EXPERIMENTAL METHODS FOR CLINICAL PRACTICE

Potassium Transport through the Erythrocyte Plasma Membrane in Patients with Insulin-Dependent Diabetes Mellitus: Effects of Insulin Therapy

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Plasma membrane potential and function of Ca^{2+}-activated K^+ channels of erythrocytes are studied in patients with insulin-dependent diabetes mellitus. A significant increase in membrane potential and in the degree of opening of Ca^{2+}-activated K^+ channels is revealed in comparison with erythrocytes of healthy donors. The degree of channel opening is higher in patients with nephropathy than in those with retinopathy, in which this parameter is normal. Insulin therapy normalize the erythrocyte plasma membrane potential in the patients and has no effect on the degree of channel opening. Ion transport disturbances and modulations of Na^+, K^+, and Ca^{2+} levels in erythrocytes may impair their function in insulin-dependent diabetes mellitus.

Key Words: *insulin-dependent diabetes mellitus; erythrocyte; Ca^{2+}-activated K^+ channels*

Hemostatic disorders as well as structural and functional changes in blood cells, including erythrocytes, are thought to be the principal factor in the etiology of diabetic angiopathies. The molecular basis of changes in functional characteristics of erythrocytes so far remains unclear. Changes in cellular pH, lowered ATP content in erythrocytes, and increased concentration of intracellular Ca^{2+} have been considered as a possible candidate [12]. Recent studies have shown that physicochemical restructuring occurring in erythrocytes in diabetes mellitus (DM) is closely associated with the damage to membrane structure and ion transport disorders [2,10-12,14]. A hypothesis that some membrane defects are hereditary, for example, enhanced Na^+/Li^+ metabolism in erythrocytes in DM, was put forward. Changes in this parameter were proposed as a marker for the identification of diabetics with the risk of nephropathy [6].

Our goal was to assess the permeability of the erythrocyte plasma membrane to K^+, function of Ca^{2+}-activated K^+ channels, and membrane potential of erythrocytes in patients with insulin-dependent diabetes mellitus (IDDM) and to examine the effect of insulin therapy on these parameters.

MATERIALS AND METHODS

The study included 17 patients (5 women and 12 men) aged 20-43 years and 15 healthy donors aged 25-46 years. Sixteen patients were in the subcompensation phase (mean glycemia level after an overnight fast 9.56±0.27 mmol/liter, glycosylated hemoglobin, HbAlc, 9.21±0.32%) and one patient was in the compensation phase (glycemia after an overnight fast 4.4 mmol/liter, HbAlc 6.5%). Vascular

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complications were diagnosed in 10 patients: 5 patients had nephropathy (transitory proteinuria) and 5 patients had retinopathy (diabetes-relatedangiopathy of the retina). The patients were injected insulin (Lilly, Humulin S, L, and M3) twice a day. Blood was collected from the ulnar vein after an overnight fast prior to treatment and after 2, 6, and 12 weeks of therapy in plastic vials with 3.8% sodium citrate (9:1). Erythrocytes were pelleted in a Beckman centrifuge (15 min, 4°C, 1500 g) and washed three times with cooled normal saline (150 mM NaCl, 5 mM sodium-phosphate buffer, pH 7.4). The membrane potential and permeability of Ca^{2+}-activated K^+ channels were measured in a Radiometer PHM-64 pH-meter equipped with an Orion electrode 91-15 with slight modifications [13]. Packed erythrocytes (100 μl) were suspended in 1.9 ml of medium containing 150 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, and 10 mM glucose and preincubated for 5 min at 37°C. After centrifugation (1500*g*, 1 min, 4°C), 1.9 ml of the medium was added again, and the suspension was thoroughly stirred and transferred to the measuring chamber, after which the electrode was installed. After the pH-meter had stabilized, 10 μl of 4 mM $CaCl_2$ was added to a final concentration of 20 μM, and 20 seconds later 20 μl of 2 mM protonophore ClCCP (carbonyl cyanide m-chlorophenyl hydrazone) was added to a final concentration of 20 μM. The pH_1 value was automatically recorded at sensitivity 50 mV and paper velocity 0.5 mm/sec. Thirty seconds after the pH-meter had stabilized, 10 μl of 100 μM Ca^{2+} ionophore A23187 was added to a final concentration of 0.5 μM, and the moment of the extremum and the pH_2 value corresponding to alkalization of the medium, opening of Ca^{2+}-dependent K^+ channels, and hyperpolarization of the erythrocyte plasma membrane were recorded. Two minutes after the start of acidification, which reflects deactivation of K^+ channels and repolarization of the membrane, the pH_3 value was recorded, which corresponds to a new stationary level. The pH_4 value reflecting the true value of cellular pH was recorded, and 20 μl 20% Triton X-100 was added to a final concentration of 0.2%. The membrane potential (mV) was calculated from the following formulas:

$E_m=(RT/F)\times(pH_4-pH_1)$ is the membrane potential before stimulation of the calcium entry.

$E_1=(RT/F)\times(pH_4-pH_2)$ is the membrane potential corresponding to the maximum membrane hyperpolarization,

$E_2=(RT/F)\times(pH_4-pH_3)$ is the membrane potential corresponding to a new stable status of the system,

$PO=E_1/E_2\times100\%$ is the percentage of open channels,

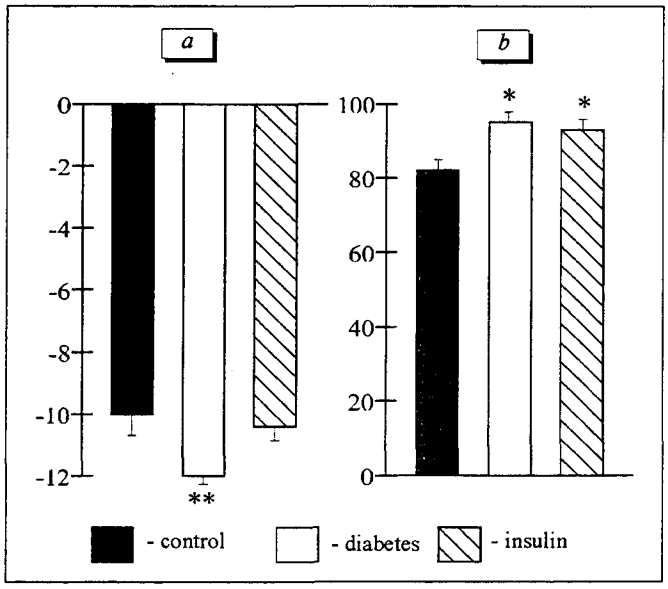

Fig. 1. Changes of membrane potential (*a*) and degree of opening of Ca^{2+}-activated K^+ channels (*b*) in erythrocytes of IDDM patients before and 12 weeks after therapy with biosynthetic insulin. Here and on Fig. 2: $*p<0.05$, $**p<0.001$ *vs.* the control.

R, *T*, and *F* are the known values.

The results were analyzed using Student's *t* test. Differences were considered to be significant at $p<0.05$.

RESULTS

Our findings indicate that the permeability of the erythrocyte plasma membrane for potassium ions is changed in IDDM. A slight (18%) statistically significant ($p<0.001$) change in the erythrocyte membrane potential and in the degree of opening of Ca^{2+}-activated K^+ channels (by 14%, $p<0.05$) was observed in IDDM patients in comparison with healthy donors (Fig. 1). These parameters did not differ in erythrocytes of IDDM patients with or without vascular complications, nor did they depend on the hereditary origin of the disease or its duration.

Insulin therapy lowered the membrane potential to the control level as early as 2 weeks after beginning (Table 1) and had no effect on the degree of opening of Ca^{2+}-activated K^+ channels (Fig. 1). Noticeable differences were revealed in IDDM patients with nephropathy ($n=5$) and retinopathy ($n=5$). The degree of erythrocyte plasma membrane hyperpolarization was almost the same in patients with these vascular complications (Table 1). The degree of opening of Ca^{2+}-activated K^+ channels was significantly higher in patients with nephropathy ($p<0.001$) than in those with retinopathy, in whom this parameter did not differ from the control (Fig. 2). Insulin therapy effectively reduced the

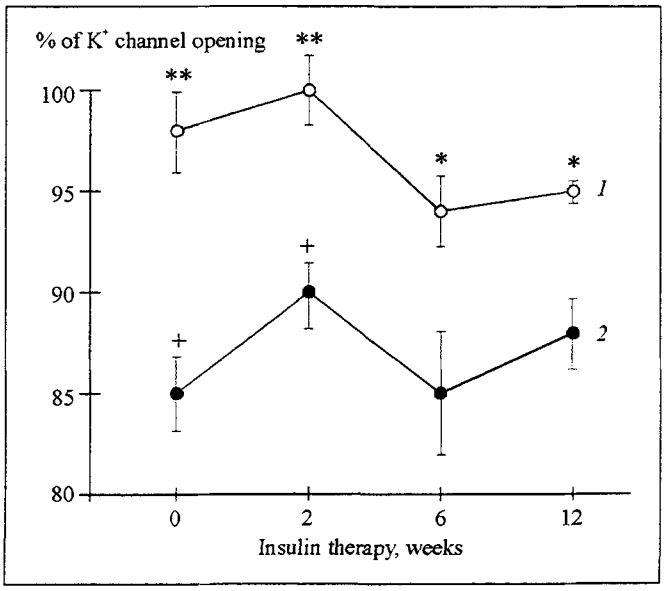

Fig. 2. Percentage of opening of Ca^{2+}-activated K^+ channels in erythrocytes of IDDM patients with nephro- (*1*) and retinopathy (2) before and during therapy with biosynthetic insulin. *$p<0.05$ *vs.* that in erythrocytes of patients with nephropathy.

membrane potential in both subgroups (Table 1) but had no effect on the degree of opening of Ca^{2+}-activated K^+ channels (Fig. 2).

The physiological role of Ca^{2+}-activated K^+ channels in erythrocytes is unclear. These channels are involved (the Hardos pathway) in dehydration of erythrocytes and in the modification of their ion content in sickle-cell anemia [5]. The permeability of human erythrocyte plasma membrane for K^+ is known to be regulated by the Ca^{2+}-dependent protein calmodulin. An increase of the intracellular concentration of free Ca^{2+} ($[Ca^{2+}]_i$) leads to membrane hyperpolarization as a result of increased K^+ conductivity [3]. The intracellular Ca^{2+} content in erythrocytes of DM patients [11] and diabetic rats [15] is reportedly increased, and Ca^{2+} transport through the erythrocyte plasma membrane is impaired. Calcium outflow from erythrocytes is provided by Ca^{2+}-ATPase [4], the activity of which in DM was shown to be lowered in erythrocytes DM [10,12] and enterocytes [7]. In DM, calcium overload of erythrocytes — cells lacking Na^+/Ca^{2+} metabolism system and intracellular Ca^{2+} transporting structures [4] — can be attributed to reduced efficiency of the plasma membrane Ca^{2+} pump. The increase in $[Ca^{2+}]_i$ is attended by a number of membrane-coupled events: opening of Ca^{2+}-activated channels, hyperpolarization, inhibition of Na^+ pump, and modulations of membrane proteins and erythrocyte morphology [4].

We observed an increase in the number of open Ca^{2+}-activated K^+ channels and hyperpolarization of the erythrocyte plasma membrane in patients with IDDM. Decreased [8], increased, and unchanged [10] activity of the erythrocyte Na^+/K^+-ATPase has been demonstrated in DM. On the basis of these findings it can be hypothesized that $[Ca^{2+}]_i$ rise induces the opening of Ca^{2+}activated K^+ channels in erythrocytes from DM patients. The differences in the degree of opening of Ca^{2+}-activated K^+ channels in erythrocytes of patients with nephro- and retinopathy imply a possible relationship between K^+ transport and Na^+/H^+ metabolism in erythrocytes, which is more intensive in diabetics with nephropathy than in those with retinopathy [2]. Activation of Na^+/H^+ and Na^+/Ca^{2+}metabolism and elevation of intracellular Na^+ concentration, which lead to an increase in $[Ca^{2+}]_i$ and in the degree of opening of Ca^{2+}-activated K^+ channels, may account for such a relationship in cells with a functioning Na^+/Ca^{2+} metabolism system. However, human erythrocytes lack this system and intracellular Ca^{2+}-transporting structures [3,4]. Presumably, $[Ca^{2+}]_i$ is higher in erythrocytes of diabetics with nephropathy than in diabetics with retinopathy. The higher $[Ca^{2+}]_i$, the higher the degree of opening of Ca^{2+}-activated K^+ channels, on the one hand, and the higher the activation Ca^{2+}-depended protein kinase C, which is

TABLE 1. Membrane Potential of Erythrocytes in IDDM Patients Treated with Insulin

Group	Before insulin therapy	Duration of insulin therapy, weeks		
		2	6	12
Healthy donors (n=15)	-10.18±0.56	-	-	-
IDDM patients (n=17)	-12.02±0.31**	-9.76±0.53*	-10.16±0.59	-10.48±0.4*
Patients without angiopathies (n=7)	-12.8±0.39*	-8.74±0.6**	-10.54±0.92	-8.99±0.73**
Patients with angiopathies (n=10)	-11.94±0.37*	-11.23±0.34	-9.76±0.53**	-10.74±0.49
Patients with nephropathies (n=5)	-12.00±0.33*	-11.38±0.59	-10.00±0.88	-10.02±0.79
Patients with retinopathies (n=5)	-12.31±0.19*	-11.08±0.44	-8.92±0.30**	-10.36±0.62*

Note. *$p<0.05$, **$p<0.001$ *vs.* the control, *$p<0.05$, **$p<0.001$ *vs.* the value before insulin therapy.

involved in the regulation of the Na^+/H^+ exchanger [2], on the other. To our knowledge, no data on $[Ca^{2+}]_i$ in the erythrocytes of diabetics with nephro- and retinopathy have been published; therefore, the above considerations so far remain hypothetical. The effects of insulin therapy on the erythrocyte K^+ channels in DM patients are even more difficult to interpret. Insulin recovers Ca^{2+}-ATPase in erythrocytes of DM patients and diabetic rats [10] and in enterocytes of diabetic rats [7], which normalizes Ca^{2+} outflow from these cells. However, even after 12 weeks of insulin therapy the number of open Ca^{2+}-activated K^+ channels remains high, probably as a result of high $[Ca^{2+}]_i$. The high $[Ca^{2+}]_i$ after prolonged insulin therapy may be due to increased entry of Ca^{2+} through a modified plasma membrane. Despite insulin therapy, lipid peroxidation in the erythrocytes remains enhanced, particularly in DM associated with angiopathies [1]. The intensification of lipid peroxidation induces the formation of peroxide clusters and Ca^{2+} entry. The membrane potential is probably associated with recovered Na^+/K^+-ATPase activity in erythrocytes and normalization of glycemic regulation as a result of insulin therapy.

Undoubtedly, the Na^+, K^+, and Ca^{2+} contents and ion transport are altered in the erythrocytes of IDDM patients. It is difficult to define the cause-and-effect relationships, because different types of ion transport are coupled to each other, and changes in one type may induce those in many others. It should be noted that many types of ion transport depend on the membrane lipid content. Correlations between the activity of Na^+/H^+ metabolism, Na^+/K^+ cotransport, Na^+/K^+-ATPase, on the one hand, and the erythrocyte plasma membrane content of cholesterol, phospholipids, and free fatty acids, on the other, has been demonstrated [9]. Profound changes in the membrane structure, primarily in its lipid composition, occur in erythrocytes of IDDM patients [14]. Presumably, modifications of lipid spectrum occurring in the erythrocyte plasma membrane in IDDM induce substantial changes in ion transport and membrane potential.

An increase in the degree of opening of K^+ channels and enhanced K^+ outflow contribute to the ion imbalance in erythrocytes of IDDM patients. Structural and functional changes persisting in the erythrocyte plasma membrane despite insulin therapy and good glycemic control may be responsible for the development of vascular complications.

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