

Alterations in the properties and isoform ratios of brain Na^+/K^+ -ATPase in streptozotocin diabetic rats

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Abstract

In this study we analysed the changes in the properties of rat cerebral cortex Na^+/K^+ -ATPase in streptozotocin induced diabetes (STZ-diabetes). Special attempt was made to determine whether insulin treatment of diabetic animals could restore the altered parameters of this enzyme. Na^+/K^+ -ATPase activity was found to be decreased by 15% after 2 weeks, and by 37% after 4 weeks in diabetic rat brains with a parallel decrease in maximal capacity of low affinity ouabain binding sites. There was no significant change in the high affinity ouabain binding sites. The K_d values did not change significantly. Western blot analysis of brain Na^+/K^+ -ATPase isoforms indicated a $61 \pm 5.8\%$ and $20 \pm 2.8\%$ decrease of the α_1 and α_3 isoforms, respectively in 4 weeks diabetic animals. Change in the amount of the α_2 isoform proved to be less characteristic. Both types of β subunit isoform showed a significant decrease in four weeks diabetic rats. Our data indicate a good correlation in diabetic rats between changes in Na^+/K^+ -ATPase activity, low affinity ouabain binding capacity and the level of α_1 isoform. While insulin treatment of diabetic animals restored the blood glucose level to normal, a complete reversal of diabetes induced changes in Na^+/K^+ -ATPase activity, ouabain binding capacity and Na^+/K^+ -ATPase isoform composition could not be achieved.

Keywords: Brain; ATPase, Na^+/K^+ -; Isoenzyme; Streptozotocin-induced diabetes

1. Introduction

Na^+/K^+ -ATPase is a transmembrane enzyme primarily responsible for the active transport of sodium and potassium in mammalian cells [1]. This protein is greatly expressed by neurons and consuming 30–60% of brain ATP stores it maintains the electrical potential necessary for excitability of this tissue [2]. The enzyme exists as a heterodimer, there are three isoforms of the catalytic alpha (α) subunit and at least two of the beta (β) chain [3,4]. The α isoforms differ from each other in several biochemical and physiological characteristics and display a cell and tissue specific distribution [4,5]. The α_1 isoform is widely

distributed in all tissues, while α_2 and α_3 expression is mostly limited to excitable cells [4,6,7]. In brain the α_3 isoform is solely expressed by neurons, but both neurons and glia cells express the α_1 or α_2 isoforms [8,9]. The distribution of β subunit isoforms in different cells is less well characterized, β_1 has a broad distribution, while β_2 is localized mostly to glia [10]. The significance of the tissue and cell specific distribution of the subunit isoforms is not known, but evidence is slowly accumulating to suggest that α isoforms may be differentially regulated by physiological and pathological stimuli [11–13]. Some data show that Na^+/K^+ -ATPase isoform ratio changes during development in brain and heart, furthermore certain hormones alter both isoform ratio and isoform abundance [14–16]. It has also been demonstrated that Na^+/K^+ -pump sites were considerably reduced in skeletal muscle and adipose tissue in streptozotocin induced diabetes mellitus [17,18], and insulin administration enhanced Na^+/K^+ -ATPase activity [19]. It seems that the α_2 isoform is mostly responsible for insulin action in these tissues [20,21]. Recent data show that streptozotocin induced dia-

Abbreviations: C, control rats; DAG, diacylglycerol; D₂, 2 weeks diabetic rat; D₄, 4 weeks diabetic rats; D₂R, 2 weeks diabetic + 2 weeks insulin reverted rats; D₄R, 4 weeks diabetic + 2 weeks insulin reverted rats; Na^+/K^+ -ATPase, Na^+/K^+ -dependent ATPase (E.C. 3.6.1.37); STZ, streptozotocin.

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betes causes a decrease of brain Na^+/K^+ -ATPase activity [22]. Insulin has been demonstrated to stimulate the synaptosomal Na^+/K^+ -ATPase activity [23]. Mayamil and his coworkers reported, that alloxan diabetes induced an initial decrease followed by an increase in rat brain Na^+/K^+ -ATPase activity which was corrected by insulin administration [24]. The questions remain whether similar changes could be detected in brain cortex Na^+/K^+ -ATPase activity in streptozotocin induced diabetes, whether the alteration of Na^+/K^+ -ATPase activity results from a quantitative decrease of a specific isoform of the enzyme protein or from inactivation of the enzyme. The aim of this study was to investigate the Na^+/K^+ -ATPase activity and the P_i facilitated ouabain binding, as well as the isoenzyme composition of brain cortex derived from normal and STZ-diabetic rats with and without insulin treatment. Some preliminary results were published elsewhere [25].

2. Materials and methods

2.1. Materials

Crystalline porcine insulin was obtained from NOVA (Denmark). Antibodies against rat Na^+/K^+ -ATPase were purchased from Upstate Biotechnology (UBI) (Lake Placid, NY, USA). Peroxidase conjugated goat anti rabbit antibodies were from DAKO (Germany), and streptozotocin, phenylmethylsulfonyl fluoride (PMSF), bovine serum albumin (BSA), ovalbumin, aprotinin were obtained from Sigma (St. Louis, MO, USA). Immobilon-P Transfer Membrane was a Millipore (Bedford, MA, USA) product, [^3H]ouabain (1.22 TBq/mmol) and ECL Western blotting detection reagent were those of Amersham (Amersham, UK), blood glucose assay kits were from Boehringer (Mannheim, Germany).

2.2. Animals

Male Sprague-Dawley rats weighing between 180–210 g were used for the experiments. Streptozotocin (STZ) (60 mg/kg body weight i.v.) in 100 mM citrate buffer pH 5.5 was given to a group of randomly selected animals [26]. Diabetes was verified 24 h later by estimating hyperglycemia and glucosuria. The first group of diabetic animals was killed after 2 weeks (D_2) and another group after 4 weeks (D_4) of STZ administration. In insulin replacement studies ultralente insulin was administered daily to the 2 and 4 weeks diabetic rats. Insulin was given in an individual dose to normalize blood glucose level during 2 weeks before the time of the assay (D_2R , D_4R). Control animals (C) were kept under the same condition as diabetic rats and used as age-matched control after 2, 4 and 6 weeks, respectively. The rats were killed by decapitation and the brains were immediately removed. The cerebral cortex was isolated and rapidly frozen in liquid nitrogen.

2.3. Analytical methods

Preparation of cerebral cortex microsomal fractions

1 g of cortex was homogenized in 10 ml of 0.25 M sucrose 10 mM Tris, pH 7.4 containing 2 mM PMSF, 1 μg aprotinin by a Janke Kungel homogenizer (Germany) for 20 s with half maximal speed. The homogenate was centrifuged at $2500 \times g$ for 10 min and the pellet was discarded. The supernatant was then centrifuged at $100\,000 \times g$ for 60 min in a Centrikon TFT 70.38 rotor. The pellet (crude microsome) was finally suspended in homogenizing medium and stored at -80°C [27].

Protein content

Protein content was assayed by the method of Bradford using ovalbumin as standard [28].

Blood glucose

Blood glucose was estimated at the same time of day using the Boehringer-Mannheim kit.

[^3H]Ouabain binding

[^3H]Ouabain binding was performed as described before [29] with minor modifications. Crude microsomal fractions (100 μg) were incubated for 2 h at 37°C in 1 ml medium containing 3 mM MgCl_2 , 3 mM imidazole/ PO_4 , pH 7.25, [^3H]ouabain (1.22 TBq/mmol) and unlabelled ouabain in final concentrations of $2 \cdot 10^{-8}$ – $5 \cdot 10^{-5}$ M (spec. act. 500–15 000 cpm/pmol). Nonspecific [^3H]ouabain binding was measured in the presence of 1 mM unlabelled ouabain. The samples were filtered on Whatman GFC filters. Filters were washed and counted by liquid scintillation. Maximal binding (B_{max}) expressed in pmol per mg of protein, and the dissociation constant (K_d) expressed in nmol or μmol per l, were calculated according to the methods of Scatchard [30] using a computer program ENZFITTER (version 1.05 EGA single ligand two binding sites model, Elsevier-Biosoft, Cambridge, UK).

Na^+/K^+ -ATPase activity

Na^+/K^+ -ATPase activity was assayed according to Somogyi [31] with minor modifications. Briefly, the assay mixture in a final volume of 1 ml contained microsome (50 μg) and a buffer of 50 mM Tris/HCl pH 7.4, 20 mM KCl, 100 mM NaCl, 1 mM EGTA, 5 mM MgCl_2 and 5 mM ATP. The control tubes contained the same reaction mixture and 1 mM ouabain. The reaction was started by the addition of ATP and the tubes were incubated at 37°C for 10 min. The reaction was stopped by the addition of 500 μl of 20% TCA, and the amount of inorganic phosphate was determined [31]. The Na^+/K^+ -ATPase activity (100%) represents the difference between the activity in the presence and absence of 1 mM ouabain. Inhibition percentages were calculated by comparing the activities in the presence of different concentration of ouabain to the

100% value. The experimental data were fitted using ENZFITTER (Biosoft, Elsevier, Cambridge, UK). The following model was used:

$$V = V_{\max} C / (IC_{50} + C)$$

where V is the observed velocity at a given inhibitor concentration (C) and $V_{\max}$ is the maximal velocity of Na^+/K^+ -ATPase. The best fit curve was calculated using non-linear regression [32].

The amount of inorganic phosphate determined in the presence of 1 mM ouabain represents the Mg^{2+} -ATPase activity [31].

5'-Nucleotidase

5'-Nucleotidase was determined in the presence of 50 mM Tris-HCl pH 9.0, 10 mM $MgCl_2$, 10 mM AMP, 100 mM KCl, 100 μ g crude microsomal protein. The reaction was started by the addition of the microsomal protein and the tube was incubated for 20 min at 37°C. After stopping the reaction by addition of 500 μ l 20% TCA [33], the amount of inorganic phosphate was determined as above.

SDS-PAGE

Crude microsomal proteins (50 μ g) were resolved by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli [34]. Samples were solubilized in a buffer consisting of 0.125 M Tris, pH 6.7, containing 4% SDS, 1 mM EDTA, 15% glycerol, 0.1 M dithiothreitol, and 0.01% bromophenol blue. The samples were heated for 15 min in 98°C and loaded on a discontinuous polyacrylamide gel (stacking gel 3.4% acrylamide pH 6.7, resolving gel 7.5% acrylamide pH 8.9). The running buffer contained 0.2 M glycine, 2 mM EDTA, 25 mM Tris and 0.1% SDS. Molecular weight markers were the low molecular weight prestained SDS standard of Bio-Rad (Hercules, CA).

Western blot analysis

Crude microsomal proteins resolved on SDS-PAGE were transferred onto nitrocellulose sheets with a Milliblot SDE transfer system (Millipore, Bedford, MA) using a low

ionic discontinuous buffer system (anode buffer-1: 0.3 M Tris and 10% methanol, pH 10.4, anode-2: 25 mM Tris and 10% methanol, pH 10.4, cathode buffer: 25 mM Tris, 40 mM glycine, and 20% methanol, pH 9.4). Transfers were routinely carried out at 23°C for 15 min at 25 mA followed by 45 min at 200 mA. The nitrocellulose was fixed for 1 hour in a blot solution containing 5% BSA, 0.5% Tween 20, 150 mM NaCl, 20 mM Tris pH 7.5 solution followed by 16 h (4°C) incubation with antibodies of the Na^+/K^+ -ATPase subunit isoforms (UBI, USA), diluted to 1:500. Blots were washed three times and then incubated by peroxidase conjugated goat anti-rabbit secondary antibody for 2 h at 23°C. The blots were washed three times and developed with the ECL Western blotting detection reagent [35]. The film negatives were analyzed by laser densitometry.

2.4. Statistical analysis

A Student's *t*-test was used throughout this study to compare the differences in various parameters examined. The difference between the means was considered to be significant if $P < 0.05$. Data were analyzed by non-linear least squares procedures. As indicated the best fit curve was calculated using non-linear regression [32].

3. Results

3.1. Body weight and blood glucose

All animals treated with STZ, developed hyperglycemia on the following day (blood glucose level was minimum 25 mM). The body weight of STZ-diabetic rats was significantly lower after the 2 and 4 weeks than the age-matched non diabetic controls (Table 1). The blood glucose level of the STZ-diabetic groups were 3.7- and 4.2-fold higher than those of age-matched non diabetic controls. Blood glucose was decreased close to the control level by insulin administration and a relative body weight gain was also observed during two weeks of insulin administration.

Table 1
Body weight and blood glucose levels in control, STZ-diabetic and insulin-treated STZ-diabetic rats

Groups	C (17)	D2 (13)	D4 (16)	D2R (14)	D4R (14)
Body weight (g)					
initial	190 $\pm$ 24	196 $\pm$ 18	218 $\pm$ 20	187 $\pm$ 19	198 $\pm$ 20
2 weeks	211 $\pm$ 24	176 $\pm$ 18 *	196 $\pm$ 19 *	167 $\pm$ 15 *	178 $\pm$ 15 **
4 weeks	227 $\pm$ 24	—	183 $\pm$ 19.8 **	181 $\pm$ 20 **	166 $\pm$ 18 **
6 weeks	236 $\pm$ 24	—	—	—	188 $\pm$ 21 **
Glucose (mM)	7.8 $\pm$ 1.5	28.7 $\pm$ 3.5 **	32.8 $\pm$ 3.6 **	8.8 $\pm$ 2.6 **	9.8 $\pm$ 2.5 **

Experimental diabetes was induced by streptozotocin injection (60 mg/kg body weight i.v.). Diabetic rats were killed either after 2 weeks (D₂ group) or after 4 weeks (D₄ group). Insulin treatment was started after 2 or 4 weeks of STZ administration (groups D₂R, D₄R, respectively) and continued for 2 weeks as described in Materials and methods. Data are means $\pm$ S.E., n = number of experimental animals. Statistically significant ($P < 0.05$) differences between the age-matched controls and either STZ-diabetic or insulin treated STZ-diabetic rats are denoted by *. Statistically significant ($P < 0.05$) differences between STZ-diabetic (D₂ and D₄) and insulin treated STZ-diabetic (D₂R, D₄R) rats are denoted by **.

3.2. Enzyme activities of brain microsomes

We have summarized the activity of ouabain sensitive Na^+/K^+ -ATPase, Mg^{2+} -ATPase and 5'-nucleotidase in crude microsomal fractions prepared from control, 2 and 4 weeks diabetic (D_2 and D_4) and diabetic insulin treated animals (D_2R and D_4R) in Table 2. The activity of all the three enzymes was the same in the two, four and six weeks control groups, therefore C represents all the age-matched control groups in the presentation of the results. In the first two weeks of diabetes there was a small, but significant change (-15%) in the activity of brain cortex Na^+/K^+ -ATPase. A more pronounced decrease (-37%) in Na^+/K^+ -ATPase activity was observed in the 4 weeks diabetic state (D_4). Two weeks insulin treatment restored the enzyme activity in all of diabetic animals.

Mg^{2+} -ATPase activity was significantly higher in diabetic animals (near $+30\%$) independently of the duration of diabetes. Insulin treatment reduced the activity of Mg^{2+} -ATPase to the control level in both 2 and 4 week diabetic groups. 5'-Nucleotidase activity was found to be equal in all groups investigated. These data indicated that the decrease in Na^+/K^+ -ATPase activity was not attributable to nonspecific damage of the proteins during separation of microsomes.

Testing the ouabain dependence of the Na^+/K^+ -ATPase activity of brain microsomes we have found significant differences in ouabain sensitivity of Na^+/K^+ -ATPase prepared from control, 4 weeks diabetic and 4 weeks diabetic insulin treated rats (Fig. 1). The ouabain sensitivity of the microsomes derived from 4 week diabetic animals shifted to lower ouabain concentration. The ouabain concentration producing half maximal inhibition was $8.34 \pm 0.19 \mu\text{M}$ in the case of the control and $2.84 \pm 0.15 \mu\text{M}$ in 4 weeks diabetic animals. This alteration was partly diminished by insulin treatment, $4.18 \pm 0.15 \mu\text{M}$ ouabain resulted half maximal inhibition in STZ-diabetic insulin treated animals. Less pronounced differences were detected in ouabain sensitivity in 2 weeks diabetic and 2 weeks diabetic insulin treated groups (not shown).

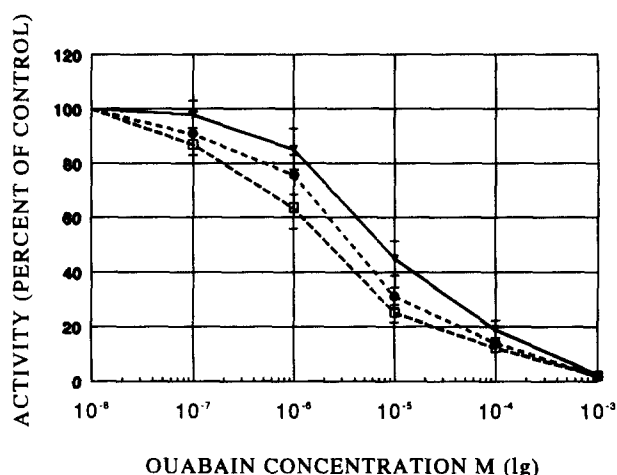


Fig. 1. Na^+/K^+ -ATPase inhibition by ouabain in cerebral microsomal membranes from control and from four weeks diabetic rats without and with insulin treatment. Data are expressed as percent of maximal ouabain sensitive Na^+/K^+ -ATPase activity in control (∇) four weeks diabetic rats without ($\square$) and with ($\bullet$) insulin treatment, respectively. The data are means $\pm$ S.E. of minimum of three determinations performed in duplicates. Computed IC_{50} values are $8.34 \pm 0.19 \mu\text{M}$ in the case of the control and $2.84 \pm 0.15 \mu\text{M}$ in 4 weeks diabetic and $4.18 \pm 0.15 \mu\text{M}$ in insulin treated diabetic animals. Mathematical analysis was performed in Materials and methods. $P < 0.001$ for differences from control; $P < 0.01$ for differences from insulin treated diabetic group.

3.3. [^3H]Ouabain binding capacity

The basic characteristic of [^3H]ouabain binding to brain cortex microsomes from control, diabetic and diabetic insulin treated groups were assessed in a series of experiments. We defined relative [^3H]ouabain affinities over the concentration $2 \cdot 10^{-8}$ to 10^{-5} M to evaluate the binding sites and to determine the K_d values. In this concentration range Scatchard type plot gives two populations of binding sites with apparent dissociation constant in the control group ($K_{d1} = 62.4 \pm 5.9 \mu\text{mol}$ in the case of low affinity binding site and $K_{d2} = 112.3 \pm 14.8 \text{ nmol}$ in the case of high affinity binding sites). We did not investigate individually the high and very high affinity binding sites. Maxi-

Table 2

Ouabain sensitive Na^+/K^+ -ATPase, Mg^{2+} -ATPase and 5'-nucleotidase activities in cerebral cortex microsomes from controls, STZ-diabetic and insulin treated STZ-diabetic rats

Experimental group	C (n = 17)	D_2 (n = 5)	D_4 (n = 6)	D_2R (n = 5)	D_4R (n = 5)
Na^+/K^+ -ATPase ($\text{nmol P}_i \text{ min}^{-1} \text{ mg protein}^{-1}$)	589.2 ± 41	$500.8 \pm 55^*$	$371.5 \pm 36^*$	$561.1 \pm 47^{**}$	$614.4 \pm 6^{**}$
Mg^{2+} -ATPase ($\text{nmol P}_i \text{ min}^{-1} \text{ mg protein}^{-1}$)	255.6 ± 25	$319.2 \pm 34^*$	$325.2 \pm 29^*$	$236.8 \pm 31^{**}$	$243.3 \pm 21^{**}$
5'-Nucleotidase ($\text{nmol P}_i \text{ min}^{-1} \text{ mg protein}^{-1}$)	72.1 ± 8.1	65.9 ± 7.2	67.5 ± 7.15	69.5 ± 8.2	75.9 ± 5.2

Cerebral cortex microsomal Na^+/K^+ -ATPase, Mg^{2+} -ATPase and 5'-nucleotidase activities were determined using the methods described in Materials and methods. Values are means $\pm$ S.E., n = number of experiment. The activity of all of the enzymes investigated was the same in the 2, 4 and 6 weeks control groups, therefore C denotes all the age-matched control groups. Statistically significant ($P < 0.05$) differences between controls and either STZ-diabetic (D_2 and D_4) or insulin treated STZ-diabetic rats (D_2R , D_4R) are denoted by *. Statistically significant ($P < 0.05$) differences between STZ-diabetic (D_2 and D_4) and insulin treated STZ-diabetic (D_2R , D_4R) rats are denoted by **.

Table 3

Maximal ouabain binding capacity and K_d values of cerebral cortex microsomes from control, STZ-diabetic and insulin treated STZ-diabetic rats

Groups	Low affinity sites		High affinity sites	
	K_d (μ mol)	B_{max} (pmol/mg protein)	K_d (nmol)	B_{max} (pmol/mg protein)
C ($n = 16$)	62.4 ± 5.9	43.1 ± 4.3	112.3 ± 14.8	35 ± 4.3
D ₄ ($n = 7$)	65.6 ± 9.7	23.2 ± 3.9 *	117.7 ± 17.2	34.6 ± 3.6
D ₄ R ($n = 5$)	70.2 ± 8.3	38.8 ± 4.2 * * * * *	99.5 ± 18.3	43.9 ± 4.6 * * * * *

Maximal ouabain binding capacity (B_{max}) of brain cortex microsomes were determined as described in Materials and methods. Values are means $\pm$ S.E., n = number of experiments. The B_{max} and K_d values were the same in the 2, 4 and 6 weeks control groups, therefore C denotes all the age-matched control groups. Statistically significant ($P < 0.05$) differences between control and STZ-diabetic (D₄) are denoted by *. Statistically significant ($P < 0.05$) differences between control and insulin treated STZ-diabetic (D₄R) rats are denoted by **. Statistically significant ($P < 0.05$) differences between STZ-diabetic (D₄) and insulin treated STZ-diabetic (D₄R) rats are denoted by ***.

mal [³H]ouabain binding capacity of the brain tissue was reduced in STZ-induced diabetes compared to the control and to the diabetic insulin treated animals. Changes in ouabain binding capacity proved to be significant in four week diabetic animals. The reduction in ouabain binding was particularly pronounced at the low affinity ouabain binding site and was mostly reversed by insulin treatment, although the K_d values did not change significantly (Table 3). No significant changes were detected between the control and 2 weeks diabetic as well as 2 weeks diabetic insulin treated groups (data are not shown).

3.4. Na^+/K^+ -ATPase isoforms of brain cortex microsomes

The α subunit isoform composition of Na^+/K^+ -ATPase was altered in STZ-induced diabetes. Fig. 2A shows a representative Western blot of subunit isoforms, while the B part contains the densitometric analysis of all the data. After two weeks of streptozotocin treatment there was a significant decrease ($23 \pm 3.1\%$) in the amount of the α_1

isoform. At the same time the amount of α_2 isoform increased by $24 \pm 3.1\%$ and the level of α_3 isoform did not change significantly.

The isoform pattern of the 4 weeks diabetic samples differed from the 2 weeks diabetic and also from control. The relative amount of α_1 decreased to a greater extent after 4 weeks than in 2 weeks diabetes (61 ± 5.8 and $23 \pm 3.1\%$, respectively). The amount of α_2 was the same as in the control samples, but $24 \pm 3.1\%$ lower than in D₂ samples. The amount of α_3 was $20 \pm 2.8\%$ lower than in the control ones.

Two weeks insulin treatment of diabetic animals did not restore the original isoform pattern completely (D₂R, D₄R). The relative amount of α_1 isoform in diabetic insulin treated animals remained at the same level as in the untreated diabetic samples. The amount of α_2 decreased during 2 weeks insulin treatment to the same level as in the control. The main difference between 2 weeks diabetic animals with and without insulin treatment was found in the relative amount of the α_3 isoform. In D₂R samples the quantity of α_3 isoform increased by $39 \pm 3.1\%$ compared

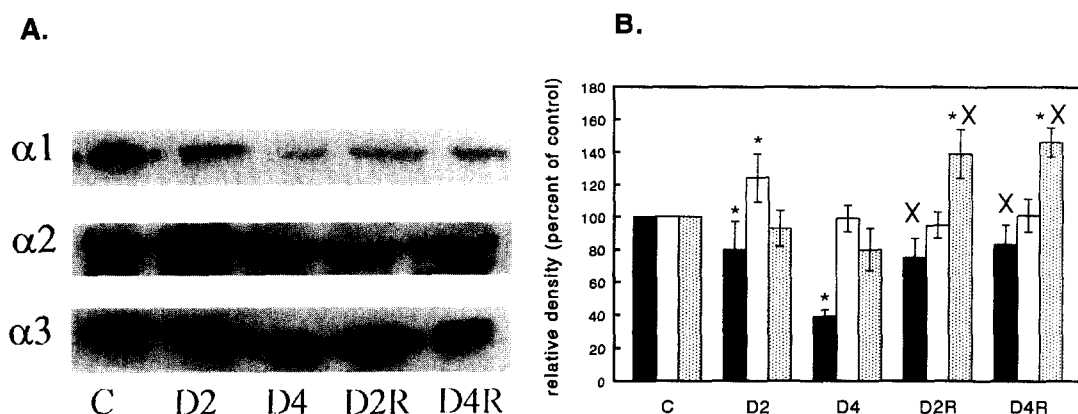


Fig. 2. Relative abundance of Na^+/K^+ -ATPase α subunit isoforms in cerebral cortex of control, STZ-diabetic and STZ-diabetic insulin treated rats. Microsomal membranes (50 μ g) of cerebral cortex from control (C), 2 weeks STZ-diabetic without (D₂) and with insulin treatment (D₂R), 4 weeks STZ-diabetic without (D₄) and with insulin treatment (D₄R) were subjected to SDS-PAGE. Relative abundances of α_1 , α_2 and α_3 were quantitated by immunoblotting using isoform specific antiserum and peroxidase conjugated antirabbit IgG as primary and secondary antibodies, respectively. (A) A representative photo of an ECL developed Western blot of subunit isoforms. (B) Relative densities of Na^+/K^+ -ATPase α subunit isoforms. Closed bars: α_1 , open bars: α_2 , dotted bars: α_3 . Data are means $\pm$ S.E. of 5 experiments. Statistically significant ($P < 0.05$) differences between controls and either STZ-diabetic or insulin treated STZ-diabetic rats are denoted by *. Statistically significant ($P < 0.05$) differences between STZ-diabetic and insulin treated STZ-diabetic rats are denoted by x.

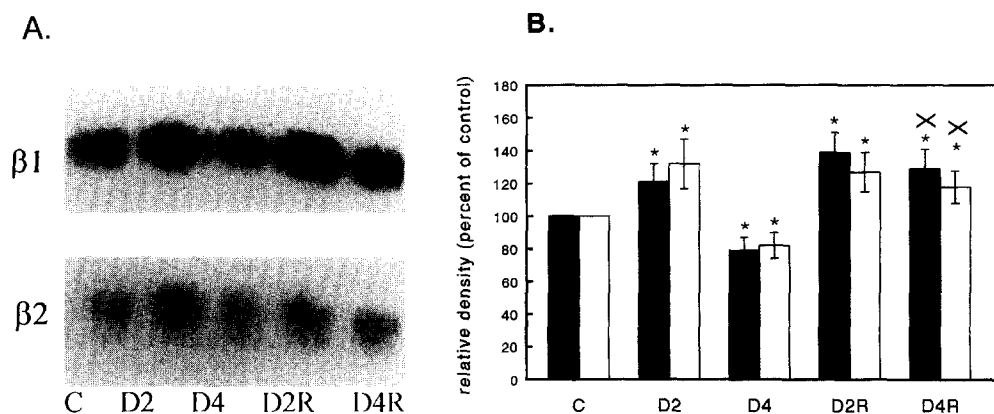


Fig. 3. Relative abundance of Na^+/K^+ -ATPase β subunit isoforms in cerebral cortex of control, STZ-diabetic and STZ-diabetic insulin treated rats. Microsomal membranes (50 μg) of cerebral cortex from control (C), 2 weeks STZ-diabetic without (D_2) and with insulin treatment (D_2R), 4 weeks STZ-diabetic without (D_4) and with insulin treatment (D_4R) were subjected to SDS-PAGE. Relative abundance of β_1 and β_2 were quantitated by immunoblotting using isoform specific antiserum and peroxidase conjugated antirabbit IgG as primary and secondary antibodies, respectively. (A) A representative photo of an ECL developed Western blot of β subunit isoforms. (B) Relative densities of Na^+/K^+ -ATPase β subunit isoforms. Closed bars: β_1 , open bars: β_2 . Data are means $\pm$ S.E. of 5 experiments. Statistically significant ($P < 0.05$) differences between controls and either STZ-diabetic or insulin treated STZ-diabetic rats are denoted by *. Statistically significant ($P < 0.05$) differences between STZ-diabetic and insulin treated STZ-diabetic rats are denoted by x.

to the age-matched control, and $46 \pm 5.1\%$ compared to the untreated diabetic group, respectively. The relative amount of all the isoforms changed similarly in D_4R than in D_2R rats. The amount of α_1 increased, but did not reached the control level. α_3 isoform was over the control (Fig. 2B).

A representative experiment and the densitometric analysis of Western blots of Na^+/K^+ -ATPase β subunit isoforms of control, diabetic and diabetic insulin treated animals can be seen in Fig. 3A and B, respectively. The quantity of both the β_1 and β_2 isoforms enhanced significantly after two weeks diabetes by 23 ± 3.1 and $32 \pm 4.1\%$ respectively. On the other hand, the level of both β_1 and β_2 decreased by nearly 20% compared to the control in 4 weeks diabetic animals. When we compared the relative amount of β isoforms in D_2 and D_4 diabetic animals, more than 40% decrease could be observed in the case of both β isoforms within a 2 weeks period of diabetic state, namely from the second to the fourth week of the disease. Insulin treatment did not have any significant effect on 2 weeks diabetic animals but restored the relative abundance of β subunits to the control level in the case of 4 weeks diabetes.

4. Discussion

It is well established that the Na^+/K^+ -ATPase activity in the peripheral nervous system is decreased in STZ-induced diabetes [36,37]. Less experimental data are available of diabetic alterations in the central nervous system. According to an earlier report brain Na^+/K^+ -ATPase activity decreases in diabetes by 5–30% depending on the region of the brain examined [22]. Our study shows that

the Na^+/K^+ -ATPase activity is significantly reduced (37%) in brain cortex microsomes in STZ-induced diabetes.

The reduction of diabetic Na^+/K^+ -ATPase activity does not derive from the altered purity of the microsomes prepared from the animals of different experimental groups, because the 5'-nucleotidase activity is the same in the preparations and Mg^{2+} -ATPase activity changes to the opposite direction than Na^+/K^+ -ATPase. A decrease in Na^+/K^+ -ATPase activity from 589.2 ± 41 to 371.5 ± 36 nmol P_i /mg protein per min can be observed in 4 weeks diabetic animals. If the molecular activity of Na^+/K^+ -ATPase is assumed 7500/min [38] this change corresponds to the decrease in Na^+/K^+ -pump concentration from 78.5 to 49.5 pmol/mg protein.

As reported earlier, the α subunit of Na^+/K^+ -ATPase is responsible for ouabain binding, and the different isoforms of α subunit show different ouabain binding affinities [39]. There is only one low affinity ouabain binding isoform (α_1) and two high affinity ouabain binding isoforms (α_2 and α_3), the latter two isoforms have very similar affinity constants for ouabain [4,6]. According to our results in diabetes we have got a reduction in ouabain binding capacity of the low affinity binding sites, which corresponds to a reduction in the level of the α_1 isoform. The high affinity binding sites do not change significantly. The diabetes induced reduction of low affinity [^3H]ouabain binding site from 43.1 ± 4.3 to 23.2 ± 3.9 pmol/mg protein is in the same range as the change in Na^+/K^+ -pump concentrations (78.5 and 49.5 pmol/mg protein in control and 4 weeks diabetic groups, respectively).

In contrast to skeletal muscle [17], in brain cortex the number of low affinity binding sites decreases in diabetes. The high affinity binding sites do not change significantly,

however we have not analyzed separately the high and the very high affinity binding sites. Insulin treatment resulted in a small enhancement of low affinity sites, while it caused a significant increase in high affinity binding sites, similarly to the results of Brodsky in brain synaptosomal preparation [23].

Similar conclusion can be drawn from the changes in the ouabain sensitivity of the enzyme after STZ-treatment (Fig. 1). The ouabain concentration producing 50% inhibition of Na^+/K^+ -ATPase activity was $8.34 \pm 0.19 \mu\text{M}$ in the case of the control and $2.84 \pm 0.15 \mu\text{M}$ in 4 weeks diabetic animals. This alteration was partly diminished by insulin treatment, the IC_{50} value enhanced to $4.18 \pm 0.15 \mu\text{M}$, but remained significantly different from the control. This phenomenon suggests the reduction of the less ouabain sensitive isoform of Na^+/K^+ -ATPase in diabetes. Insulin treatment partly restores original ouabain sensitivity of Na^+/K^+ -ATPase in brain cortex.

The question arises whether the changes in Na^+/K^+ -ATPase activity, ouabain sensitivity, and ouabain binding capacity in diabetes are due to alterations of biochemical properties of the enzyme or derive from the quantitative changes of the isoforms of the transport molecule. Data available on peripheral nerve tissue suggest that the reduction of Na^+/K^+ -ATPase activity is a consequence of a decrease of the enzyme protein [36,37].

To evaluate the expression of Na^+/K^+ -ATPase isoform, antisera specific for α_1 , α_2 , α_3 , β_1 and β_2 subunits were used to probe Western blots of cerebral cortex microsomes prepared from control, STZ treated diabetic and STZ treated insulin injected rats. A significant decrease was observed in the level of the diabetic α_1 isoform. This finding correlates with the enzyme activity and ouabain binding data. Change in the levels of diabetic α_1 isoform is in disagreement with the earlier idea, that the α_1 isoform functions as a basal Na^+/K^+ -ATPase and is not supposed to be hormonally affected [40]. However, there are other data showing that the synthesis and degradation rate of α_1 isoform are enhanced in the nervous system of spontaneously diabetic rats [41]. In contrast to other tissues, where α_2 β_1 isoform composition was regulated by insulin [17,21], our results show that both α_1 and α_3 as well as β_1 and β_2 isoforms change in diabetic brain and are affected by insulin. However, the amount of β subunit did not change parallel with the Na^+/K^+ -ATPase activity. This finding is in agreement with other data obtained on muscle [13].

The most important observations of the present study are: (1) changes in Na^+/K^+ -ATPase enzyme activity are correlated with the reduction of [^3H]ouabain binding capacity; (2) the change in enzyme activity and ouabain binding correlate with a reduction in the level of α_1 isoform detected by Western blot analysis; (3) there was no correlation between changes in Na^+/K^+ -ATPase activity and the amount of its β subunit; (4) there are more than one type of α (α_1 , α_3) and β (β_1 and β_2) isoforms

which can be affected in diabetes and by insulin administration.

The physiological significance of these alterations of brain cortex Na^+/K^+ -ATPase in diabetes remains to be clarified [42]. A variety of structural and biochemical alterations have been described in the central nervous system of diabetic patients and animals [43,44]. Since the α_1 isoform localizes to both glia cells and neurons, and α_3 can be detected only in neurons [8,9], our results suggest that the reduction of Na^+/K^+ -ATPase activity in diabetes may participate in changes of both neuron and glia cell functions.

The question whether the observed alteration in experimental diabetes is derived from the absence of insulin, from the relative overflow of glucagon and other hormones or from other metabolic disorders is not resolved in this study [45,46]. One possibility to explain the changes in diabetic Na^+/K^+ -ATPase function is that through the enhanced synthesis of sorbitol, the *myo*-inositol turnover is damaged, resulting in the inactivation of Na^+/K^+ -ATPase [47–49]. Several reports show that in rat brain in streptozotocin induced diabetes there is a high *myo*-inositol and glucose level [50]. According to other reports there is a defect in PMA and DAG responsive Na^+/K^+ -ATPase in peripheral nerve of diabetic animals, and this corresponds to an altered *myo*-inositol turnover [47].

Another explanation is that insulin exerts a rapid effect on gene transcription through upstream insulin response elements (IRE) [51]. However, there are also data showing a prolonged latency of changes in the synthesis of Na^+/K^+ -ATPase catalytic subunit after insulin administration [41], and these results suggest that the regulation of Na^+/K^+ -ATPase activity in diabetes does not directly take place through insulin response elements. Besides changes in the transcription of Na^+/K^+ -ATPase we also have to consider the possible changes in the translation, translocation and degradation of the enzyme in diabetes. The investigation of these aspects is in progress in our laboratory.

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