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RED CELL MEMBRANE (Na^++K^+)-ATPase IN DIABETES MELLITUS

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SUMMARY : The red cell membrane (Na^++K^+)-ATPase activity is significantly elevated in diabetes mellitus. The osmotic fragility of diabetic red cells is also increased. In vivo insulin treatment restores the enzyme activity and the osmotic fragility to the normal level. In vitro insulin treatment of diabetic red cells was found to inhibit the further increase in its activity, but it failed to restore the activity to the normal level as in vivo. In diabetes increased Km of (Na^++K^+) ATPase for ATP was observed but Vmax remained the same. Arrhenius plot of this enzyme was also altered in diabetes.

It has been known for many years that the chronic diabetic state may be associated with changes in certain enzyme activities affecting the direction of major metabolic fluxes (1). Erythrocytes earlier regarded as 'non-target' cells for insulin have been shown to carry specific receptors for this hormone (2). Many alterations in the erythrocyte such as reduced deformability (3), increased red cell aggregation (4) and reduced membrane content of cholesterol and sialic acid (1) have been reported in diabetes. Some of the reported changes have been found to be reversible on insulin treatment. Exposure to insulin has been shown to cause an altered generation of pH gradient and ion transport in erythrocytes (5). As our laboratory is actively engaged in studies on red cell membrane, we found it interesting to study the role of insulin in modulating the red cell membrane (Na^++K^+)-ATPase activity in diabetes mellitus, particularly in view of available reports that insulin may play a significant role in the regulation of ionic events in its target cells and that diverse ATPases may be involved in regulating transmembrane ionic gradients (6).

MATERIALS AND METHODS

All reagents were of highest purity available. Disodium adenosine triphosphate ($ATPNa_2$), ouabain, tris-HCl(Trizma Hydrochloride) and bovine serum

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albumin were purchased from Sigma Chemical Company, USA. Imidazole was procured from Fluka AG, Buchs, Switzerland.

Patient selection : All patients with diabetes mellitus had fasting blood glucose level between 250 to 300 mg per 100 ml, were not receiving any hypoglycemic agents and having no clinical evidence of pancreatitis. These patients received no blood transfusions within the previous two months, and their hemoglobin content was about 14g/100 ml. Insulin treated diabetic patients had fasting blood glucose levels between 80 to 90 mg per 100 ml and were receiving 40 to 80 units of insulin per day. Blood samples were collected in heparin (10 u/ml) by venipuncture with informed consent.

Preparation of erythrocyte ghosts : Ghost membranes were prepared at $4^{o}C$ following the method of Marchesi and Palade (7). The blood sample was centrifuged for 15 min at 1000g, the plasma and buffy coat were removed and so obtained erythrocytes were washed 4 to 5 times with 0.154M NaCl. Enzyme assays were performed within 24 hours of ghost preparation.

Assay of $(Na^{+}+K^{+})$-ATPase : ATPase activity was assayed by incubating 2.5 ml of a solution containing 0.4 to 0.9 mg ghost protein per ml, 140 mM NaCl, 20 mM KCl, 3mM $MgCl_2$, 30 mM imidazole (pH 7.25), $\pm 5x10^{-4}$M ouabain and 6mM ATP, for 30 min at $37^{o}C$. The reaction was stopped after 30 min by the addition of 3.5 ml of a solution containing 0.5M H_2SO_4, 0.5% ammonium molybdate and 2% SDS. After vortexing, 0.1 ml of a solution containing 1.2% sodium metabisulphite, 1.2% sodium sulphite and 0.2% 1-amino-2-naphthol-4-sulfonic acid was added. The colour was allowed to develop for 30 min before measuring the absorbance at 650nm, during the development time the samples were centrifuged for 5 min at the maximum speed of a clinical centrifuge. Appropriate phosphate standards demonstrated the absorbance (at 650 nm) linear with inorganic phosphate concentration from 0 to 0.5 umole of phosphate.

Effect of insulin and high glucose loading : In vitro studies were carried out by isolating red cells and suspending in Krebs-Ringer phosphate buffer (hematocrit 40%) containing 0.01% (w/v) sodium azide. Four units of Lente insulin (Boots, India) was added to 5 ml of diabetic red cell suspension and incubated for 18 hours at $37^{o}C$ with constant shaking. In another experiment 5 ml of normal red cell suspension containing glucose to a final concentration of 250 mg/100 ml was incubated as above. Prior to $(Na^{+}+K^{+})$-ATPase assay, the incubated ghost membranes were extensively dialyzed against 10 mM Tris-HCl to remove any trace of inorganic phosphate.

Osmotic fragility experiments were performed following the method of Dacie and Lewis (8).

Blood glucose values were determined by using Ames Glucometer, Miles Laboratories Inc., U.S.A. Protein was estimated following the method of Lowry et al. (9) using bovine serum albumin as a standard. Absorbance was measured on a Schimadzu Spectrophotometer at 690 nm.

RESULTS

The osmotic fragility of diabetic erythrocytes was increased however insulin treated patients showed almost normal osmotic fragility profile of their red cells (Fig.1).

The activity of red cell membrane $(Na^{+}+K^{+})$-ATPase during diabetes mellitus, insulin treated diabetes and normal humans is shown in table I. The activity of this enzyme was observed to be significantly ($p<0.001$) elevated in untreated

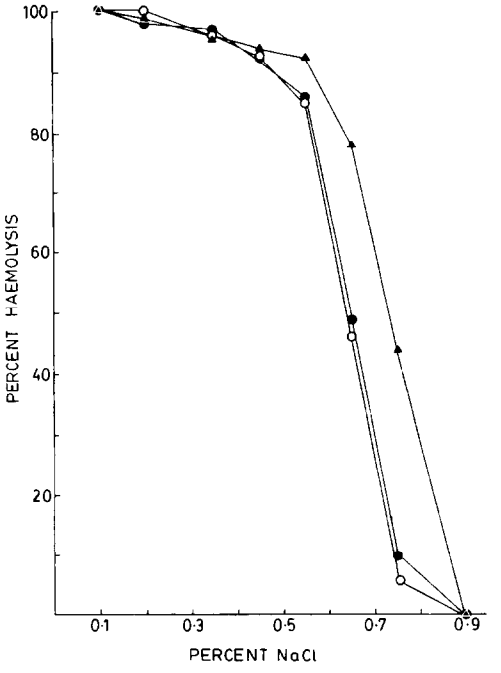


Fig. 1. Osmotic fragility of erythrocytes (—O— normal individuals; —▲— diabetic; —●— insulin treated diabetes). Each point is the mean of at least three experiments.

Table 1 . Membrane $(Na^+ + K^+)$-ATPase activity of normal, high glucose loaded and diabetic (insulin treated and untreated) red blood cells

	In vivo effect of insulin		
	Normal	Diabetic	Insulin treated
	0.0403 ± 0.005	0.1004 ± 0.007	0.039 ± 0.007
	In vitro[a] effect of high glucose loading and insulin		
	Fresh	Incubated (-glucose)	Incubated (+glucose)
Normal RBC	0.0403 ± 0.005	0.3962 ± 0.012	0.3810 ± 0.009
	Untreated fresh	(-insulin)	(+insulin)
Diabetic RBC	0.1004 ± 0.007	0.3715 ± 0.009	0.1061 ± 0.007

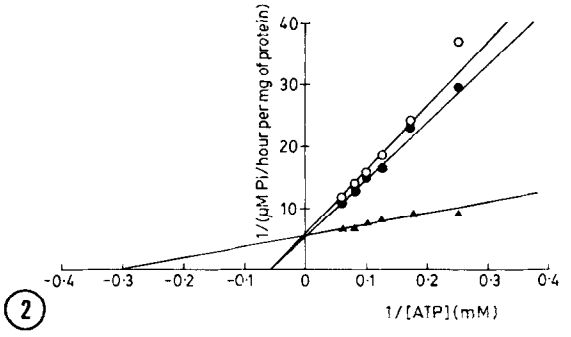
$(Na^+ + K^+)$-ATPase activity expressed in terms of umole Pi liberated per hour per mg of protein. Values are means of 4 to 5 experiments ± S.D.

a. In vitro experiments were carried out as described in Materials and Methods Section.

diabetic patients whereas this activity was found to be restored to the normal level in insulin treated patients.

In vitro incubation of red cells for 18 hours at 37°C in Krebs-Ringer phosphate buffer (100 vol. of 0.9% NaCl, 4 vol. of 1.15% KCl, 3 vol. of 1.22% $CaCl_2$, 1 vol. of 3.82% $MgSO_4.7H_2O$, 20 vol. of 0.1 M sodium phosphate buffer pH 7.4) resulted in an increased activity of $(Na^+ + K^+)$-ATPase both in normal and diabetic membrane preparations. Decrease in the activity of most of the red cell enzymes has already been reported (10) during aging process. But an increase in the activity of Ca^{++}-Mg^{++} ATPase has been reported by our laboratory as a result of in vitro aging of red cell ghost membrane under physiological conditions (11). Addition of insulin to the incubation medium resulted in a significant ($p < 0.001$) decrease of $(Na^+ + K^+)$-ATPase activity, but insulin in contrast to its in vivo effect failed to restore back the elevated activity to the normal level of non-diabetic red cell membrane. However high glucose loading of normal red cells was found to have no effect on the activity of this enzyme. To characterize further the elevated $(Na^+ + K^+)$-ATPase activity observed in erythrocyte membranes of patients with diabetes mellitus, some kinetic parameters of the enzyme were determined. The $(Na^+ + K^+)$-ATPase activities of erythrocyte ghost membranes were measured as a function of ATP concentration and the data are plotted as the double reciprocal transformation (Lineweaver & Burk, 1934) of the Michaelis Menten relation for the determination of Km and Vmax (Fig.2). There appeared to be no consistent difference in the value of Vmax for the three types of preparation (normal, diabetic and insulin treated diabetes mellitus), however the value of Km was considerably decreased in case of diabetes mellitus.

The response to the temperature change in the range of 20-40°C on the $(Na^+ + K^+)$-ATPase activity of erythrocyte ghost membrane was examined. Arrhenius plots (Fig.3) clearly revealed an elevated enzyme activity at all temperatures between 20-40°C in membrane samples from diabetes mellitus.



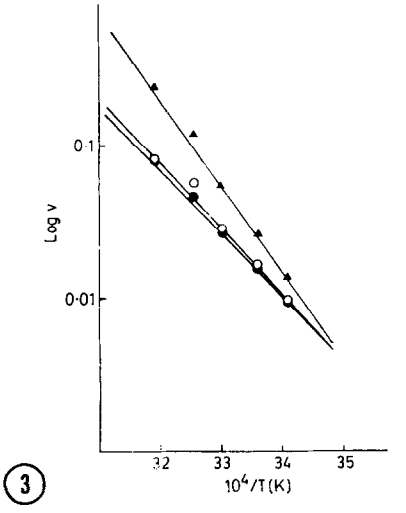


Fig. 2. $(Na^+ + K^+)$-ATPase activity at 37°C of erythrocyte ghost membranes as a function of ATP concentation. The data are plotted as a Lineweaver-Burk plot of 1/V against 1/ S . The intercept on the ordinate is equal to 1/Vmax and the intercept on the absicca is equal to -1/Km. (-○-normal individuals; -▲- diabetic; -●- insulin treated diabetes). Each point is the mean of at least three experiments.

Fig. 3. Arrhenius plots of the response to temperature of $(Na^+ + K^+)$-ATPase activities of erythrocyte ghosts. (-○-normal individuals; -▲- diabetic; -●- insulin treated diabetes). Each point is the mean of at least three experiments.

DISCUSSION

Our results clearly show that the activity of the enzyme $(Na^+ + K^+)$-ATPase of red cell membrane is significantly increased in diabetes mellitus. This is in contradiction to the earlier reports of Agarwal et al. (12) on alloxan induced diabetes in rats showing a decrease in the activity of red cell membrane $(Na^+ + K^+)$-ATPase.

Our in vitro insulin treatment results are in agreement with the findings of Luly et al. (13) that insulin treatment in vitro inhibits $(Na^+ + K^+)$-ATPase activity of human erythrocyte membranes. We may mention here that Luly et al. have observed the insulin effect with erythrocyte ghost membrane whereas we have treated the intact red cells with insulin, thereafter ghost membranes were isolated and $(Na^+ + K^+)$-ATPase activity was assayed. The insulin effect in vivo was found to be the same (inhibitory) as with ghost membrane reported by Luly et al. and this may lead to an inference that insulin receptor sites are not affected and their vicinal residues remain the same during the prepara-

tion of ghost membrane from the intact red cells. The observed increase in the activity of this enzyme, during our in vitro experiments with intact red cells, above the both normal and diabetic basal levels is probably due to aging process during the incubation period. Aging process is known to bring about structural and compositional changes in the erythrocyte membrane (14-16). Several reports (16,17) confirm that the aging erythrocytes, release their membrane glycopeptides which are important constituents of the receptor sites. Baumann and MacCart (18) have shown that in mature erythrocyte the number of insulin receptors decreases as an exponential function of cell age. Our in vitro observations with intact red cells (diabetic) show that although insulin can inhibit the increase in $(Na^+ + K^+)$-ATPase activity but it fails to restore back the activity to the normal level as in vivo. This anomalous behaviour of insulin in vitro may be due to loss of insulin receptors from the cell surface during the incubation period, however this is still speculative and further work in this field is needed to elucidate the mechanism clearly. Since high glucose loading in normal red cells did not elicit any effect on the activity of this enzyme, this suggests that the increased activity observed in case of diabetes is due to low level of insulin in blood. The role of insulin on red cell membrane is further emphasized from our osmotic fragility results.

Interpretation of altered Arrhenius plot observed during our experiments on diabetes suggest the possibility of an altered lipid domain in the erythrocyte membrane of diabetic patients. In vitro interaction of the hormone has been shown to negatively affect membrane fluidity (13), this alteration of the membrane fluidity might thus be responsible for the decrease of $(Na^+ + K^+)$-ATPase activity observed after insulin treatment of diabetic patients. Decreased membrane fluidity has been frequently associated with a decrease in both $(Na^+ + K^+)$-ATPase and Ca^{2+} ATPase activities (19,20). Moreover increased fluidity has been found to stimulate sodium pumping activity in human erythrocytes (21). $(Na^+ + K^+)$-ATPase being an integral protein spans the red cell membrane, the conformational state and hence its catalytic activity may be dictated by the vicinal activating phospholipids since a direct interaction between the enzyme

and phospholipids has been shown by Muczynsky et al (22). The low Km for ATP of the enzyme, observed in diabetes could be due to the 'masking' of the enzyme active site as a result of altered membrane phospholipid and protein composition. An altered phospholipid composition has already been reported in diabetes (23). The unchanged Vmax of the enzyme indicates that the number of enzyme molecules remains the same.

A functional linkage has been found between the membrane $(Na^+ + K^+)$-ATPase and glyceraldehyde-3-phosphate dehydrogenase (G3PD) at the cytoplasmic face of the red cell membrane (24), and the binding of G3PD has been reported to be dependent upon the ionic strength (25). These reports indicate that an altered activity of $(Na^+ + K^+)$-ATPase could possibly affect the partitioning of G3PD between the membrane and cytosol in red cells of diabetic individual. The role of $(Na^+ + K^+)$-ATPase in red cells assumes greater importance because evidences indicate that the ATP formed by phosphoglycerate kinase which is also membrane bound is used by the Na: K pump in preference to cytoplasmic ATP (26).

Our studies thus clearly indicate that the deficiency of insulin during untreated diabetes mellitus brings about certain membranal alterations in red cells. In view of our observation regarding the alterations in Km and temperature dependance of $(Na^+ + K^+)$-ATPase in diabetic patients and also the reversibility of these changes after insulin treatment, we may state that insulin plays a vital role in the maintenance of the membrane microenvironment of this enzyme.

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