PMS) on ouabain-sensitive Rb^+ uptake and the intracellular contents of ATP and/or Na^+ in HeLa cells in the presence of respiratory inhibitors or under partial anaerobic conditions. With NaCN: a) control; b) 20 μM PMS; c) 5 mM NaCN; d) 20 μM PMS plus 5 mM NaCN. With rotenone or antimycin A: a) control; b) 20 μM PMS; c) 10 μM rotenone; d) 20 μM PMS plus 10 μM rotenone; e) 20 μM antimycin A; f) 20 μM PMS plus 20 μM antimycin A. Under anaerobic conditions: a) air; b) 20 μM PMS in air; c) 99.9% N_2 in place of 95% of the air; d) 20 μM PMS in the same atmosphere as for c. Columns and bars are means ± SD for four samples.

icantly affect Rb^+ uptake (Fig. 2). The intracellular ATP content is also unaffected by this treatment. Similar findings are obtained when the cells are placed in a partially anaerobic atmosphere of 95% N_2 and 5% air. However, respiratory inhibitors or anaerobic conditions enhance the stimulation of ouabain-sensitive Rb^+ uptake by PMS. The intracellular ATP content is also somewhat decreased by addition of PMS in the presence of the respiratory inhibitors, but the Na^+ content is not significantly affected in the presence of PMS and 5 mM NaCN. NaCN or rotenone alone does not influence active Rb^+ uptake, but antimycin A inhibits it in parallel with the decrease in the ATP content. The inhibitory effect of antimycin A is observed even in the presence of PMS.

TABLE II. Effects of Phenazine Methosulfate (PMS) and Respiratory Inhibitors on the Contents of Nicotinamide-Adenine Dinucleotide in HeLa Cells

Addition[a]	Nucleotide (nmol/mg prot) NADH	NAD	NADH/NAD
None	1.55	6.39	0.24
PMS, 20 μM	0.02	7.52	0.00
NaCN, 5 mM	2.16	7.30	0.30
PMS, 20 μM + NaCN, 5 mM	0.02	7.59	0.00
None	1.22	5.79	0.21
PMS, 20 μM	0.06	6.50	0.01
Rotenone, 10 μM	1.14	6.06	0.19
PMS, 20 μM + Rotenone, 10 μM	0.13	6.31	0.02

[a]Experiments with NaCN and rotenone were carried out independently.

The cellular NAD and NADH contents are not affected appreciably by treatment with NaCN or rotenone (Table II). Treatment with 20 μM PMS decreases the NADH content without significantly affecting the total content of dinucleotide, ie, NAD plus NADH. Hence, PMS greatly decreases the NADH/NAD ratio. The decrease in the NADH content is accompanied by a marked increase in the Q_{O_2} (Table I). In contrast, PMS treatment suppresses the glycolytic activity, even in the presence of respiratory inhibitors that stimulate the activity, when the inhibitors are added alone.

Effect of Ascorbate

Ouabain-sensitive Rb^+ uptake stimulated in the presence of 50 μM PMS is reduced by ascorbate in a concentration-dependent manner. This reduction reaches a steady level with more than about 5 mM ascorbate (Fig. 3). The addition of 5 mM ascorbate has no significant effect on Rb^+ uptake; but in the presence of low concentrations of PMS, it stimulates ouabain-sensitive Rb^+ uptake. This stimulation is maximal in the presence of 10 μM PMS, decreases with an increase in the PMS concentration beyond 10 μM, and drops to a subnormal level with 100 μM PMS.

Effects of Other Inhibitors

Sodium vanadate (1 μM-1 mM) added extracellularly does not inhibit ouabain-sensitive Rb^+ uptake even in medium containing a low Na^+ concentration (25 mM) and after a 2-hr preincubation period (data not shown). Inhibitors of NADH dehydrogenase in the plasma membrane, such as quinacrine (2 mM) and triiodothyronine (0.1 mM), also have no effect on Rb^+ uptake by HeLa cells (data not shown).

DISCUSSION

Intracellular Na^+ strongly stimulates active Rb^+ uptake by HeLa cells [18]. This Rb^+ uptake is proportional to the intracellular ATP content [19]. Therefore, in this work on the mechanism of action of PMS, we first examined the effects of PMS on the intracellular Na^+ and ATP contents. However, our results show that stimulation of active Rb^+ uptake by PMS is not obviously related to any effects on the intracellular Na^+ and ATP contents.

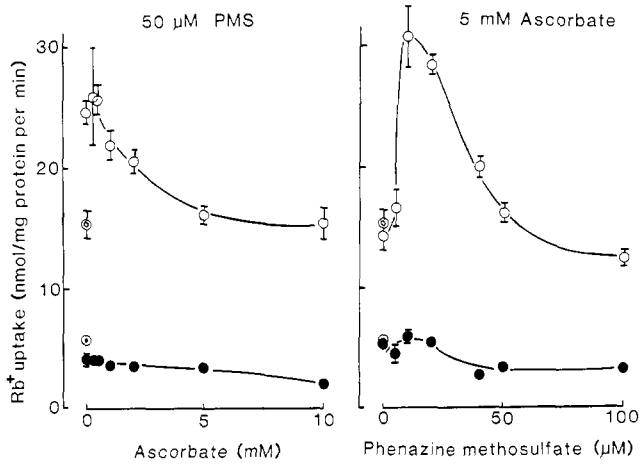

Fig. 3. Effects of ascorbate and phenazine methosulfate (PMS) on Rb^+ uptake by HeLa cells. Rb^+ uptake at various ascorbate concentrations: (◎), control; (◉), 10 μM ouabain; (○), 50 μM PMS; (●), 50 μM PMS plus 10 μM ouabain. Rb^+ uptake at various PMS concentrations: (◎), control; (◉), 10 μM ouabain; (○), 5 mM ascorbate; (●), 5 mM ascorbate plus 10 μM ouabain. Points and bars are means ± SD for four samples.

Phenazine dyes have been used to link NADH, a major hydrogen donor in mammalian cells, to associated dehydrogenases [3,4,20]. Therefore, we next examined the effect of PMS on the cellular NADH content. Results showed that PMS decreases both the NADH content and the NADH/NAD ratio without changing the total dinucleotide content significantly. In contrast to its effect on cellular NADH, it greatly increases cellular respiration, consistent with reports of PMS-mediated linkage between NADH and oxygen [21–23]. The increase in cell respiration and decrease in NADH content suggest that the flux of reducing equivalents increases somewhat in the presence of PMS. These reducing equivalents probably originate from NADH and other physiological reductants and are probably donated to terminal electron acceptors, including oxygen molecules, via PMS.

In a partially anaerobic atmosphere, the PMS-mediated flux of reducing equivalents to oxygen should be limited, and the flux should become directed to other terminal acceptors, resulting in an increase in flux with these acceptors. This assumption is supported by the finding that the PMS-mediated reduction of an artificial electron acceptor, tetrazolium, is greatly reduced by atmospheric oxygen [24]. Similarly, in the presence of sufficient concentrations of respiratory inhibitors, the blocked pathway of reducing equivalents is probably bypassed via PMS. Ouabain-sensitive Rb^+ uptake occurs under the same conditions as enhancements of PMS-mediated flux of reducing equivalents. How then are the two related? One way to solve this problem is to identify the key electron acceptor(s) whose molecular state is related to the regulation of Na/K-pump activity. A possible candidate is vanadate, which has been reported to inhibit ouabain-sensitive Rb^+ uptake by human red cells [25]. This reagent has been shown to become ineffective when reduced to vanadyl ion and bound to protein [26]. Kaniike [27] reported that the Ki for vanadate to the activity of Na^+,

K^+-ATPase from guinea pig kidney is increased 10 to 100 times by addition of PMS with NADH or ascorbate. The concentration of PMS used in the present study was effective in these previous studies. However, it seems premature to explain our results in terms of reduction of vanadate, because we have not obtained evidence for vanadate-induced inhibition of active Rb^+ uptake in HeLa cells.

Figure 3 shows that the PMS-stimulated ouabain-sensitive Rb^+ uptake is inhibited in the presence of high concentrations of ascorbate with PMS. This implies that, when flux of reducing equivalents via PMS is markedly increased, active Rb^+ uptake is inhibited rather than accelerated. Addition of ascorbate with PMS has been reported to inhibit K^+-dependent phosphatase activity in a membrane preparation of Na^+, K^+-ATPase [28]. This inhibition was reversed by chelators of bivalent cations and was related to the reduction of a ferric compound to a toxic ferrous compound. Our results on active Rb^+ uptake are consistent with the reported results for the concentration ranges and mode of actions of PMS and ascorbate used. However, it is difficult to determine whether the inhibition of PMS-stimulated active Rb^+ uptake is related to the reduction of heavy metals, since the cells become detached from the culture dishes in the presence of chelators such as EDTA and EGTA, making it impossible to perform these experiments with living attached cells.

Our results do not provide decisive information of the mechanism of stimulation of active Rb^+ uptake by PMS, but they suggest clearly that certain intracellular redox systems participate in regulation of Na/K-pump activity.

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