Changes in Na,K-Adenosine Triphosphatase (ATPase) Concentration and Na,K-ATPase–Dependent Adenosine Triphosphate Turnover in Human Erythrocytes in Diabetes

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The concentration of Na,K-adenosine triphosphatase (ATPase) and Na,K-ATPase–dependent adenosine triphosphate (ATP) turnover was measured in fasting blood samples of 20 subjects with insulin-dependent diabetes mellitus (IDDM), 22 subjects with non–insulin-dependent diabetes mellitus (NIDDM), and 20 nondiabetic subjects. [^{3}H]ouabain binding was used to determine Na,K-ATPase concentration. There were 471 ± 70 (mean ± SD) ouabain binding sites per erythrocyte, normally distributed in the nondiabetic subjects. The number of ouabain sites per cell was lognormally distributed in the two populations of diabetic subjects. The mean of lognormal distributions of ouabain sites per cell was significantly lower in the IDDM group. The mean of the lognormal distribution for the NIDDM group was not significantly different from that of the nondiabetic subjects. Na,K-ATPase–dependent ATP turnover (molar activity) was 9,580 ± 742 mol/mol minute (mean ± SD) normally distributed in the nondiabetic population. A lognormal distribution was observed in the diabetic population. Means of the lognormal distributions were significantly different: 3.98 ± 0.05 for the nondiabetic population and 3.13 ± 0.48 for both diabetic populations. Changes in the concentration of Na,K-ATPase (ouabain sites per cell) and Na,K-ATPase–dependent ATP turnover did not correlate with hemoglobin A_{1c} (HbA_{1c}) or with blood glucose. This would suggest that elevated glucose concentrations do not directly cause decreased Na,K-ATPase function in the diabetic erythrocyte.

IN MAMMALIAN CELLS, intracellular Na^+ concentrations are low (15 to 30 mmol/L) and intracellular K^+ concentrations are high (120 to 135 mmol/L). In extracellular fluids, Na^+ concentrations are high (135 to 145 mmol/L) and K^+ concentrations are low (5 to 6 mmol/L). Because the Na^+ and K^+ gradients are used for movement of metabolites into and out of the cell, they are essential for cell viability. The gradients are maintained across the cell membrane by the membrane-bound Na,K-adenosine triphosphatase ([ATPase] Na^+ pump), which moves Na^+ ions out of the cell and K^+ ions into the cell against the existing Na^+/K^+ gradients. The energy for this process is supplied by the hydrolysis of adenosine triphosphate (ATP). The stoichiometry for the process is three Na^+ and two K^+ ions per ATP molecule.[1,2]

Fairly well-controlled diabetic humans and animals suffer complications such as blindness (cataract and retinopathy), kidney disease (nephropathy), and nervous system disease (neuropathy). These complications of diabetes are devastating. They affect the quality of life and shorten the life-span of the individual.

Acute hyperglycemia would appear to lead to time-dependent increases in Na,K-ATPase activity,[3-5] whereas partial inhibition of Na,K-ATPase would appear to be general in chronic diabetes. In rats (spontaneously diabetic BB Wistar rats or rats made diabetic by streptozotocin injection), Na,K-ATPase is inhibited in skeletal muscle,[6] peripheral nerve,[7] kidney,[8] retinal pigment epithelium,[9,10] and heart.[11] In the peripheral nerve and retina pigment epithelium, there are concomitant changes in intracellular Na^+ and K^+ levels. Na,K-ATPase in the lens epithelium,[12] renal medulla,[13] and erythrocyte[14] of human diabetics is likewise inhibited.

The sodium pump (Na,K-ATPase) is critical for absorption processes (nutrient transfer from blood to cells), filtration processes (waste elimination), and electric impulse generation (nerve conduction). A change in the activity of membrane-bound Na,K-ATPase would have pleiotropic consequences in tissues. For example, changes in cell Na^+ might be expected to result in changes in cell Ca^{2+}, due to alteration of Na^+/Ca^{2+} exchange.[15,16] This has been postulated to account for diabetic autonomic neuropathy. Concentrations of cellular metabolites, such as taurine, for which the transport into tissues is dependent on the steepness of the Na^+ gradient[17] might also be altered. Cellular taurine concentrations decrease almost 50% in diabetic animals and in rats fed a high-galactose diet. Since taurine, like sorbitol, is one of the small organic molecules that act as cellular osmoregulators,[18] this change in the ability of a cell to concentrate taurine could have profound effects on tissue response to changes in extracellular osmolarity.[18] *myo*-Inositol is also transported into cells using the sodium gradient.[19] *myo*-Insoitol is used as an osmoregulator.[18,19] It is also used in the synthesis of phosphatidylinositides, a class of membrane phospholipids used in cell-signaling pathways.[20] From this brief discussion, it is clear that sodium-pump dysfunction could play a major role in most diabetic complications.

There are two possible routes by which Na,K-ATPase inhibition could occur: (1) inactivation by posttranslational modification of Na,K-ATPase (protein kinase phosphorylation,[7,21-23] oxidation, or nonenzymatic glycation[24]); and (2) altered synthesis and/or degradation of Na,K-ATPase.[4-6] Both routes would lead to decreased Na,K-ATPase activity, route one by decreased ATP turnover (molar activity = moles ATP hydrolyzed by 1 mol Na,K-ATPase

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per minute) and route two by decreased Na,K-ATPase concentration.

An analysis of erythrocyte Na,K-ATPase concentration and Na,K-ATPase–dependent ATP turnover in 42 diabetic subjects and 20 nondiabetic subjects is reported herein. The data suggest that inactivation by posttranslational modification is the primary cause of inhibited Na,K-ATPase activity in the diabetic erythrocyte. The data also indicate a shift from a normal distribution for Na,K-ATPase concentration in nondiabetic subjects to a lognormal distribution in diabetic subjects.

SUBJECTS AND METHODS

Subjects

After a 12-hour fast, blood samples were obtained by venipuncture from 20 nondiabetic subjects aged 21 to 65 years and 42 idiopathic diabetic subjects aged 21 to 68 years with diabetes duration of at least 6 months. Both insulin-dependent (IDDM) and non–insulin-dependent (NIDDM) diabetic subjects were included. Excluded were individuals using phenytoin, tricyclic antidepressants, or anticoagulants, as well as pregnant or nursing women. Excluded from the nondiabetic population were individuals with a history of hypertension. Individuals with significant peripheral vascular disease were excluded from the diabetic population. Informed consent was obtained from the donors. Some of the individuals had venipuncture at 4-week intervals over a 9-month period. The initial sample collected from these individuals is used for the population statistics.

Preparation of Erythrocytes and Erythrocyte Ghosts

After removal of 100 μL of each sample for determination of cell counts, the cells were sedimented by centrifugation. The buffy white layer of cells was carefully removed and discarded. The packed erythrocytes were washed twice by resuspension and recentrifugation in cold phosphate-buffered saline containing 1 mmol/L EDTA (Sigma, St Louis, MO). Erythrocyte cell number was determined directly with a hemocytometer for each blood sample; the cell number was confirmed for each sample from measurements of hemoglobin concentrations following hemolysis, during ghost isolation. Blood glucose was determined by glucometer or by a commercially available colorimetric assay (315-100; Sigma). Hemoglobin A_{1c} (HbA_{1c}) was determined by a commercially available kit (441-B; Sigma).

Erythrocyte membrane (ghost) isolation was accomplished using the procedure of Blostein.[25] Washed erythrocytes were lysed by repeated freeze-thaw cycles in 10 vol 5-mmol/L Tris/phosphate buffer, pH 8.2. Following hemolysis, the ghosts were precipitated by centrifugation for 20 minutes at 35,000 × *g*. The pellets were resuspended in 5 mmol/L Tris/phosphate buffer, pH 8.2, and rinsed.[25] For measurement of ouabain binding and ATP hydrolysis, ghosts were detergent-activated.

Ouabain Binding

Since cardiac glycosides form a 1:1 complex with Na,K-ATPase, they can be used to determine cellular Na,K-ATPase concentrations.[8,24,26] Therefore, previously described procedures[8,26] with ^{3}H-labeled ouabain were used to determine erythrocyte Na,K-ATPase concentration. Both intact erythrocytes and erythrocyte ghosts of each blood sample were used for these measurements.[8,26] The values obtained from intact erythrocytes and erythrocyte ghosts were within experimental error of one another in all cases.

ATP Hydrolysis

ATP hydrolysis by detergent-activated ghosts was measured at 37°C in imidazole buffer, pH 7.4, containing 130 mmol/L NaCl, 20 mmol/L KCl, 7 mmol/L $MgCl_2$, and 0.05 to 6 mmol/L ATP (sodium salt; Boehringer Mannheim, Indianapolis, IN) in the presence or absence of 10^{-3} mol/L ouabain, with 2 to 20 μCi γ^{32}P-labeled ATP used as a tracer. The concentration of released phosphate was determined after formation of a phosphomolybdate complex.[13] Na,K-ATPase–dependent ATP hydrolysis was computed as the difference between the rates determined in the absence and presence of 10^{-3} mol/L ouabain, and is reported as activity per milliliter blood.

Na,K-ATPase–Dependent ATP Turnover

Decreased or increased Na,K-ATPase activity per milliliter blood can result from three parameters: (1) increased or decreased erythrocytes per milliliter, (2) increased or decreased Na,K-ATPase molecules per cell, and (3) stimulation or inhibition of ATP turnover, which is defined as moles of ATP hydrolyzed by 1 mol Na,K-ATPase per minute (units = minutes^{-1}). ATP turnover was determined for each sample using the following formula: ATP turnover = (activity per milliliter)/(ouabain sites per erythrocyte) × (erythrocytes per milliliter blood) × (1/N), where N is Avogadro's number. Alternatively, ATP turnover was computed as the activity per sample divided by bound ouabain per sample.

Calculations and Statistical Analysis

All data are expressed as the mean of quadruplicate determinations (SE, 10%). The Abacus program, Statview, was used for computing the means for each measurement and for unpaired comparisons and covariance and regression analysis of data collected from the three populations.

RESULTS

The mean age, fasting blood glucose (FBG) concentration, and HbA_{1c} concentration of IDDM and NIDDM subjects were higher than those of nondiabetic subjects (Table 1). Erythrocyte count was comparable among the three populations. In agreement with previous studies, there was a positive correlation between FBG and HbA_{1c} for NIDDM and IDDM populations. The *P* value for the correlation was less than .0002 and less than .05 for NIDDM and IDDM populations, respectively.

Na,K-ATPase Concentration (pumps per cell)

Nondiabetic subjects. There were 471 ± 70 ouabain binding sites per erythrocyte normally distributed (Fig 1). The value of 519 ± 65 sites obtained for the male subjects

Table 1. Demographics of the Diabetic and Nondiabetic Subjects

Characteristic	NIDDM	IDDM	Nondiabetic
Age (yr)	56 ± 7	48 ± 14	40 ± 15
Sex (M/F)	14/8	11/9	10/10
Erythrocyte count (× 10^9 per mL blood)	5.1 ± 0.8	4.9 ± 0.6	5.0 ± 0.5
FBG (mmol/L)	12.7 ± 3.7*	10.0 ± 5.3*	4.55 ± 0.22
HbA_{1c} (%)	6.5 ± 1.2*	6.1 ± 1.3*	4.6 ± 0.7
Diabetes duration (yr)	7 ± 5	18 ± 12	NA

NOTE. Values are the mean ± SD.

Abbreviation: NA, not applicable.

*$P < .05$ for diabetic × nondiabetic subjects (unpaired *t* test).

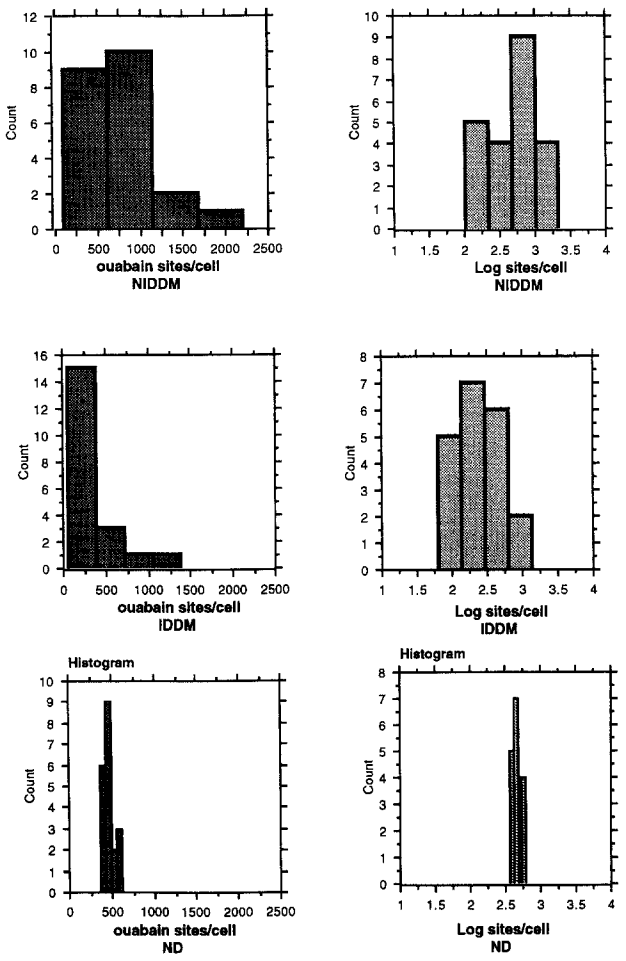

Fig 1. Plots describing the frequency distribution of values obtained for Na,K-ATPase concentration per erythrocyte (pumps per cell) as determined by ouabain binding for 22 NIDDM subjects (top left), 20 IDDM subjects (center left), and 20 nondiabetic subjects (bottom left). Panels at right represent log-transformation for NIDDM (top), IDDM (center), and nondiabetic (bottom) subjects, respectively.

was significantly higher than the value of 383 ± 47 sites obtained for female subjects ($P < .005$, unpaired Student's t test). There was no correlation between age and number of ouabain binding sites in the nondiabetic female population, but there was a negative correlation between ouabain binding sites per erythrocyte and age in the nondiabetic male population (intercept = 671 ± 41, $P < .0001$; slope = −3.57 ± 0.95, $P < .006$; $R^2 = .636$; Fig 2).

Diabetic subjects. For the two diabetic populations, values for ouabain binding sites per cell were not normally distributed (Fig 1). Instead, the distributions were lognormal. The means for log values (ouabain binding sites per cell) were 2.67 ± 0.07, 2.42 ± 0.34, and 2.71 ± 0.35 for nondiabetic, IDDM, and NIDDM populations, respectively. The mean of the lognormal distribution was significantly lower for the IDDM population ($P < .05$). The mean of the lognormal distribution of the NIDDM population was not different from that of the nondiabetic population. This is in agreement with a previous study[26] of a NIDDM population aged 52 ± 2 years with no neuropathy. This result is in disagreement with previous studies of older NIDDM populations and NIDDM populations with neuropathy.[26,27] There was no correlation between ouabain binding sites per cell or log(ouabain binding sites per cell) and age for the IDDM population, but ouabain binding sites per cell and age were negatively correlated for the NIDDM population (intercept = 2,722 ± 793 ouabain binding sites per cell, $P < .005$; slope = −37.7 ± 14.0, $P < .02$; $R^2 = .379$; Fig 2). Whether the negative correlation is related to reduced insulin action[14] remains to be elucidated.

ATP Hydrolysis

In nondiabetic subjects, ouabain-sensitive ATP hydrolysis at 4 mmol/L ATP was 223 ± 63 nmol/min for ghosts isolated from 1 mL blood (Na,K-ATPase activity per milliliter blood). There was a positive correlation between ouabain binding sites per cell (pumps per cell) and Na,K-ATPase activity per milliliter blood (Fig 3). ATP turnover was calculated for each subject from the activity values, the cell number, and the values obtained for ouabain binding sites per cell, assuming a 1:1 ratio for ouabain binding sites per Na,K-ATPase molecule.[1,2] The mean value for ATP turnover (calculated as described earlier) was 9,580 ± 742 mol/mol minute. Values for ATP turnover that were normally distributed were comparable to values for ATP turnover reported previously for Na,K-ATPase.[1,2,8,24,28]

For the two groups of diabetic subjects, ouabain-sensitive ATP hydrolysis at 4 mmol/L ATP was 40 ± 49 nmol/min for ghosts isolated from 1 mL blood. The values were lognormally distributed. As with the data for log(ouabain binding sites per cell) (pumps per cell), log(activity per milliliter) values were normally distributed. The means for nondiabetic and diabetic subjects were significantly different (data not shown). There was a weak correlation between Na,K-ATPase activity per milliliter blood and log(pumps per cell) (Fig 3). ATP turnover, computed as

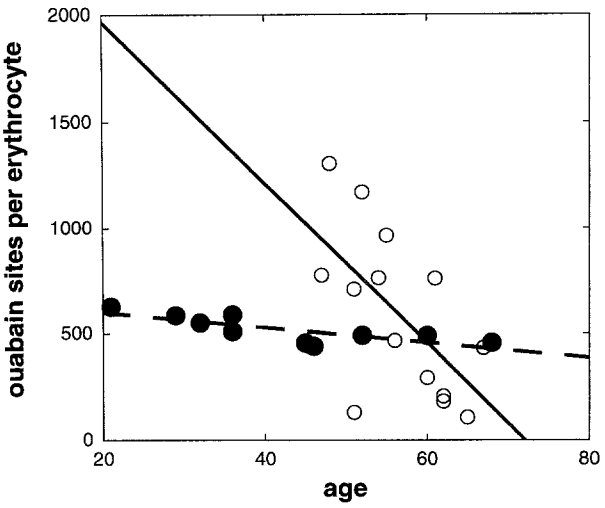

Fig 2. Regression plots demonstrating the correlation between ouabain binding sites per erythrocyte and subject age for the nondiabetic male population (●) and the NIDDM male population (○). No such correlation was observed for nondiabetic females, NIDDM females, or IDDM subjects (male and female).

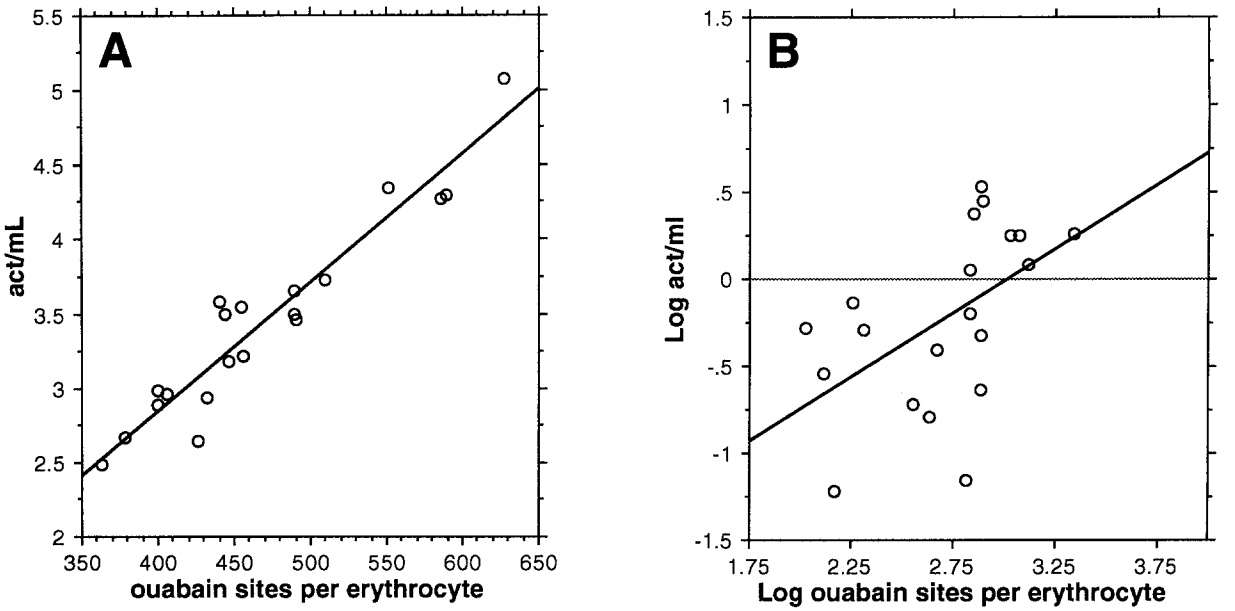

Fig 3. (A) Regression plot of the correlation between Na,K-ATPase activity per milliliter blood (act/mL) and ouabain binding sites per cell for the nondiabetic population (slope = 8.6 ± 0.6 × 10^{-3}, $P < .001$, $R^2 = .909$). (B) Regression plot of the correlation between log act/mL and log ouabain sites per cell for the NIDDM population (slope = 0.74 ± 0.29, $P < .02$, $R^2 = .267$).

described earlier, was lognormally distributed. The means of lognormal distributions for ATP turnover of 3.10 ± 0.45 and 3.15 ± 0.45 for IDDM and NIDDM populations, respectively, were significantly different from the mean of 3.98 ± 0.05 obtained for the nondiabetic population ($P < .05$). This result indicates that an inhibition of existing Na,K-ATPase rather than a change in Na,K-ATPase concentration is the major cause for the lower erythrocyte Na,K-ATPase activity, regardless of diabetes type.

Previous studies of the Na,K-ATPase of the renal medulla and lens epithelium[12,13] of diabetic subjects suggest that whereas ATP turnover is inhibited at ATP concentrations of 3 mmol/L or greater, ATP turnover is normal or stimulated at ATP concentrations less than 3 mmol/L. Experiments were performed to determine if the erythrocyte Na,K-ATPase of the two diabetic populations had similar properties. Na,K-ATPase activity was measured at nine ATP concentrations ranging from 0.050 to 6 mmol/L. All measurements were performed in the presence of 7 mmol/L $MgCl_2$ under conditions where $[Na^+] + [K^+] =$ 150 mmol/L at a $Na^+:K^+$ ratio of 6.5.[28] The results are presented in Fig 4. The curves through the experimental points were obtained from fits of the data to the equation, $v = V1/(1 + (K1/[ATP])^n) + (Vmax - V1)/(1 + (K2/[ATP])(K2/[ATP])^n)$, which assumes that ATP turnover is a function of a high-affinity and low-affinity process,[2,29] where Vmax is the rate of hydrolysis as [ATP] approaches saturation, V1 is the rate of turnover at the high-affinity ATP site as [ATP] approaches saturation, (Vmax − V1) is the rate of turnover at the low-affinity site as [ATP] approaches saturation, and K1 and K2 are the K_{50} values for the high- and low-affinity sites, respectively. The Hill coefficient, n, should be 1 under normal circumstances.[29] The values for Vmax, V1, K1, K2, and n were 11,400 mol/mol minute, 1,720 mol/mol minute, 0.15 mmol/L, 1.32 mmol/L, and 0.99, respectively, for nondiabetic subjects. The values for Vmax, V1, K1, K2, and n were 1,610 mol/mol minute, 13,800 mol/mol minute, 0.61 mmol/L, 1.65 mmol/L, and 2.5, respectively, for IDDM and NIDDM subjects. In the diabetic subjects, ATP binding at the low-affinity site inhibits ATP hydrolysis, as indicated by a Vmax value (rate of hydrolysis at ATP concentrations where both the low- and high-affinity sites are saturated) less than the V1 value (rate of ATP hydrolysis at the high-affinity site). This result is identical to that reported previously for human diabetes,[12,13] for the IDDM BB Wistar rat model,[8] and for in vitro nonenzymatic glycation of Na,K-ATPase.[12,24]

There was no correlation between pumps per cell or log (pumps per cell) and blood glucose for either diabetic population. There was no correlation between pumps per cell or log (pumps per cell) and HbA_{1c} for either diabetic population. This would suggest that the shift from a normal distribution in nondiabetic subjects to a lognormal distribution in diabetic subjects is not the result of elevated blood glucose levels. Similarly, there was no correlation between log(ATP turnover) and blood glucose or HbA_{1c} for either diabetic population. This would suggest that the decrease in ATP turnover and the shift to a lognormal distribution in diabetes is not the direct result of elevated blood glucose levels or nonenzymatic glycation.

Repetitive Determinations in Nondiabetic and Diabetic Subjects

Fasting blood samples were taken at 4-week intervals from 10 nondiabetic, four IDDM, and five NIDDM subjects. For nondiabetic subjects, values for pumps per cell, Na,K-ATPase activity per milliliter blood, and ATP turn-

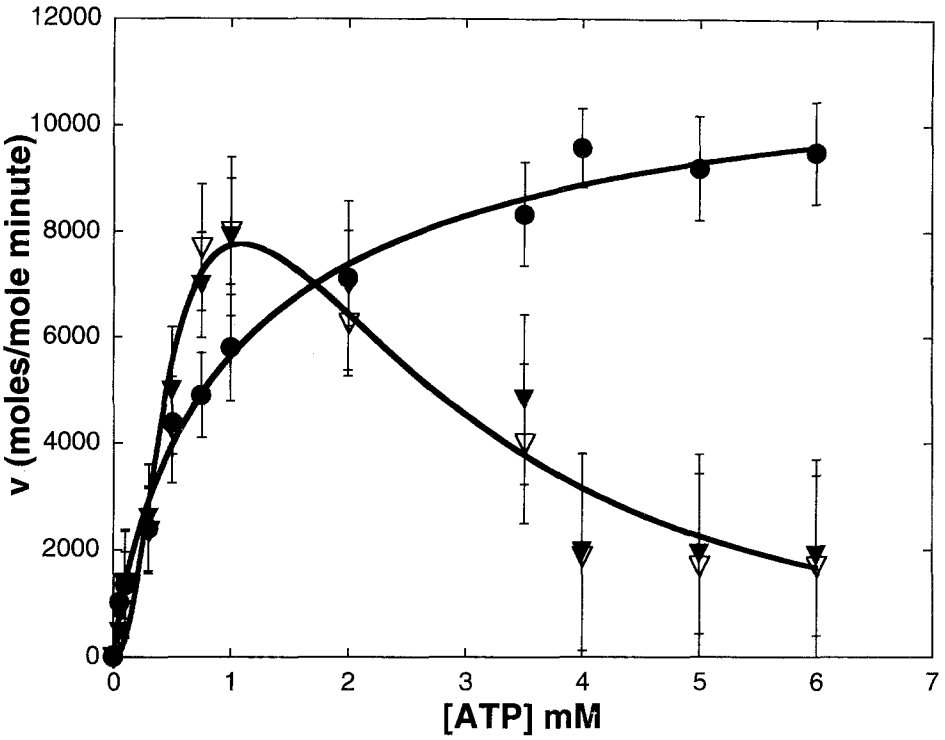

Fig 4. Steady-state kinetics of ATP hydrolysis. With the exception of 1- and 4-mmol/L concentrations, data are presented as means ± SD for 4 nondiabetic (●), 4 NIDDM (▼), and 5 IDDM (▽) subjects. The 1- and 4-mmol/L data points are means ± SD for all subjects listed in Table 1 for each respective group. There was insufficient sample to perform the complete set of measurements for all individuals of each group.

over were normally distributed, with means similar to those of the larger populations discussed earlier. Each nondiabetic individual had a characteristic number of pumps per cell (Fig 5), which was invariant for the 8- to 9-month period of the study. No diabetic individual had a characteristic number of pumps per cell (Fig 5). The range of values for each diabetic subject mirrored the large range of values observed for the diabetic population discussed earlier.

DISCUSSION

For the nondiabetic population, our results are in agreement with previous studies. Na,K-ATPase activity per milliliter blood increases with increasing Na,K-ATPase concentration (as determined by ouabain binding). This agrees with a previous study of the correlation of Na,K-ATPase–dependent Rb^+ transport and ouabain binding[30] and a previous study of Na,K-ATPase–dependent ATP hydrolysis and ouabain binding.[31] Each subject had a characteristic level of ouabain binding sites per erythrocyte, a result similar to that reported previously for five nondiabetic subjects.[31] However, in contrast to previous results,[30,31] there were more erythrocyte ouabain binding sites in males than in females in our population of nondiabetics. Furthermore, there was a negative correlation with age in the male, but not in the female, population.

There is general agreement that Na,K-ATPase activity is decreased in erythrocytes of both IDDM and NIDDM subjects.[14,26,27,30-35] Our results support this conclusion. However, there is disagreement on the degree of inhibition, as well as the cause of the decreased activity. For nondiabetic subjects, values of 3 to 445 nmol $Pi \cdot mg^{-1}$ protein $\cdot min^{-1}$ have been reported with ATP concentrations of 1 mmol/L and greater.[14,27,32-36] For IDDM subjects, values of 16 to 460 nmol $P_i \cdot mg^{-1} \cdot min^{-1}$ have been reported for ATP concentrations of 2 mmol/L and greater.[14,32,34,35] For NIDDM subjects, values of 4 to 18 nmol $P_i \cdot mg^{-1} \cdot min^{-1}$ have been reported for ATP concentrations of 2 mmol/L and higher.[27,34,35] These values represent specific activities of erythrocyte ghost preparations from several different laboratories. The different magnitudes of specific activity are related to ghost purity. Specific activity, which delineates preparation purity, is not a good measure of Na,K-ATPase function. The more appropriate value is the ATP turnover value ([{moles P_i released per minute in the absence of ouabain} − {moles P_i released per minute in the presence of ouabain}]/[ouabain sites per assay]). For a normally functioning Na,K-ATPase, ATP turnover should be at least 8,000 mol/mol minute at near-saturating ATP concentrations.

From the results presented herein for ATP turnover, where corrections for erythrocyte number and Na,K-ATPase concentration were made, the primary cause of the decreased activity would appear to be inhibition of existing erythrocyte Na,K-ATPase. The inhibition is apparent at ATP concentrations of at least 3 mmol/L and is less apparent at lower ATP levels. One cause of these disparate results would appear to be related to a change in the function of the low-affinity ATP site. In a previous study, different results were obtained. When the assay solution $MgCl_2$ was 3 mmol/L, $[Na^+] + [K^+]$ was 95 mmol/L, and the Na^+:K^+ ratio was 5.3, results for NIDDM subjects with polyneuropathy included a 29% decrease in Vmax, a 29% decrease in ouabain binding sites, and no change in the K_{50} for ATP.[27] The difference at the lower Mg^{2+} and monovalent cation concentrations[27] may result from the significant changes in monovalent cation regulation of the substrate-

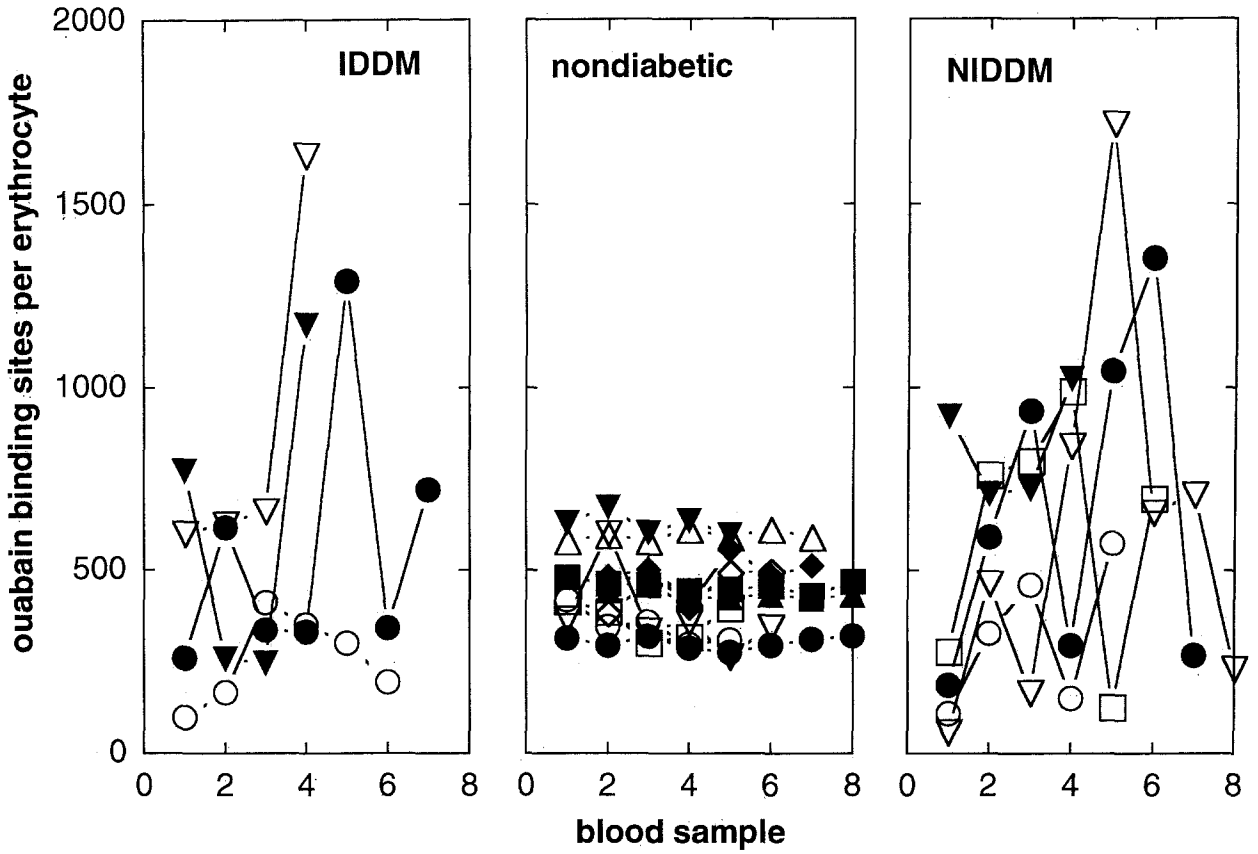

Fig 5. Plots of the number of ouabain binding sites per cell (pumps per cell) over an 8- to 9-month period for 10 nondiabetic, 4 IDDM, and 5 NIDDM subjects. Blood sample and month of sampling are synonymous.

inhibited pump,[8,24] or may be the result of differences in free Mg^{2+} in the assay solutions.[28] Future studies should clarify this issue.

The effects of changes in ATP turnover on Na,K-ATPase–dependent Na^+ and K^+ exchange remain to be fully elucidated. Since there was no correlation between ATP-dependent ATP turnover and FBG or HbA_{1c}, it would appear unlikely that glucose, via nonenzymatic glycation, is the causative agent. This is unexpected, since nonenzymatic glycation of Na,K-ATPase in vitro does cause changes in the mechanism of action similar to those observed in diabetes.[12,24] Glycation by other aldehydes cannot be excluded currently. Since lipid peroxidation is increased, aldehydes such as malonaldehyde and 4-hydroxynonenal, which are formed from unsaturated lipid under conditions of oxidative stress in the presence of Cu^{2+} or Fe^{3+}, are likely candidates if nonenzymatic glycation is the mechanism by which erythrocyte Na,K-ATPase function is altered in diabetes.

The lognormal distribution of Na,K-ATPase molecules per cell (ouabain binding sites per cell) and the variability in Na,K-ATPase molecules per cell in individual NIDDM and IDDM subjects were unexpected, and suggest that a change in erythropoiesis overlays the change in the erythrocyte Na,K-ATPase mechanism of action. Na,K-ATPase concentration and Na,K-ATPase activity are highest in reticulocytes.[37,38] As reticulocytes mature, Na,K-ATPase concentrations decrease due to degradation by a nonlysosomal ATP-dependent mechanism.[39] Therefore, Na,K-ATPase concentrations are lowest in the oldest cells. In nondiabetic subjects, in whom the erythrocyte life-span is 120 days, ouabain binding sites per cell were constant and unique to each subject whose blood was tested monthly over a 9-month period. The range of individual variation was normally distributed, with a mean of 433 ± 85 ouabain binding sites per cell. In both diabetic populations, the variability in the number of ouabain binding sites per cell for each individual in monthly samples was as great as that observed for the entire population. However, the means for each individual were comparable to those for nondiabetic individuals. This would suggest that at certain times during the 9-month period, blood samples of diabetic subjects had unusually high levels of aged erythrocytes. At other times during the 9-month period, blood samples of diabetic subjects may have had unusually high levels of younger cells such as reticulocytes.

There is other experimental evidence to support this hypothesis. Hyperglycemia induces a decrease in erythrocyte membrane phospholipid asymmetry.[40] In other words, phosphatidylserine increases in the outer membrane leaflet

and sphingomyelin increases in the inner membrane leaflet. The asymmetry of erythrocyte membrane lipids is maintained by an aminophospholipid-specific Mg^{2+} ATPase (a flipase), which transports phosphatidylserine and phosphatidylethanolamine to the inner leaflet. From studies of Percoll gradient–separated erythrocytes of healthy donors, the flipase is less active in older erythrocytes and outer-leaflet phosphatidylserine is increased.[41] The older cells of nondiabetic blood samples are more dense, have decreased cell volume, decreased cell size, decreased deformability, increased osmotic fragility, and increased in vivo clearance rates. Furthermore, the older cells display increased adhesiveness to endothelial cells and reticuloendothelial cells and also display measurable levels of lipid peroxidation. Many of the properties defined for aging erythrocytes of nondiabetic subjects are characteristic of erythrocytes of diabetic subjects.[40] Since hyperglycemia does not inhibit the flipase,[40] the decrease in phospholipid asymmetry in diabetes may be the result of phospholipid peroxidation and/or glycation of membrane proteins.

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