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EVIDENCE FOR STIMULATION OF THE K–Cl COTRANSPORT SYSTEM BY PHENAZINE METHOSULFATE

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Abstract—The effects of phenazine methosulfate (PMS), a known generator of oxygen free radicals, on ion transport in human erythrocytes were studied. The treatment of the red blood cells with 0.1–0.8 mM PMS caused the concentration-dependent increase of K loss from the cells in K-free sodium chloride medium. The PMS-dependent K efflux from the cells in oxygen-free medium, being in equilibrium with argon, did not differ from the control. After substitution of Cl^- ions in the medium by NO_3^-, PMS caused only a small activation of K loss. PMS at a concentration of 0.8 mM was found to increase passive K transport in erythrocytes (ouabain–furosemide-resistant ^{86}Rb influx) and at the same time to decrease active transport of K (ouabain-sensitive influx of ^{86}Rb). Furosemide-sensitive cotransport of K was not affected by 0.8 mM PMS. The influx of ^{22}Na in the red blood cells was also independent of the presence of 0.2–1.0 mM PMS in the bath medium. The results obtained suggest that PMS stimulated the reversible K–Cl cotransport in human erythrocyte membranes. This effect of PMS resembles the action of the well-known SH-alkylating agent, *N*-ethylmaleimide.

In the last few years the effect of phenazine methosulfate (PMS)*, on the ion transport across the human red blood cell membrane has been studied [1–3]. According to these authors, PMS induces the generation of the free oxygen radicals, followed by peroxidation of the membrane lipids. The addition of PMS to erythrocyte suspensions increases the rate of K efflux from the cells, especially in the presence of superoxide dismutase inhibitors.

The aim of the present work is to study the effect of PMS on K transport by human erythrocytes, depending on the PMS concentration, anion composition of the incubation medium and oxygen dissolved in the cell suspension. The attention was focused on the efflux and influx of K through the erythrocyte membrane.

MATERIALS AND METHODS

Measurements of the K *loss from the red blood cells.* The heparinized donor's blood was used 2 hr after it was obtained from the humans. The blood was centrifuged at 2700 *g* for 5 min at 4° and the buffy coat was aspirated. The cells were resuspended and washed three times in the chilled flux medium containing (mM): 145 NaCl, 10 Tris–HCl (pH 7.4, 37°) or 145 $NaNO_3$, 10 Tris–HNO_3 (pH 7.4, 37°).

To study the efflux of K, the same solution with 10 mM glucose was used as a flux medium. The cells (haematocrit 40–50%), suspended in the flux medium, were stored at 4° for no longer than 30 min and then added to the flux media (final haematocrit 4–8%) and put into the shaking water bath at 37°. After incubation, cell suspensions were centrifuged (2700 *g*, 3 min at 4°) and the supernatants were removed to measure K. The concentration of K was assayed using a Flapho-40 model Flame Photometer (Jena). Standard solutions were prepared using flux medium supplemented with various amounts of K.

Measurements of K *and* Na *influx.* To measure ion fluxes, washed erythrocytes were added to six tubes containing flux medium (mM): 140 NaCl, 5 KCl, 10 Tris–HCl (pH 7.4, 37°), 10 glucose; 0.1 ouabain + 0.5 furosemide (two tubes), 0.1 ouabain (two tubes). For the inhibitors to bind to the erythrocyte membrane the cell suspensions were preincubated at 37° for 30 min. Then PMS at a final concentration of 0.8 mM was added to the tubes containing ouabain, ouabain plus furosemide and the control, followed by ^{86}Rb (10 μCi/mL medium). After 60 min of incubation the suspensions were centrifuged (2700 *g*, 3 min at 4°) and the medium radioactivity was determined. Cells were washed twice with an ice-cold 145 mM NaCl solution, buffered at pH 7.4 with Tris–HCl. The washed erythrocytes were diluted with distilled water and the radioactivity of the lysate was measured using a gamma-counter. The magnitude of K influx was calculated using the value of cell uptake of ^{86}Rb and the medium concentration of K.

Erythrocyte deoxygenation. The experiments were carried out to study PMS effect on K loss from deoxygenated red cells incubated in K-free medium. To ensure a well-defined starting point for the flux measurements, the identical pairs of cell suspensions were preincubated for 60 min at 37° in equilibrium with air and in argon atmosphere provided by bubbling argon through the suspensions. Then, after the samples of the suspensions were taken to determine K loss, PMS was added to half of the probes to a final concentration of 0.8 mM. Further incubation lasted 60 min with deoxygenation being continued.

* Abbreviations: PMS, phenazine methosulfate; NEM, *N*-ethylmaleimide.

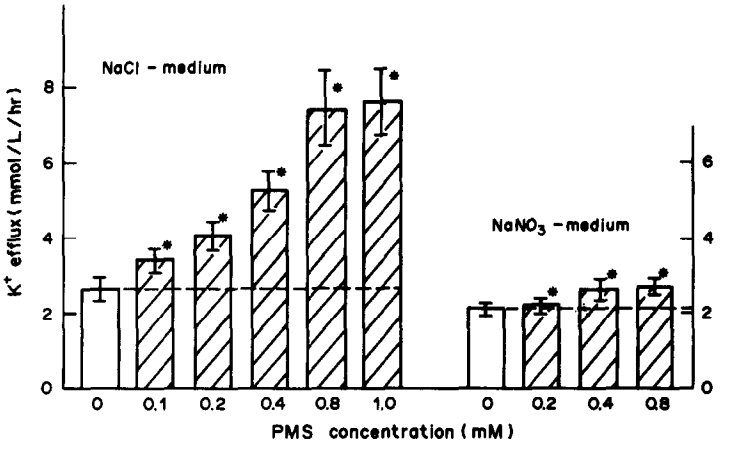

Fig. 1. Concentration-dependent effect of PMS on K^+ loss from human erythrocytes. Washed red blood cells were incubated in K-free sodium chloride or sodium nitrate media at 37° for 60 min in the presence of various concentrations of PMS. K^+ loss was calculated from K^+ accumulation in the bath media. Results are means ± SEM for seven experiments on the cells from different donors. * Significantly different from the control value (paired *t*-test); $P < 0.001$.

Reagents. PMS, ouabain and furosemide were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). PMS and ouabain were dissolved in the flux medium, furosemide (100 mM) was dissolved in dimethyl sulfoxide. The other chemicals were of analytical grade.

The values are presented as means ± SE.

RESULTS

The effect of PMS on the loss of K *in human red blood cells*

The incubation of erythrocytes in K-free solution resulted in K loss which under control conditions was 2.66 ± 0.61 mmol/L cells · hr. The leak of K was stimulated by the presence of PMS in concentrations above 0.1 M (Fig. 1). At 0.1 mM PMS there was a small but statistically significant increase of K loss which was revealed using the Student's paired test (PMS-induced component was 0.75 ± 0.12 mmol/L cells·hr). At PMS concentrations of 0.8–1.0 mmol/L the K efflux reached the maximal value (4.83 ± 0.64 mmol/L·hr). Figure 2 shows the time course of K loss at different PMS concentrations in the incubation medium. It can be seen that in the presence of PMS increased K loss retained during 3 hr of incubation compared to control.

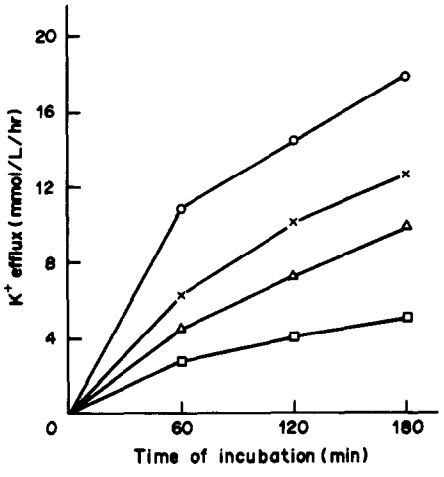

Fig. 2. Time dependence of K^+ loss from human erythrocytes in the presence of PMS. Red blood cells were incubated in K-free sodium chloride media at 37° for 180 min. The samples of cell suspension were taken at 60, 120 and 180 min and K loss was determined. Each point represents the mean of duplicate assays of the cells from two donors. Key: (□) control; PMS concentrations (mM): (△) 0.2, (×) 0.4, (○) 0.8.

The effect of substitution of Cl^- *ions in cells and medium by* NO_3^- *on the PMS-induced* K *loss*

Incubation of cells in the K-free medium containing nitrates showed a small increase in K loss in the presence of 0.2 mM PMS (paired difference of 0.15 ± 0.03 mmol/L·hr). As seen from Fig. 1, PMS enhanced K loss (0.58 ± 0.04 mmol/L·hr) to only 12% of PMS-induced K efflux in the chloride medium. Therefore, almost the whole K loss from human erythrocytes stimulated by PMS was Cl-dependent.

The effect of PMS on the influx of K *and* Na

This set of experiments was performed to study the passive influxes of these monovalent cations as well as ouabain- and furosemide-sensitive K influx.

The uptake value of ^{86}Rb was used to calculate the K influx. The results are presented in Table 1. In the presence of ouabain or ouabain plus furosemide, PMS led to a marked increase in ^{86}Rb flux while in the absence of inhibitors, PMS gave a

Table 1. Effects of 0.8 mM PMS on K influx in human erythrocytes

	K^+ influx (mmol/L cells/hr)	
	Control	PMS
Net flux	2.08 ± 0.16	1.92 ± 0.17
Ouabain-resistant	0.77 ± 0.06	1.47 ± 0.10*
Furosemide-ouabain resistant	0.36 ± 0.031	1.02 ± 0.13*
Ouabain-sensitive	1.31 ± 0.24	0.45 ± 0.11*
Furosemide-sensitive	0.41 ± 0.070	0.45 ± 0.12

Red blood cells were preincubated at 37° for 30 min in the media containing 140 mM NaCl, 4 mM KCl with and without 0.1 mM ouabain or 0.1 mM ouabain + 0.5 mM furosemide. Then ^{86}Rb and PMS to a final concentration of 0.8 mM were added to the suspensions and the cells were incubated for 60 min. K influx was determined using ^{86}Rb accumulation in the erythrocytes.

Results are the means ± SEM (N = 5–6).

* Significantly different from the control values ($P < 0.001$).

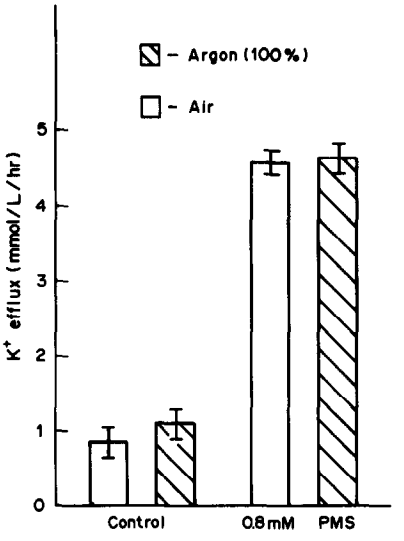

Fig. 3. Effect of PMS on K^+ loss from cells incubated in an oxygen-free medium. The cell suspension was divided into two parts, one of which was preincubated for 60 min at 37° with argon bubbling to remove dissolved oxygen. The other part was preincubated in the same conditions in contact with air. Aliquots of the suspensions were then taken to measure K loss and the incubations were made in the presence of 0.8 mM PMS or without it in the control (four variants). K efflux was calculated from K accumulation in the incubation media. Results are means ± SEM for four experiments on the cells from different donors.

small reduction in ^{86}Rb uptake by red blood cells. In addition, PMS also caused an inhibition of the active transport of K but had no effect on the furosemide-sensitive cotransport. Thus, PMS acted on the erythrocyte membrane in two different ways: it stimulated the passive (ouabain–furosemide-resistant) K influx and inhibited K influx through the Na–K pump.

To investigate cation selectivity of PMS effect on the erythrocyte membrane, Na transport was studied. Net influx of ^{22}Na was determined using the same procedure as described above for the experiments with ^{86}Rb. The red cells were incubated for 60 min with ^{22}Na at 37° without or with 0.2, 0.4, 0.8 and 1.0 mM PMS. There was no significant difference in the cell uptake of ^{22}Na in the presence of PMS as compared with control. The average value of ^{22}Na influx for five experiments was 2.02 ± 0.48 mmol/L·hr in control and 2.32 ± 0.41 mmol/L·hr in the presence of 1.0 mM PMS.

The effect of PMS in the oxygen-free medium

In previous papers, the mechanism of PMS action on the erythrocyte membrane was proposed to be related to the formation of free oxygen radicals inside the cells [1–3]. Therefore it seems worth studying the effects of PMS on K efflux in an oxygen-free medium. Figure 3 shows that treatment of the cell suspension with argon did not affect K loss by erythrocytes. The PMS-induced K efflux from the cells incubated in oxygen-free medium (3.5 ± 0.66 mmol/L·hr) did not differ significantly from that of the control (3.9 ± 0.64 mmol/L·hr).

DISCUSSION

One of the unexpected results of this study is that the addition of PMS to human red blood cells induced an increase of K–Cl cotransport. Coupled K–Cl cotransport has been studied in various tissues [4]. In human erythrocytes K–Cl cotransport is stimulated by cell swelling [5–7], by high hydrostatic pressure [8] and by *N*-ethylmaleimide (NEM) treatment [9–12]. In normal human red cells, the activity of the K–Cl system is minimal if any but it is enhanced in reticulocytes [12–14] and sickle cells [14]. Recently, a specific inhibitor of this transport system was found [11].

Unfortunately, relatively little information is available on the effect of PMS upon erythrocyte membrane. In the previous studies by Maridonneau *et al.* [1–3] the action of PMS on erythrocyte membrane was ascribed to the formation of free oxygen radicals and membrane lipid peroxidation. It was shown [15, 16] that human erythrocyte proteins exhibit high sensitivity to various oxidation systems while lipids, on the contrary, remain relatively stable. Also, oxidation of hemoglobin to methemoglobin in the presence of PMS was observed [15, 16].

Taking into account the activation of specific K–Cl cotransport by PMS it could be suggested that PMS effect is associated with modifications in membrane proteins. This suggestion is confirmed by the similarity of PMS effects and such a well-known alkylating agent as NEM. The effects of NEM on erythrocyte membrane have been extensively studied, although the mechanisms of K–Cl cotransport activation remain uncertain. The capability of PMS to cause thiol group oxidation of erythrocyte membrane proteins has been reported [17, 18].

Hebbel *et al.* [18] have demonstrated that human

red cells treated with 0.5 mM PMS become similar to sickle cells in such properties as dehydration, increase in K efflux and in adherence to endothelium. Moreover, sickle cells also have a decreased temperature of vesiculation and a similar change occurs in normal cells treated with PMS or NEM [17]. It has been suggested that K–Cl cotransport increase and vesiculation temperature decrease in sickle cells might be due to the oxidation of thiol groups of cytoskeleton proteins. The process of vesiculation was shown to follow cross bonding of erythrocyte membrane which is based on cross-linking of spectrine molecules [19]. Such a modification of spectrin is reported to be caused by NEM and some other thiol-oxidized agents.

One cannot neglect the possibility of participation of methemoglobin in K–Cl cotransport activation due to PMS treatment. In this context, it is of interest to note that the effect of another oxidative agent, phenylhydrazine, on human erythrocyte membrane results in the formation of cross-linked spectrin through disulfide exchange with denaturated methemoglobin [16].

Our data indicate that PMS activates the K–Cl cotransport even in deoxygenated red cells (Fig. 3). The effect of NEM on K–Cl cotransport is also retained in deoxygenated human erythrocytes [14].

All these data lead us to the conclusion that PMS, like NEM, may promote modification of cytoskeleton protein spectrin accompanied by activation of K–Cl cotransport. In the present investigation, PMS at a dose of 0.8 mM partially inhibits active transport of K in red blood cells. However, no evidence has been found for the effect of PMS on the influx of Na and Na–K–Cl cotransport. These data contradict the earlier findings of Maridonneau *et al.* [1] who demonstrated the inhibition of Na–K–Cl cotransport by PMS. This difference could be due to the various approaches used to evaluate the cotransport system. In our experiments, furosemide-sensitive ^{86}Rb influx was measured whereas Maridonneau *et al.* [1] determined bumetanide-sensitive K efflux. The activity of Na–K–Cl cotransport has been shown to be strongly dependent on the ion composition of red cells and the medium [20–23]. Recently, Kracke *et al.* [21] revealed the asymmetry of this transport system and found that only influxes of K and Na (but not effluxes) had true furosemide-sensitive cotransport characteristics. Some controversial results for the activity of Na–K–Cl cotransport, obtained in human erythrocytes, may be attributed to alternative modes of operation of the cotransporter [23].

It should be pointed out that as the PMS concentration increases from 0.1 to 0.8 mM, K loss from human erythrocytes increases almost linearly (Fig. 1). Maximal values of K efflux induced by PMS in the chloride medium (4–5 mmol/L·hr) are remarkably similar to those observed in swelled erythrocytes (4.7 mmol/L·hr) and inhibited by the specific inhibitor of K–Cl cotransport [11]. In the present investigation, PMS at a dose of 0.8 mM partially inhibited the active transport of K in the red blood cells alongside the stimulation of the passive transport of K.

REFERENCES

1. Maridonneau I, Braquet P and Garay RP, Na and K transport damage induced by oxygen-free radicals in human red cells membranes. *J Biol Chem* **258**: 3107–3113, 1983.
2. Maridonneau I, Garay RP and Braquet P, The effect of lipid peroxidation on transport functions in human erythrocytes. *Biomed Biochim Acta* **42**: S58–S62, 1983.
3. Maridonneau-Parini I, Braquet P and Garay R P, Heterogenous effect of flavonoids on K loss and lipid peroxidation induced by oxygen-free radicals in human red cells. *Pharmacol Res Commun* **18**: 61–72, 1986.
4. Dunham PB and Benjamin MA, Cl-dependent cation transport in mammalian erythrocytes. *Fed Proc* **43**: 2476–2478, 1984.
5. Kaji DM, Kinetics of volume-sensitive K transport in human erythrocytes. *Am J Physiol* **256**: C1214–C1223, 1989.
6. O'Neil WC, Volume-sensitive Cl-dependent K transport in human erythrocytes. *Am J Physiol* **253**: C883–C888, 1987.
7. Stewart GW, Co-ordinated variations in chloride-dependent potassium transport and cell water in normal human erythrocytes. *J Physiol (Lond)* **401**: 1–16, 1988.
8. Hall AC and Ellory JC, Effects of high hydrostatic pressure on "passive" monovalent cation transport in human red cells. *J Membrane Biol* **94**: 1–17, 1986.
9. Kaji D and Kahn T, Kinetics of Cl-dependent K influx in human erythrocytes with and without external Na: effect of NEM. *Am J Physiol* **249**: C490–C496, 1985.
10. Lauf PK, Adragna NC and Garay RP, Activation by *N*-ethylmaleimide of a latent K–Cl flux in human red blood cells. *Am J Physiol* **246**: C385–C390, 1984.
11. Garay RP, Nazaret C, Hannaert PA and Cragoe EJ, Demonstration of a (K–Cl)-cotransport system in human red cells by its sensitivity to [(dihydro-indenyl)oxy]alcanoic acids: regulation of cell swelling and distinction from the bumetanide-sensitive Na–K–Cl-cotransport system. *Mol Pharmacol* **33**: 696–701, 1988.
12. Berkowitz LR, Walstad D and Orringer EP, Effect of *N*-ethylmaleimide on K transport in density-separated human red blood cells. *Am J Physiol* **253**: C7–C12, 1987.
13. Canessa M, Fabry ME, Blumenfeld N and Nagel RL, Volume-stimulated, Cl-dependent K^+ efflux is highly expressed in young human red cells containing normal hemoglobin or HbS. *J Membrane Biol* **97**: 97–105, 1987.
14. Canessa M, Fabry ME and Nagel RL, Deoxygenation inhibits the volume stimulated, Cl-dependent K^+ efflux in SS and young AA cells: a cytosolic Mg^{2+} modulation. *Blood* **70**: 1861–1866, 1987.
15. Davies KJA and Goldberg AL, Oxygen radicals stimulate intracellular proteolysis and lipid peroxidation by independent mechanisms. *J Biol Chem* **262**: 8220–8226, 1987.
16. Vilsen B and Nielsen H, Reaction of phenylhydrazine with erythrocytes. Cross-linking of spectrin by disulfide exchange with oxidized hemoglobin. *Biochem Pharmacol* **33**: 2739–2748, 1984.
17. Rank BH, Moyer NL and Hebbel RP, Vesiculation of sickle erythrocytes during thermal stress. *Blood* **72**: 1060–1063, 1988.
18. Hebbel RP, Ney PA and Foker W, Autoxidation, dehydration, and adhesivity may be related abnormalities of sickle erythrocytes. *Am J Physiol* **256**: C579–C583, 1989.
19. Fischer TM, Role of spectrin in cross bonding of the cell membrane. *Blood Cells* **13**: 696–701, 1988.
20. Shennan DB and Chipperfield AR, The dependence

on chloride ions of the loop diuretic sensitive component of passive sodium efflux from human red cells. *Pflügers Arch* **406**: 333–339, 1986.

21. Kracke GR, Anatra MA and Dynham PB, Asymmetry of Na–K–Cl cotransport in human erythrocytes. *Am J Physiol* **254**: C243–C250, 1988.
22. Dagher G, Brugnara C and Canessa M, Effect of metabolic depletion on the furosemide-sensitive Na and K fluxes in human red cells. *J Membrane Biol* **86**: 145–155, 1985.
23. Canessa M, Brugnara C, Cusi D and Tosteson DC, Modes of operation and variable stoichiometry of the furosemide-sensitive Na and K fluxes in human red cells. *J Gen Physiol* **87**: 113–142, 1986.