Cerebral metabolism of glucose, one of the determinants of tissue ATP level, is crucial for central nervous system function. The activity of P-type pumps, namely Na⁺, K⁺ - ATPase, Ca²⁺ - ATPase and Mg²⁺ - ATPase were examined in brain synaptosomes of 5 - day, 3 - month and 18 - month - old rats to determine if changes in enzyme activity related to aging are potentially associated with alterations in glucose homeostasis.

Activities of all the ATPases studied in isolated brain synaptosomes were expressed in µmol of P₅₀ liberated from ATP by 1 mg of synaptosome protein during one hour. Serum glucose concentration was measured by the glucose oxidase method and insulin level was estimated by the RIA. Our results demonstrate that 18 - month - old rats are characterized by hyperglycemia and hyperinsulinemia. Their serum glucose concentration was significantly increased approx. 62.3% and 135.8 % as compared to 3 - month - old rats and 5 - day, newborn rats, respectively. An enormous increase in serum insulin concentration in the old, hyperglycemic rats was observed concomitantly. As a result of these changes the insulin - to - glucose ratio in the old rats was greatly increased approx. (270% and 230%) compared to young, mature and newborn rats. Hyperglycemia and hyperinsulinemia occurring in the old rats, had a different impact on activities of the ATPases tested. Our results have revealed that Na⁺, K⁺ - ATPase activity remains almost unchanged with age, the activity of Ca²⁺ - ATPase decreases, whereas that of Mg²⁺ - ATPase increases significantly in old, insulin resistant rats. In conclusion it seems that changes in activity of different P-type pumps may differ with aging and that adaptation of specific ATPases to internal environment alterations is not identical.

Key words: Na⁺, K⁺-ATPase, Ca²⁺-ATPase, Mg²⁺-ATPase, brain synaptosomes, hyperglycemia, hyperinsulinemia, aging.
INTRODUCTION

It is generally accepted that cerebral metabolism of glucose, which appears one of the determinants of tissue ATP level, is crucial for central nervous system (CNS) activity. Na\(^+\), K\(^+\) - stimulated ATPase (E.C. 3.6.1.3.) is known to be involved in the maintenance of sodium and potassium gradients across plasma membranes at the expense of ATP hydrolysis, with very high activity in electrically excitable tissues. The activity of this enzyme has been found to increase in the developing rat brain and to decrease during aging (1).

A study on the effect of aging on Na\(^+\), K\(^+\) - ATPase activity in crude synaptosomal fractions from the rat brain parietal cortex, hippocampus and striatum revealed a progressive decline in enzyme activity from 12 months to 24 months of age (2). Zaidi and co-workers (3) found that also Ca\(^{2+}\)-ATPase activity in F344/BFN1 rat brain synaptic plasma membranes exhibited a progressive age-dependent decrease which might be accounted for some loss of PMCA from the membranes and for age-related structural changes of calmodulin.

By contrast, Kennedy et al., (4) showed that aging-related changes in energy utilization demonstrated in brain slices and homogenates were not associated with alterations in the Na\(^+\), K\(^+\)-ATPase enzyme system. Moreover as shown by Mooradian et al., (5) sodium - potassium or magnesium ATPase activity was not altered with aging or diabetes but exhibited reduced inhibition by NEM (N-ethylmaleimide), the known (H\(^+\)) - ATPase inhibitor.

Insulin resistance in old, compared with young, humans and animals has been well documented to lead finally to hyperglycemia and hyperinsulinemia.

Insulin cannot be synthesized by the central nervous system and is transported from the blood to the brain acting as a multiple functioning molecule, i.e. growth factor during development and modulator of electrical activity via phosphorylation of voltage-gated ion channels (6). According to Baura et al., (7), the delivery of plasma insulin into the CNS is saturable and is facilitated by an insulin - receptor mediated transport process. The results of Zai and Piantanelli (8) showed a significant decrease in the number and increase in affinity of insulin binding sites in brains of old animals that may be responsible for alterations in central insulin action, among them neuromodulation.

As shown by Aragno et al., the hyperglycemia-induced damage of central nervous system is brought about by a detrimental effect of chronic hyperglycemia on the integrity of synaptic membranes due to overproduction of free radicals (9).

The aim of our study was to elucidate if there is any relationship between hyperglycemia and hyperinsulinemia found in old animals and changes in activity of several ATPases, including Na\(^+\), K\(^-\)-ATPase, Ca\(^{2+}\)-ATPase and Mg\(^{2+}\)-ATPase in synaptosomes isolated from rat brain during aging. Our review of the literature revealed no study on the activity of ATPases in the CNS along with blood glucose and insulin concentrations during aging, performed under identical conditions at the same time.
MATERIAL AND METHODS

The experiments were performed on male Wistar rats and conducted following the experimental protocol approved by the Committee for Research and Animal Ethics of the University of Medical Sciences in Poznań. The animals were housed in cages at normal room temperature and maintained on standard laboratory chow (LSM) with free access to food and water. All the experiments were carried out between 9-11 a.m. The rats were sacrificed by decapitation and after complete exsanguination brains were removed immediately and used for isolation of synaptosomes. In the present study brain synaptosomes were isolated from three groups of rats: newborn (5-day-old), young (3-month-old) and old (18-month) animals.

The tissue was homogenized in 20 vol of 0.32 M sucrose at 4°C.

Preparation of synaptosomes

Synaptosomes were isolated from rat brains by the method of Lin and Way (10, 11). Briefly, a whole-brain homogenates in 0.32 M sucrose - 5 mM HEPES buffer, pH 7.5, were centrifuged at 1000 x g for 10 min. The resultant supernatant was centrifuged for 30 minutes at 17 000 x g to obtain the crude synaptosomal fraction. The fraction, washed twice and suspended in 0.32 M sucrose HEPES - buffer was subjected to Ficoll gradient centrifugation (63 000 x g for 1 hour). Synaptosomes collected at the interfaces between Ficoll layers (7.5% and 12% in sucrose - HEPES solution) were pelleted and suspended in 0.32 M sucrose solution for enzyme assays within 2 hours. All the procedures described above were carried out at 4°C.

ATPases Assay

The Na⁺, K⁺-ATPase activity was estimated by the method of Muszbek et al., (12). The enzyme activity was determined by measuring the amount of inorganic phosphate (Pᵢ) liberated from ATP during the incubation of synaptosomal fraction. The reaction mixture contained 100 mM NaCl, 20 mM KCl, 2mM ATP (disodium salt), 30mM Tris-HCl buffer (pH 7.4) and the synaptosomes (50 µg of protein) in a final volume of 1 ml. After a 10-min. preincubation at 37°C to allow the ouabain (1.5 mM) to react with the ATP-ase, the reaction was initiated by addition of ATP, and terminated after 15 min. incubation by addition of 500 µl of 15% (w/v) trichloroacetic acid. The released inorganic phosphate was assayed by the spectrophotometric method of Goldberg (13). Na⁺, K⁺-ATPase activity was calculated from the difference between amounts of inorganic phosphate found after incubation in the absence and presence of 1.5 M ouabain.

The Mg²⁺-ATPase activity and Ca²⁺-ATPase activity were measured by the method of Lin and Way (11). The assay medium (1 ml) contained 50 mM imidazole-HCl buffer (pH 7.5), 0.4 mM MgCl₂ or 0.4 mM CaCl₂, 2 mM ATP and the synaptosomal fraction (50 µg protein) suspended in 0.32 M sucrose. After 15 minutes of incubation at 37°C the reaction was stopped by adding 1.2 ml of ice cold 10% TCA. The Ca²⁺ - ATPase (or Mg²⁺ - ATPase) activity represented the difference between the enzyme activity in the presence and absence of Ca²⁺ (or Mg²⁺) cations.

Activities of all the ATPases tested, were expressed in µmol Pᵢ liberated from ATP by 1 mg of synaptosome protein during one hour (µmol Pᵢ/mg protein/hour). Protein concentration was assayed by the method of Lowry et al., (14) using bovine serum albumin as a standard.

Other analyses

Serum insulin was measured by a standard radioimmunoassay (RIA) using kits for rat insulin (Linco, Research Inc. USA). Glucose was determined by the glucose oxidase method (Sigma).
Student's t-test was used for statistical evaluation and differences were considered to be significant at a level of p<0.05.

RESULTS

The results summarized in Tab. 1 demonstrate that 18-month-old rats, weighing 595 - 630 g, can be regarded as hyperglycemic and hyperinsulinemic animals. Their serum glucose concentration was significantly increased by about 62.3% and 135.8% as compared to 3-month-old rats (weighing 220 - 250 g) and 5-day-newborn rats (5.8 - 6.0 g), respectively. As shown in the same table, an enormous increase in serum insulin concentration was observed in the old, hyperglycemic rats in comparison to insulin levels measured in serum of newborn and young, mature rats. As a result of these changes the insulin-to-glucose ratio in the old rats was greatly increased, by about 270% as compared to young rats, and by about 230% in comparison to the newborns. These findings confirm that the 18-month-old rats, used in our experiments, are characterized by an age-dependent insulin resistance.

Tab. 1. Serum glucose and insulin concentrations and the insulin/glucose ratio in newborn (5 days), young (3 months) and old (18 months) rats.

<table>
<thead>
<tr>
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<th>NEWBORN RATS</th>
<th>YOUNG RATS</th>
<th>OLD RATS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol/l)</td>
<td>4.35 ± 0.50</td>
<td>6.32 ± 0.30</td>
<td>10.26 ± 0.30</td>
</tr>
<tr>
<td>Insulin (IRI) µU/ml</td>
<td>14.88 ± 3.71</td>
<td>19.00 ± 1.91</td>
<td>114.06 ± 5.59</td>
</tr>
<tr>
<td>IRI/glucose µU/mol</td>
<td>3.35 ± 0.66</td>
<td>3.01 ± 0.98</td>
<td>11.16 ± 0.56</td>
</tr>
</tbody>
</table>

Results are presented as mean ± SEM (n = 10)
*Significantly different from newborn rats (p < 0.01)

Tab. 2. Activities of Na⁺, K⁺, Mg²⁺ and Ca²⁺ - ATPases (expressed in µmol P, x mg of protein⁻¹ x hour⁻¹) in synaptosomes isolated from brains of newborn (5 days), young (3 months) and old (18 months) rats.

<table>
<thead>
<tr>
<th></th>
<th>NEWBORN RATS</th>
<th>YOUNG RATS</th>
<th>OLD RATS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺, K⁺ - ATPase</td>
<td>6.88 ± 2.08</td>
<td>15.80 ± 1.30</td>
<td>13.50 ± 1.20</td>
</tr>
<tr>
<td>Ca²⁺ - ATPase</td>
<td>6.67 ± 2.04</td>
<td>10.40 ± 0.80</td>
<td>7.40 ± 0.60</td>
</tr>
<tr>
<td>Mg²⁺ - ATPase</td>
<td>2.31 ± 0.90</td>
<td>8.30 ± 0.90</td>
<td>11.60 ± 0.80</td>
</tr>
</tbody>
</table>

Results are presented as mean ± SEM (n = 10)
*Significantly different from newborn rats (p < 0.001)
**Significantly different from young rats (p < 0.001)
Tab. 2 activities of Na\(^+\), K\(^+\), Ca\(^{2+}\) and Mg\(^{2+}\) - ATPases (expressed in µmol P\(_i\) x mg protein x hour) in synaptosomes isolated from brains of newborn (5-day-old) rats (A) and 18-month-old rats (B) expressed in % of control activity. Values obtained from young mature rats (3-month-old) were accepted as control (100%).

As depicted in Fig. 1 activities of Na\(^+\), K\(^+\), Ca\(^{2+}\) and Mg\(^{2+}\)-ATPases (expressed in µmol P\(_i\) x mg protein x hour) in synaptosomes isolated from brains of newborn, young and old rats differ among the groups tested. The activities of Na\(^+\), K\(^+\)-ATPase and Ca\(^{2+}\)-ATPase were found to be the highest in brain synaptosomes isolated from young, mature rats (3-month-old) whereas surprisingly, the activity of Mg\(^{2+}\)-ATPase was significantly elevated in the group of 18-month-old rats.

When values of ATPase activities estimated in young, mature rats, commonly used as experimental animals, were accepted as 100% (Fig. 1) it was found that greater relative differences can be observed in the group of newborn rats than in the old rat group. These results may also suggest that changes in activities of all synaptosomal ATPases tested, could provide an index of brain maturity not only in the developing rat brain, as shown previously, but also in the postnatal period. On the contrary, relative differences in activities of Ca\(^{2+}\)-ATPase and Na\(^+\), K\(^+\)-ATPase in synaptosomes isolated from brains of the old, 18-month animals were smaller or even insignificant, except of those concerning Mg\(^{2+}\)-ATPase activity that appeared significantly elevated.
DISCUSSION

It has been proved that either glycemia per se or glucose metabolites are responsible for the blood-brain barrier abnormality which occurs in diabetes. Knudsen and Jacobsen (15) suggest that the specific decrease of sodium permeability could be the result of glucose-mediated inhibition of the Na⁺, K⁺-ATPase localized at the blood-brain barrier. However in some conditions hyperglycemia can be beneficial for the organism. For example data of Ekholm et al., (16) indicate that hyperglycemia retards the loss of ion homeostasis by leading to production of additional ATP supporting Na⁺, K⁺-driven ATPase activity during ischemia.

In the present study we have shown that serum glucose concentration was significantly increased in old, 18-month rats as compared with the newborn animals. These changes were accompanied by an increase of immunoreactive serum insulin concentration in old rats. As a result of these changes IRI/glucose ratio in the old rats was greatly elevated obviously showing insulin resistance. Hyperglycemia and hyperinsulinemia observed in our study had a different impact on activities of the ATPases tested. There are striking differences among Na⁺, K⁺-ATPase, Ca²⁺-ATPase and Mg²⁺-ATPase in the synaptosomal fraction isolated from brains of old hyperglycemic animals. Our results revealed that Na⁺, K⁺-ATPase activity remains almost unchanged with age, the activity of Mg²⁺-ATPase increases significantly whereas activity of Ca²⁺-ATPase decreases. The changes in the latter enzyme can be accounted for the hyperglycemia found in old rats, that in turn may lead to the enzyme protein glycation as shown by Janicki et al., (17).

Another pathway, in addition to generation of free radicals that is involved in age-related cellular degeneration is the accumulation of advanced glycosylation end-products (AGE). Under in vivo conditions AGE formation depends mainly on the blood glucose concentration that has been proved to be elevated with age (18). There is a growing number of data showing that hyperglycemia may inhibit the activity of several enzymes due to enzyme protein glycation (19,20).

It has been shown that also experimental diabetes is associated with reduction of activity of P-type pumps, e.g. Na⁺, K⁺-ATPase in the central nervous system (21). In contrast, the marked hyperglycemia caused by the administration of 2-DG to satiated rats was associated with significant increase in Na⁺, K⁺-ATPase activity and in [3 H]-ouabain and [3 H]-mazindol binding to hypothalamic sites which are functionally coupled in their response to circulating glucose (22). Plasma membrane Ca²⁺-ATPase (PMCA), a regulator of intracellular calcium, is known to be an enzyme, the activity of which undergoes early developmental changes in rat brain as a function of its maturity (23).

According to Zaidi and Michaelis, (24) PMCA appears to be very sensitive to the inhibitory effect of reactive oxygen species (ROS) due to the age dependent oxidative modification of PMCA and the related chronic oxidative stress.
Alterations in the capacity to maintain normal calcium homeostasis have been suggested to underlie the reduced cellular function bound with the aging process. It seems likely that in the brain, multiple methionines within the calmodulin molecule become oxidized to methionine sulfoxides, resulting in an inability to activate a range of target proteins, including plasma membrane Ca\(^{2+}\)-ATPase. (25).

Recent evidence indicates, that chronic hyperglycemia may inhibit plasma membrane Ca\(^{2+}\)-ATPase (PMCA) in cells of various tissues. The P-type plasma membrane PMCA, a regulator of cytosolic Ca\(^{2+}\) has a diminished activity in erythrocyte and renal cortical cell membranes in uncontrolled diabetes.

As shown by Janicki et al., (17) PMCA pumping activity in SPM vesicles prepared from the cerebra of hyperglycemic rats was depressed by about 8.4%, compared to control normoglycemic rats. The brain synaptic PCMA inhibition was accompanied by glycation of hemoglobin in rats with streptozocin-induced diabetes. In addition, PCMA activity in synaptic plasma membranes from normoglycemic rats was inhibited by prior incubation with glucose. This may indicate that hyperglycemia inhibits brain Ca\(^{2+}\)-ATPase by glycation, particularly because brain glucose concentration has been shown to be increased ten-fold in diabetic rats.

Our present study reveals that activity of Ca\(^{2+}\)-ATPase in synaptosomes prepared from the brains of 18 - month - old rats decreased by about 28.8% as compared to the activity of the enzyme in brains of young, mature rats (3-month-old). These changes in PCMA activity can be accounted for the hyperglycemia found in old rats, that in turn may generate formation of reactive oxygen species and/or lead to glycation, causing damage of the enzyme protein.

ATPase II, a vanadate-sensitive and phosphatidylserine-dependent Mg\(^{2+}\)-ATPase, is a member of a subfamily of P-type ATPases and is presumably responsible for aminophospholipid translocation activity in eukaryotic cells. (26). The aminophospholipid translocation activity plays an important physiological role in the maintenance of membrane phospholipid asymmetry that is observed not only in plasma membranes but also in membranes of other cellular organelles.

As shown by Nedeljkovic and co-workers (27) Mg\(^{2+}\)-ATPase is not uniformly distributed and differs in respect to affinity for ATP in rat brain regions. It has been confirmed that the enzyme is activated by millimolar concentrations of Mg\(^{2+}\) and that it cannot be effectively inhibited by known ATPase inhibitors.

Comparison of Na\(^{+}\), K\(^{+}\)-ATPase and Mg\(^{2+}\)-ATPase activities in the synaptic plasma membrane from various regions of rat brain reveals that moderate hypoxia increases the activity of synaptosomal Mg\(^{2+}\)-ATPase whereas activities of both Ca\(^{2+}\)-ATPase and Na\(^{+}\), K\(^{+}\)-ATPase are decreased (28).

In our present study it was only the Mg\(^{2+}\)-ATPase, the activity of which has been shown to become gradually higher with age. As compared to newborn rats the activity of this enzyme was 5-fold higher in old animals, and this may confirm
its crucial role in maintaining the membrane phospholipid asymmetry as well as in the process of signal transduction via controlling ATP concentration.

It thus may be concluded that changes in activity of different P-type ATPases, including Na\(^+\), K\(^-\)-ATPase, Ca\(^{2+}\)-ATPase and Mg\(^{2+}\)-ATPase, may differ even in the same conditions and that their adaptation to internal and/or external environmental changes is not identical.

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