Effect of Handling on ATP Utilization of Cerebral Na,K-ATPase in rats with Trimethyltin-Induced Neurodegeneration.

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Research Article

Abstract

Previously it was shown that for reduction of anxiety and stress of experimental animals, preventive handling seems to be one of the most effective methods. The present study was oriented on Na,K-ATPase, a key enzyme for maintaining proper concentrations of intracellular sodium and potassium ions. Malfunction of this enzyme has an essential role in the development of neurodegenerative diseases. It is known that this enzyme requires approximately 50% of the energy available to the brain. Therefore in the present study utilization of the energy source ATP by Na,K-ATPase in the frontal cerebral cortex, using the method of enzyme kinetics was investigated. As a model of neurodegeneration treatment with Trimethyltin (TMT) was applied. Daily handling (10 min/day) of healthy rats and rats suffering neurodegeneration induced by administration of TMT in a dose of (7.5 mg/kg), at postnatal days 60-102 altered the expression of catalytic subunits of Na,K-ATPase as well as kinetic properties of this enzyme in frontal cerebral cortex of adult male Wistar rats. Everyday handling of rats, beside the previously published beneficial effect on spatial memory was accompanied by improved maintenance of sodium homeeostasis in frontal cortex of brains. The key system responsible for this proces, the Na,K-ATPase was able to utilize better the energy substrate ATP. In rats with TMT-induced neurodegeneration handling promoted the expresion of α2 isoform of the enzyme which is typical for glial cells. In healthy rats the handling was followed by increased expression α3 subunit which is typical for neurons.

Introduction

In the brain the intracellular homeostasis of sodium and potassium ions is essential for regulating neuronal excitability and for multiple cellular functions. In maintenance of proper gradient of sodium ions across the cell surface membrane, the pivotal role is ascribed to Na,K-ATPase, called also as sodium pump. This enzyme located in plasma membrane exchanges 3 intracellular ions of sodium for 2 extracellular potassium ions consuming the energy of one molecule of ATP [1, 2]. The enzyme consists of the catalytic α-subunit insuring the ATP hydrolysis with transmembrane transport of ions and the β-subunit which is responsible for the correct embedding of the sodium pump into the surface membrane. In the brain three isoforms of catalytic α-subunit (α1- α3) are generally recognized [3]. Malfunction of Na,K-ATPase in brain is probably involved in development of several neurological disorders. Post mortem studies of the Na,K-ATPase showed alterations in brains of patients suffering from various encephalopathies, like Alzheimer's disease [4, 5], epilepsy [6] or bipolar disease [7]. For getting deeper insight into the molecular basis of neurodegeneration various experimental animal models were introduced. Mutation of the α3-subunit contributed to development of rapid-onset dystonia parkinsonism in mice [8]. In the experimental model of aluminium chloride-induced Alzheimer's disease abnormal increase of Na,K-ATPase activity in hippocampus of rats was observed [9]. On the other side, decreased activity of the enzyme was connected to experimentally induced epilepsy in rodents [10, 11]. In another model of neurodegeneration, induced by administration of Trimethyltin (TMT) the decrease of Na,K-ATPase activity may be implicated as documented by inhibition of the enzyme in synaptosomes from the forebrain of mice when measuring the direct effect of TMT in vitro [12]. When studying the influence of

various interventions on experimental animals for reduction of anxiety and stress, preventive handling seems to be one of the most effective methods [13–17]. Positive effects of handling on the behavior of experimental animals were accompanied with large variability in the response of cerebral Na,K-ATPase, from decrease to increase of the enzyme activity [18–20]. The functionality of the Na,K-ATPase is very important also for maintaining of energy in the brain. It is known that this enzyme requires approximately 50% of the energy available to the brain [21]. Therefore the present study was oriented to estimation of ATP utilization by Na,K-ATPase in the brain, using the method of enzyme kinetics as an experimental tool.

Materials And Methods

Animals

Experimental animals (male Wistar rats) in age of 7 weeks were obtained from the breeding station Dobrá Voda (Slovak Republic, reg. No. SK CH 24016) and maintained in our animal care facility (12 h/12 h light/dark cycle with free access to food and water, relative humidity of 55 ± 10% and the temperature 22 ± 2°C). After 10 days lasting quarantine, rats were randomly divided into four experimental groups and kept (by fours) in cages with wood shavings and with environment enriched with paper rolls. All procedures with animals were performed in compliance with principles of laboratory animal care issued by EU Directive 2010/63/EU for animal experiments, proved and controlled by the State Veterinary and Food Administration of Slovak Republic (No. 4030/10–221) and with agreement of the Ethical Committee of the Centre of Experimental Medicine, Slovak Academy of Sciences.

Experimental groups

The animals (n = 32) were randomly divided into four experimental groups: CN group: non-handled control rats (n = 8); CH group: handled control rats (n = 8); TN group: non-handled with TMT (n = 8); TH group: handled rats with TMT (n = 8).

TMT administration

At 12 weeks of age, TMT rats received a single *i.p.* dose of TMT chloride (Sigma-Aldrich; 7.5 mg/kg dissolved in 0.1% DMSO in the volume of 0.2 ml/100g of rat body weight). Control rats received a single *i.p.* dose of saline (with 0.1% DMSO). Adult rats weighed 285 ± 5 g at the time of TMT/saline administration.

Handling process

Six weeks lasting handling (10 min/daily) was performed during a period of postpartum days 60–102, as it was described previously [17]. First four weeks the handling was applied prior to any treatment of rats and was prolonged for other 2 weeks after TMT or vehicle administration. The same person manipulated the rats in the van, took them to the hands, laid them on chest or let them climb out of the van allowing to rats explore the surroundings of the table. Non-handled rats were placed in separated room and without the possibility of getting out of the cage. After six weeks of handling procedure, the animals were left for

additional two weeks without any experimental manipulation. Terminating the experiment animals were anesthetized by ether and unconscious were sacrificed by decapitation. Frontal cortexes of the brains were promptly removed, rapidly rinsed with ice-cold physiological saline, immediately frozen in liquid nitrogen and stored at -60°C until used.

Preparation of tissue fraction for electrophoresis and immunochemical Western blot analysis

Brain frontal cortex samples were re-suspended in homogenizing buffer (50 mmol l-1 Tris-HCl, 250 mmol l-1 sucrose, 1 mmol l-1 dithiothreitol, 1 mmol l-1 phenylmethylsulfonylfluoride; pH 7.4) and homogenized with piston-hand homogenizer in ice, followed by ultrasonic homogenization (UP100H, Hielscher Ultrasonics, Germany) in repetitions 3 x 5 sec in ice. The homogenates were centrifuged (Eppendorf centrifuge 5804 R, Eppendorf, Germany) at 800 x g for 5 min at 4°C. After that, pellets were discarded and the supernatants were centrifuged again (16 100 x g, 30 min., 4°C). Following this second centrifugation, the supernatants were discarded and the pellets were re-suspended in homogenizing buffer complemented with 0.2 % Triton X-100 and centrifuged again (16 100 x g, 5 min., 4°C). The Triton X-100 soluble supernatants represented the membrane fraction. The protein concentrations were estimated by the method of Bradford [22].

Prepared samples of membrane fractions were plated and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the equivalent volume corresponding to 30 μg protein per lane. Separated proteins were transferred from gel to a nitrocellulose membrane overnight at 4°C (Biorad Mini-PROTEAN, USA). The quality of the transfer was controlled by Ponceau S staining of nitrocellulose membranes after the transfer.

Specific primary antibodies against α1 (mouse monoclonal antibody, Sigma, A-277, in dilution 1: 250, RRID:AB_258030) α2 (polyclonal antibody, Millipore, # 07-674, in dilution 1:1000, RRID:AB_390164), α3 (rabbit polyclonal antibody, Millipore, # 06-172, in dilution 1: 1000, RRID:AB_11213338) subunits of Na,K-ATPase were used for the primary immunodetection. As the secondary antibodies were used peroxidase-labeled anti-rabbit (Cell Signaling, #7074S, in dilution 1:1000, RRID:AB_2099233) and anti-mouse (Cell Signaling, #7076S, in dilution 1:1000, RRID:AB_330924) immunoglubulins. Bound antibodies were detected by the enhanced chemiluminescence detection method (Amersham Imager 600, United Kingdom). Densitometrical quantification of protein levels was performed by comparison to loading control beta-actin (mouse monoclonal antibody, Abcam, ab6276, in dilution 1: 1000, RRID:AB_2223210) using a ImageJ software (RRID:SCR_003070).

Estimation of ATP utilization by Na,K-ATPase using the method of enzyme kinetics

The plasmalemmal membrane fractions from cerebral cortexes and cerebellums were isolated according to Jørgensen [23]. Amount of proteins was determined by the procedure of Lowry [24] using bovine serum albumin as a standard. All assays of the Na,K-ATPase activity were performed at 37 ºC using 20 μg·ml^{-1}

of protein from homogenate in an assay buffer containing (in mmol·l^{-1}): 4 MgCl$_2$, 100 NaCl, 10 KCl and 50 TRIS (pH = 7.4). The samples were pre-incubated for 20 min in substrate-free medium. The enzyme reaction was initiated by addition of increasing amount of TRIS-ATP in the range of 0.16−8.00 mmol·l^{-1}. The reaction was stopped after 20 min by adding 12% ice−cold trichloracetic acid. The inorganic phosphorus generated from ATP hydrolysis was estimated according to Taussky and Shorr [25]. In order to establish the enzyme activity, specific inhibitor of the Na,K-ATPase ouabain in concentration of 3 mmol·l^{-1} was applied. Kinetic parameters V_{max} and K_m were evaluated according to Michaelis-Menten equation from the data by direct nonlinear regression. The parameter V_{max} represents the maximal velocity of enzyme reaction, K_m value refers to the concentration of energy substrate ATP necessary for half maximal activation of the enzyme.

Statistical analysis

All investigated parameters are expressed as means ± standard errors of mean (SEM). Two-way ANOVA and Holm-Sidak tests were used for statistical analysis in Sigma plot software (RRID:SCR_003210). The differences were considered to be significant when the *p*-value was less than 0.05.

Results

Studying the influence of TMT administration *in vivo* on the Na,K-ATPase in brain homogenate by activation of the enzyme with increasing concentrations of ATP we observed higher activities in the whole concentration range in non-handled TMT rats (TN) when comparing to non-handled controls (CN) (Fig. 1). The difference decreased stepwise with increasing concentrations of substrate from 28% observed in the presence of 0.16 mmol.l^{-1} of ATP, to 8% in the presence of 8 mmol.l^{-1}. Analysis of the data according to Michaelis-Menten equation resulted in significantly lowered K_m value by 22% in the TN group without significant changes in the V_{max} value (Fig. 2).

Studying the influence of handling on the Na,K-ATPase in control rats without TMT-treatment we observed higher activities especially in the lower concentration range of ATP in handled control rats (CH) when comparing to non-handled controls (CN) (Fig. 1). The highest increase amounting 48% was observed in the presence of 0.16 mmol.l^{-1} of ATP and it decreased stepwise with increasing concentrations of substrate. In the presence of ATP above 2.4 mmol.l^{-1} of ATP the enzyme activity was similar in both control groups without TMT. Analysis of the data resulted in significantly lower K_m value by 47% in the CH, as compared to CN group without significant changes in the V_{max} value (Fig. 2).

In animals treated with TMT the handling induced also increase of Na,K-ATPase activity in the lower concentration range of ATP when comparing to non-handled TMT-treated rats (TN) (Fig. 1). The highest increase amounted 25% in the presence of 0.16 mmol.l^{-1} of ATP and it decreased stepwise with increasing concentrations of substrate. In the presence of ATP above 0.8 mmol.l^{-1} of ATP the enzyme activity was similar as compared to non-handled group with TMT. Analysis of the data resulted in

significantly lower K_m value by 37% in the TH, as compared to TN group without significant changes in the V_{max} value (Fig. 2).

Investigation of the protein expression of catalytic α-subunits of Na,K-ATPase by Western blot showed that α1, α2 and α3 subunits were affected variously in the frontal cortex of handled or non-handled, and TMT or saline affected rats (Fig. 3). While the expression of α1 subunit of Na,K-ATPase remained unaffected after TMT treatment as well as after handling, the expression of α2 subunit was higher after regular daily handling of experimental animals in rats subjected to TMT as compared to non-handled rats with TMT. The TMT did not significantly affect the expression of α2 subunit in non-handled control animals. Concerning the expression of α3 subunit, significant influence of handling was observed resulting in higher protein expression in the CH control group when compared to the CN group (Table 1).

Table 1
Relative abundance of α1−3 subunits of Na,K-ATPase from frontal cortex in control non-handled rats (CN), in control handled rats (CH), in non-handled rats with TMT administration (TN) and in handled rats with TMT administration (TH). Data represent mean ± SEM, n = 5 in each group. Significance a: $p < 0.05$ vs. CH and TN, b: $p < 0.05$ vs. CN.

	CN control nonhandled	CH control handled	TN TMT nonhandled	TH TMT handled
α1	100 ± 11.3	109.1 ± 10.7	90.2 ± 5.5	116.5 ± 13.6
α2	100 ± 5.8	114.5 ± 11.2	98.2 ± 5.3	182.3 ± 18.7 [a]
α3	100 ± 3.5	135.4 ± 13.2 [b]	107.9 ± 11.8	124.5 ± 8.1

Discussion

Studies devoted to various neurodegenerative disorders pointed out to malfunction of certain isoforms of the Na,K-ATPase in the brain. Due to the presence of 3 various isoforms of the catalytic α-subunit in the brain tissue the data obtained by our investigation of enzyme kinetics represent the cumulative activity of all 3 isoforms of the enzyme. Previously it was documented that administration of TMT to rats induced neurodegeneration [26]. By *in vitro* studies it was shown that TMT was able to inhibit the Na,K-ATPase in synaptosomes from the forebrain of mice [12] indicating that in neurotoxic action of trimethyltin (TMT) this enzyme might be involved. In the present study we tried to broaden the information concerning the molecular principles of expected alterations of Na,K-ATPase *in vivo* in rats, four weeks after administration of TMT. Our investigations using measurements of enzyme kinetics showing higher Na,K-ATPase activities in the lower concentration range of ATP indicate better utilization of the energy substrate confirmed also by lowered value of K_m in TMT-treated non-handled animals when compared to non-handled healthy controls. The number of active Na,K-ATPase molecules remained unchanged as indicated by similar values of V_{max} in TMT and the control group, both without handling. So the effect of TMT on the Na,K-ATPase from frontal cortex seems to be different when measured *in vitro* and *in vivo*. Our measurement four weeks after *in vivo* administration of TMT showed higher effect in the presence of

physiologically relevant concentrations of ATP (0.16–0.80 mmol·l⁻¹), while the *in vitro* measurements of Costa [12] were done at higher concentration (2 mmol·l⁻¹). Another important fact probably contributing to the difference between *in vivo* and *in vitro* experiments is the probably reduced presence of TMT due to urinary excretion of TMT during the *in vivo* experiment. Previously, it was documented that after 8 days the TMT presence decreased almost by 50% in mice and rats [27].

Previous study of TMT-induced neurodegeneration showed that handling of young adult male rats improved the spatial memory in healthy and also in TMT-treated rats and prevented the memory impairment induced by trimethyltin [17]. Therefore the second aim of the present study was oriented to the effect of handling of rats on the Na,K-ATPase properties in healthy rats and also in rats with TMT-induced neurodegeneration.

Our present results of enzyme kinetic investigations indicate that the Na,K-FATPase might be involved in positive effects of handling in healthy rats as well as in rats with TMT-induced neurodegeneration. While in both groups of handled rats the number of active enzyme molecules remained unchanged, as indicated by unaltered V_{max} value when comparing to respective controls, the ability of the enzyme to bind and utilize the energy substrate ATP was markedly improved as indicated by lowered K_m values. So, the handling of rats may affect similarly the energy utilization and extrusion of superfluous sodium from cells in frontal cortex in healthy rats and in rats with TMT-induced neurodegeneration. The positive impact of enhanced Na,K-ATPase functionality in the brain tissue probably plays an important role in improvement of spatial memory in healthy non-treated rats and rats with TMT-induced neurodegeneration when handled for 6 weeks. The importance of Na,K-ATPase in prevention of neurodegeneration was supported also in the experimental model of epilepsy where stimulation of Na,K-ATPase with specific antibody (DRRSAb) restored the crossing activity of pilocarpine-treated mice in the open-field test [28].

This study provides also new information concerning the expression of individual α subunits of Na,K-ATPase (α1, α2, α3) in the frontal cerebral cortex in consequence of handling of healthy rats or rats with TMT-induced neurodegeneration. The global maintenance of intracellular sodium homeostasis was probably not affected by TMT-treatment of adult rats in handled as well as in non-handled rats as indicated by unaltered expression of α1 subunit. This ubiquitously expressed isoform is responsible for global maintenance of intracellular sodium homeostasis [29, 30].

The increased expression of glial isoform α2 in handled rats with TMT-induced neurodegeneration seems to be interesting in view of pathological alterations in consequence of poisoning by TMT. It was previously documented that malfunction of this isoform of Na,K-ATPase is probably involved in development of familial hemiplegic migraine type 2 [31]. However, the increased expression of glial isoform α2 in TMT-affected and handled rats may represent an interesting effect requiring further investigations for explaining the physiological relevance of this fact.

Based on our analysis of the protein expression of Na,K-ATPase α3 subunit it may by hypothesized that increased expression of this subunit could play a role in the neuroprotective mechanism of handling in

healthy rats whose were not poisoned by TMT. Previously it was documented that α3 subunit is exclusively specific for fully differentiated neurons [32], thus we can suppose that handling of healthy animals might act at the neuronal level. Na,K-ATPase stimulates the growth of dendrites during the development of the brain where signal growth is triggered by signal transduction and probably plays a role in neurogenesis [33]. Malfunction of α3 is probably involved in development of several neurological disorders, as it was suggested also for rapid-onset dystonia parkinsonism in mice [8]. So, it may be hypothesized that increased expression of neuronal α3 probably is involved in the mechanism of previously reported improvement in memory of handled control animals [17]. This hypothesis seems to be in agreement with previous observation of altered spatial learning, motor activity and anxiety in α3-deficient mice [34]. In addition, the animals with the haploinsuficiency gene for this subunit of Na,K-ATPase were accompanied by cognitive deficits [35].

Conclusion

It may be concluded that everyday handling of rats, beside the previously published beneficial effect on spatial memory was accompanied by improwed maintenance of sodium homeeostasis in frontal cortex of brains. The key system responsible for this proces, the Na,K-ATPase was able to utilize better the energy substrate ATP. In rats with TMT-induced neurodegeneration handling promoted the expresion of α2 isoform of the enzyme which is typical for glial cells. In healthy rats the handling was followed by increased expression α3 subunit which is typical for neurons.

Declarations

Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

Ethics declarations

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures with animals were performed in compliance with principles of laboratory animal care issued by EU Directive 2010/63/EU for animal experiments, proved and controlled by the State Veterinary and Food Administration of Slovak Republic (No. 4030/10-221) and with agreement of the Ethical Committee of the Centre of Experimental Medicine, Slovak Academy of Sciences. This article does not contain any studies involving human participants performed by any of the authors.

Data availability statement

The datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

Author´s contributions

ZG designed the study; VNS, DM and EU performed the biological model; BK, NV, DS and JV performed the analysis and evaluation of Na,K-ATPase expression and estimation of ATP utilization by Na,K-ATPase using the method of enzyme kinetics; ZG, BK, DS and NV prepared manuscript.

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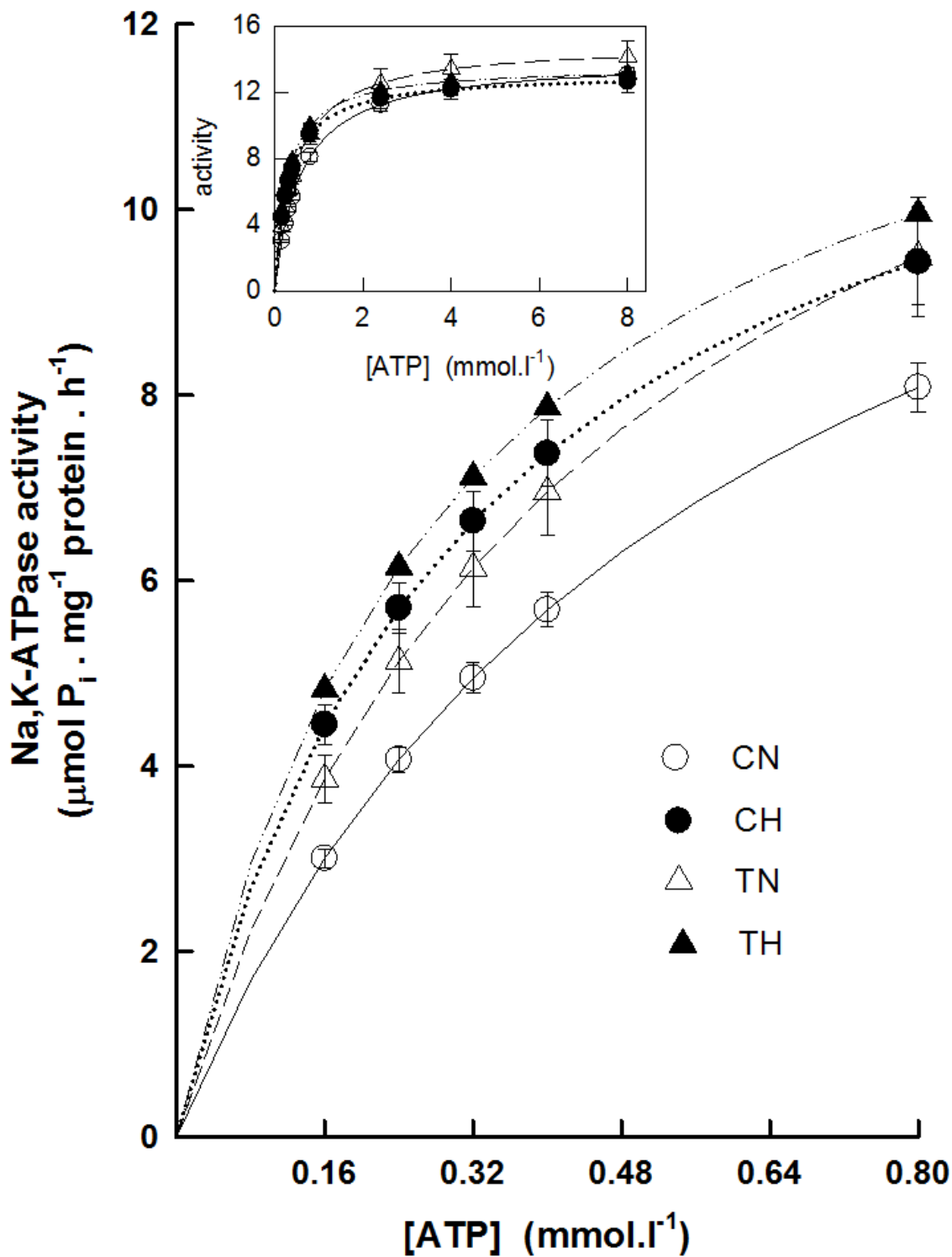
Figures

Figure 1

Activation of the Na,K-ATPase by low concentrations of substrate ATP in control non-handled rats (CN), in control handled rats (CH), in non-handled rats with TMT administration (TN) and in handled rats with TMT administration (TH). Inset: activation of the enzyme in the whole investigated concentration range of ATP. Data represent mean ± SEM, n = 8 in each group.
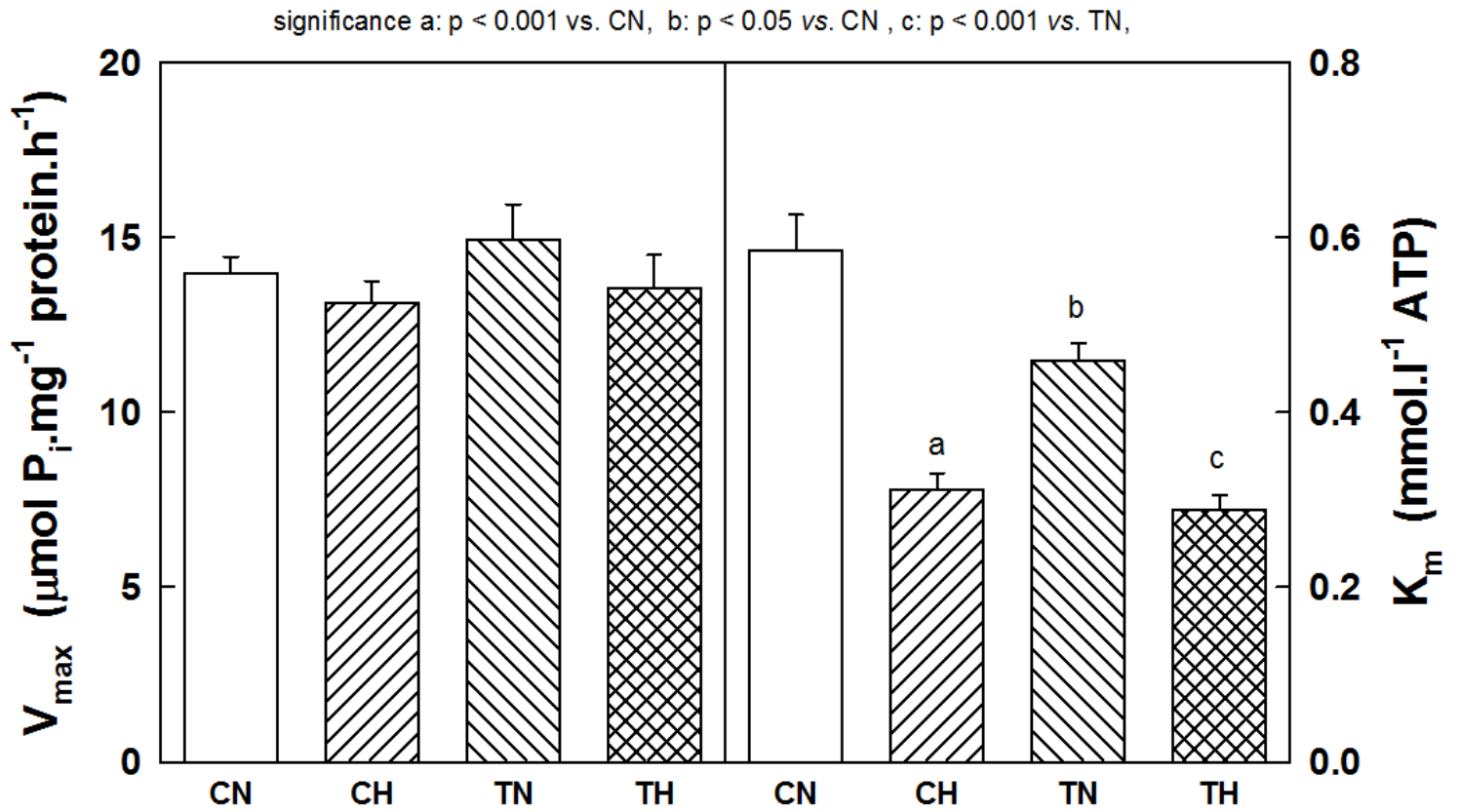
Figure 2

Kinetic parameters of the Na,K-ATPase during activation with substrate ATP in control non-handled rats (CN), in control handled rats (CH), in non-handled rats with TMT administration (TN) and in handled rats with TMT administration (TH). The parameter Vmax represents the maximal velocity of enzyme reaction Km value refers to the concentration of ATP necessary for half maximal activation of the enzyme. Data represent mean ± SEM, n = 8 in each group. Significance a: $p<0.001$ vs. CN, b: $p<0.05$ vs. CN, c: $p<0.001$ vs. TN.