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Research Article

POTASSIUM TRANSPORT IN THE ACETYLCHOLINESTERASE-DEFICIENT ERYTHROCYTES OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA (PNH) *

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The mechanism by which certain mammalian red cells accumulate potassium has been the subject of extensive investigation. Maintenance of a high intra-erythrocytic potassium concentration against a relatively low plasma concentration has been attributed to an active transport system. The precise relationship of such a transport system to cellular metabolism in general is obscure. Glycolysis, an important energy-yielding process in erythrocytes, has been held responsible for potassium reaccumulation and sodium extrusion by cold-stored human red blood cells when rewarmed to 37.5° (1, 2). Certain metabolic poisons which inhibit glycolysis also affect cation transport in red blood cells (3). These observations support the concept that the active transport mechanism may be geared to and utilize energy from glycolysis. Yet the hypothesis of an active transport system in human erythrocytes has never been absolutely established.

Some investigators have attempted to incriminate a single enzyme, acetylcholinesterase, as responsible for maintaining normal permeability in human and canine erythrocytes (4–6). These workers noted a retarding effect of acetylcholine on cation movement and on hemolysis and a reversal of this effect by physostigmine. These reports stimulated much interest in the possible role of acetylcholinesterase in governing sodium and potassium transport in erythrocytes. Other investigators, however, were unable to find evidence of a significant relationship between erythrocyte acetycholinesterase activity and potassium movement (7–10).

Recently we reported that the acetylcholinesterase activity of erythrocytes of eight patients with paroxysmal nocturnal hemoglobinuria (PNH)[1] was strikingly reduced (11, 12). Whether this defective cholinesterase activity is due to a deficiency of the enzyme *per se* or to the action of inhibitors has not been settled. Nevertheless, the PNH red cell provided an excellent experimental tool for investigating the role played by this enzyme in maintaining the potassium gradient of the human red cell. If the acetylcholinesterase enzyme plays a major role in potassium transport, the cholinesterase-deficient PNH cell should show alterations in potassium movement.

MATERIALS AND METHODS

Patient material. The clinical studies and detailed laboratory investigations regarding the finding of decreased erythrocyte acetylcholinesterase in PNH patients have been documented (11, 12). Case 1 of the current manuscript corresponds to Patient 6 of the previous publications and Case 2 to Patient 4.

Collection of blood. Sixty to 80 ml. of blood were collected from an antecubital vein into silicone-treated tubes containing heparin.[2] Saftidonor®[3] sets were employed for the collection of the blood.

Determination of acetylcholinesterase activity. A small portion of the collected blood was centrifuged at high speed (10,000 × G) for 10 minutes to pack the red cells. Following meticulous removal of the plasma, the red cells were washed three times with large volumes of 0.85 per cent sodium chloride solution and finally packed again by centrifugation at 3,000 rpm for five minutes.

Acetylcholinesterase activity of 0.2 ml. portions of

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[1] The abbreviation PNH will be used throughout the manuscript.

[2] Heparin sodium, 1,000 U.S.P. units (10 mg.) per ml., was kindly supplied by the Upjohn Company, Kalamazoo, Mich.

[3] We are indebted to Dr. E. B. McQuarrie, Biochemical Research Division, Cutter Laboratories, Berkeley, Cal., for the Saftidonor® sets.

washed, packed cells was determined manometrically using the Warburg technique. Measurements were carried out at 37.5° in a Ringer-Krebs bicarbonate medium. The final volume was 2.0 ml. Acetylcholine bromide in a final concentration of 5×10^{-3} M was employed as the substrate. The substrate had been recrystallized several times from absolute alcohol. The vessels were gassed with a mixture of 95 per cent N_2 and 5 per cent CO_2 for 10 minutes. The substrates and cells were mixed and allowed exactly three minutes to reequilibrate. Successive manometric readings were made at intervals of 10 minutes for one-half hour. The first 10 minute interval reading was considered the most accurate. This value was multiplied by six to give the rate per hour. Although a volume measurement was used for the determinations, the final expression of enzyme activity was in terms of dry weight (12).

Preparation of blood for influx and efflux studies. Following removal of the plasma, packed red cells were washed five times with Ringer-Krebs buffer.

For influx studies 6.0 ml. of washed, packed cells was mixed with 24.0 ml. of Ringer-Krebs buffer or of the subject's own heparinized plasma. (Similar results were obtained whether Ringer-Krebs buffer or the subject's own plasma were used as the diluent.) Small amounts of radioactive $K^{42}Cl$ were added to both the normal and PNH specimens simultaneously. The suspensions were mixed well and allowed to incubate at 37.5° in a Warburg bath with constant slow agitation.

The efflux studies were performed under the following experimental conditions: 8.0 ml. of washed, packed cells, 8.0 ml. of heparinized plasma, and $K^{42}Cl$ were mixed and allowed to incubate with slow, continuous agitation for four hours in silicone-treated Erlenmeyer flasks. The labeled cells were separated by centrifugation and washed thrice with Ringer-Krebs buffer (pH 7.4). To 6.0 ml. of these washed cells was added 24.0 ml. of Ringer-Krebs buffer. The suspensions were mixed and incubated at 37.5° in a Warburg bath.

Sampling of aliquots for influx studies. Four ml. samples were removed immediately and at 30 to 60 minute intervals over a four hour period following the addition of the isotope to the reconstituted mixtures. The samples were centrifuged for five minutes at 3,000 rpm to separate cells from plasma. One ml. of plasma was plated in a planchette, baked dry, and the radioactivity determined in a Geiger counter. To another 1 ml. aliquot of plasma 7.0 ml. of water was added and the protein precipitated by the addition of 2.0 ml. of 25 per cent trichloracetic acid. The filtrate was analyzed for potassium by employing a Beckman spectrophotometer model DU with a flame attachment. One-half ml. of red cells was hemolyzed by the addition of 4.5 ml. of water. One ml. of the hemolysate was plated in a planchette, dried, and the radioactivity determined. All counts were corrected for decay and background. The remaining hemolysate was rendered protein-free by the addition of trichloracetic acid. The filtrate was analyzed for potassium.

Sampling of aliquots for efflux experiments. Four ml. samples were removed from the reconstituted mixture (labeled cells and Ringer-Krebs buffer) at 30 to 60 minute intervals over a four hour period. Potassium analysis and radioactivity determinations were performed on the supernatant and on the cells by the methods described for the influx studies.

Mathematical treatment of the data. The treatment of the data is abstracted from that of Solomon (13). The following equation governs or describes these experiments:

$$\frac{dp}{dt} = -k_{ab}p + k_{ba}q \qquad 1)$$

where p = plasma concentration of K^{42}, q = red cell concentration of K^{42}, k_{ab} and k_{ba} = transfer coefficients (k_{ab} from plasma to cells and k_{ba} from cells to plasma). Multiplying this equation through by $\frac{v_p}{v_q}$, one obtains the following:

$$\frac{dP}{v_q dt} = -k_{ab}\left(\frac{v_p}{v_q}\right)p + k_{ba}\left(\frac{v_p}{v_q}\right)q \qquad 2)$$

where P is the amount of K^{42} in the plasma, and v_p and v_q are the volumes of plasma and cells, respectively. This equation gives the amount of P transported per unit time per unit volume of cells, and this quantity is independent of v_p and v_q. Therefore, $k_{ab}\left(\frac{v_p}{v_q}\right)$ and $k_{ba}\left(\frac{v_p}{v_q}\right)$ are the constants which are independent of the v_p and v_q. The efflux equations are of the same form as the influx equations.

RESULTS

Acetylcholinesterase activity of normal and PNH erythrocytes

Table I shows the acetylcholinesterase activity of the PNH red cells compared to the mean value of determinations on the cells of 20 normal subjects. Under these experimental conditions the erythrocytes of Case 1 possessed no enzyme activity. The activity of red cells of Case 2 was 17.3 per cent of normal.

TABLE I

Acetylcholinesterase activity of normal and paroxysmal nocturnal hemoglobinuria (PNH) erythrocytes

	Manometric data: $\mu L. CO_2$ per mg. dry weight per hour	% of normal
Normal (average of 20)	15.0	
Case 1	0	0
Case 2	2.6	17.3

Potassium transport data

The data obtained from two normal subjects and the two PNH patients are plotted in Figures 1 and 2. Each figure is typical of four separate experiments. It can be seen that potassium transport, which is characterized by the quantities k_{ab} (v_p/v_q) and k_{ba} (v_p/v_q) in the case of the influx studies, is virtually the same for normal and PNH cells. The computed transfer coefficients are in good agreement with those reported by Solomon (13).

The precision of the efflux experiments was not very high. Nevertheless, the curves obtained from the data appeared to have the same slopes, indicating that there is no difference in the transfer coefficients of normal and PNH cells.

DISCUSSION

Several investigators have suggested that specific acetylcholinesterase, which is situated entirely in the cell membrane, is concerned with the permeability of the red cell (4–6). When acetylcholine was added to a suspension of red cells in an isotonic bicarbonate medium, it not only reduced the loss of potassium but also delayed hemolysis. In an isotonic sodium chloride or sodium bicarbonate medium containing 35 mM of KCl per L. at pH 8.0, the addition of acetylcholine produced a small increase (5 to 10 per cent) in cellular potassium. Physostigmine, an inhibitor of acetylcholinesterase, reversed or blocked the above effects. When benzoylcholine, a substrate not appreciably hydrolyzed by acetylcholinesterase, was substituted for acetylcholine, such changes likewise did not occur. It was concluded from these studies that cholinesterase activity is responsible in some manner for maintenance of normal potassium and sodium concentrations in human red cells (4–6).

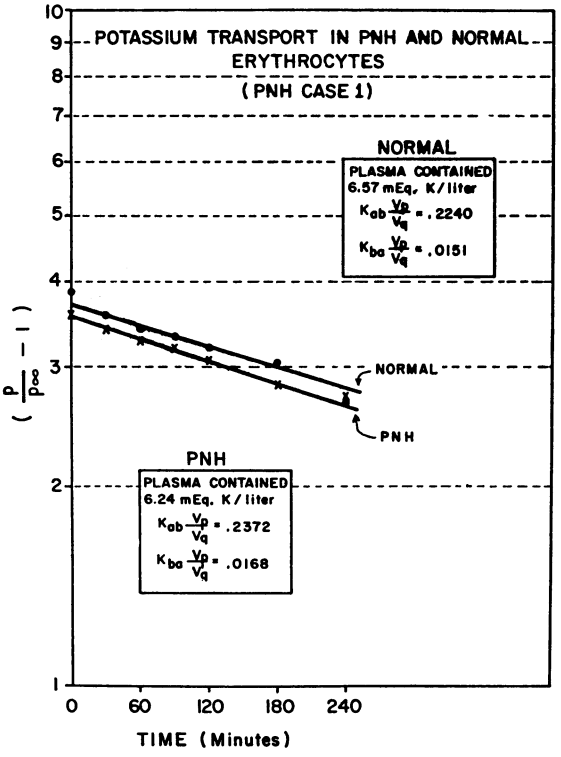

FIG. 1. POTASSIUM TRANSPORT IN PNH AND NORMAL ERYTHROCYTES (PNH CASE 1)

Parpart and Hoffman (7) repeated the experiments of Lindvig, Greig and Peterson (6). The addition of acetic acid to red cell suspensions brought about the same results as the addition of acetylcholine with respect to retarding both cellular loss of potassium and hemolysis. They concluded that the effect of acetylcholine was due to the reduction in pH produced by the addition of this substrate. Physostigmine failed to block the effect of acetic acid but did abolish the acetylcholine effect, further evidence in favor of their interpretation. These authors concluded that there was no evidence linking cation permeability of human red cells and the activity of the acetylcholinesterase system.

Taylor, Weller and Hastings studied the effect of inhibitors of cholinesterase (physostigmine, diisopropyl fluorophosphate) on the potassium concentration gradient as well as on the rate of potassium exchange of human erythrocytes (8). Cholinesterase inhibitors brought about a loss of potassium from the cells primarily by decreasing the rate at which potassium entered the cell from the plasma. No direct relationship between inhibition of the enzyme and the rate at which potassium left the cell was found. A loss of cellular potassium was noted only when concentrations of the inhibitors were at least 100 times that necessary to inactivate the enzyme.

Goodman, Marrone and Squire found no evidence of a relationship between erythrocyte acetylcholinesterase activity and the *in vivo* potassium gradient (9). Recently Gale, Brown and Eadie have demonstrated that inhibition of red cell cholinesterase with and without the addition of acetyl-

choline did not cause potassium loss from cells at 4° or at 37.5° (10). Re-entry of potassium at 37.5° in cells previously stored at 4° was also unaffected.

Our experiments revealed that human erythrocytes deficient in cholinesterase activity because of a disease state (PNH) were still capable of maintaining a normal potassium concentration gradient. The inward and outward potassium rate constants were similar for both PNH and normal erythrocytes.

The acetylcholinesterase activity of red cells of Case 2 was approximately 17 per cent of normal. Bullock, Grundfest, Nachmansohn and Rothenberg noted that a nerve may still transmit impulses even when as much as 80 per cent of the enzyme activity is inhibited (14). This suggests the possibility that the residual acetylcholinesterase activity in the PNH erythrocytes of Case 2 might account for the normal transfer coefficients. On the other hand, the red cells of Case 1 possessed *no enzyme activity* when determined by the manometric method. Yet potassium influx and efflux were not significantly altered. These observations are not compatible with the concept that small amounts of the enzyme can account for the maintenance of a high intracellular potassium concentration. It remains possible that other methods of determining erythrocyte acetylcholinesterase might have revealed traces of enzyme activity in the cells of Case 1. On the other hand, Holland and Greig demonstrated that only 60 per cent inhibition of acetylcholinesterase activity was necessary to increase the permeability of the canine erythrocyte membrane (5).

Cation transport in sickle cell anemia cells has been investigated (15). Marked changes in cation transport occurred when the transformation of the disc to the sickle shape took place. These marked changes in cation transport were not accompanied by significant alteration in acetylcholinesterase activity. Similar observations were made in our laboratory during the current studies.

Hellem and Skaug demonstrated decreased penetration of P^{32} through the PNH erythrocyte membrane (16). Whether this is related to the deficiency of acetylcholinesterase activity remains unsettled. These authors concluded that the decreased permeability to P^{32} might be due to membrane abnormalities or to altered metabolic processes in the interior of the PNH erythrocyte.

The blood platelet is another formed element bathed in the same fluid (plasma) as the red cell. Recent studies have demonstrated that like the human red cell, the human platelet contains a high concentration of potassium and a relatively low sodium concentration (17). Rather convincing histochemical and manometric studies have demonstrated that normal human platelets contain no measurable cholinesterase (18). These observations suggest that despite the absence of acetylcholinesterase activity, the platelet is capable of maintaining a high internal potassium concentration. In a similar vein the apparent lack of acetylcholinesterase in the erythrocytes of some mammalian species has led certain investigators to conclude that the enzyme cannot play a universal role in maintaining cell permeability (18).

The acetylcholinesterase-deficient PNH red cell provided an excellent opportunity to test the hypothesis that this enzyme is responsible for maintaining normal permeability of the human erythro-

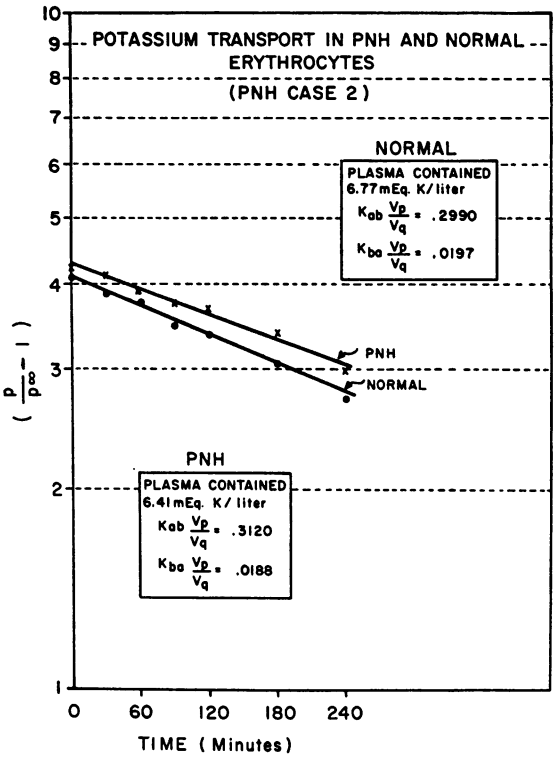

FIG. 2. POTASSIUM TRANSPORT IN PNH AND NORMAL ERYTHROCYTES (PNH CASE 2)

cyte. It was not necessary to inhibit acetylcholinesterase activity by drugs (e.g., physostigmine) which in turn might have produced nonspecific effects. That the PNH cell still showed normal potassium influx and efflux rates provides evidence against the above hypothesis. It remains possible that the acetylcholinesterase enzyme may play some role in permeability of normal human erythrocytes. Nevertheless, the present study indicates that this enzyme is *not essential* for normal permeability in human erythrocytes.

SUMMARY

The influx and efflux rates of potassium in normal human and PNH (paroxysmal nocturnal hemoglobinuria) erythrocytes have been investigated. PNH erythrocytes markedly deficient in acetylcholinesterase activity still had normal potassium influx and efflux rates. The computed transfer coefficients showed virtually no difference. These findings suggest that erythrocyte acetlycholinesterase activity is not essential for the maintenance of normal human red cell permeability.

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