Effect of the pyridoindole antioxidant stobadine on ATP-utilisation by renal Na,K-ATPase in rats with streptozotocin-induced diabetes

N. Vrbjar[1], S. Strelková[1], V. Javorková[1], J. Vlkovičová[1], L. Mézešová[1], M. Štefek[2], Z. Kyseľová[2] and A. Gajdošíková[2]

[1] *Institute for Heart Research, Department of Biochemistry, Slovak Academy of Sciences, 840 05 Bratislava 45, P.O.Box 104, Slovakia*

[2] *Institute of Experimental Pharmacology, Slovak Academy of Sciences, Dúbravská cesta 9, 841 04 Bratislava, Slovakia*

Abstract. The effect of the pyridoindole antioxidant stobadine on diabetes-induced changes of Na,K-ATPase, especially those concerning the utilisation of its substrate ATP, was investigated. Sixteen weeks of streptozotocin-induced diabetes (single i.v. dose of streptozotocin; 55 mg/kg) was followed by decrease in the enzyme activity. This effect was emphasised in the presence of higher concentrations of substrate and in the presence of 8 mmol·l^{-1} ATP it represented 20%. It might be a consequence of altered functional properties of Na,K-ATPase as suggested by 20% decrease in the V_{max} value along with decrease in the K_m value by 20%. Administration of 0.05% (w/w) stobadine in the diet to diabetic rats improved the function of renal Na,K-ATPase with respect to utilisation of ATP as suggested by significant increase in the enzyme activity in the whole concentration range of ATP investigated as a consequence of V_{max} elevation to the level comparable to absolute controls. In conclusion, stobadine may play a positive role in restoring the functional properties of renal Na,K-ATPase, especially concerning the utilisation of energy derived from hydrolysis of ATP, improving thus the maintenance of ionic homeostasis during diabetes.

Key words: Sodium-pump — Kidney — Diabetes — Stobadine

Introduction

Mammalian kidneys play a major role in maintenance of body fluid and electrolyte homeostasis. This function of kidneys requires the presence of numbers of specific carriers able to transport a large variety of ions across the plasmalemmal membrane. Among these systems, Na,K-ATPase was ascribed to exert an important function in the renal plasmalemma. This enzyme transforms the chemical energy from ATP to osmotic work, utilising 20–30% of the current ATP production in mammals for active Na,K transport in kidney (Jorgensen and Pedersen 2001).

The Na,K-ATPase activity and expression are altered during the development of various diseases (Breier et al. 1998; Vrbjar et al. 1999; Ondrejickova et al. 2000; Lehotsky et al. 2002; Matejovicova et al. 2002; Vrbjar et al. 2002; Das Evcimen et al. 2004; Vlkovicova et al. 2006). Insulin-dependent diabetes mellitus is a condition characterised by hyperglycaemia resulting from lack of insulin secretion by the pancreatic β-cells. Insulin has been shown to regulate the intracellular homeostasis of Na^+ ions *via* stimulating the activity of Na,K-ATPase (Feraille et al. 1992; Gupta et al. 1996; Tack et al. 1996). The mechanism of the above stimulation seems to be a specific effect targeted on the Na,K-ATPase molecule, since it is independent of sodium entry through amiloride-sensitive sodium channels (Feraille et al. 1995). Due to the fact that insulin did not alter the number of active enzyme units, an increase in turnover of Na,K-ATPase activity was proposed (Feraille and Doucet 2001).

Hyperglycaemia, a key clinical manifestation of diabetes mellitus, is often accompanied with increased generation of reactive oxygen species (ROS) (Larkins and Dunlop 1992; Miyata et al. 1997; Suzuki et al. 1999). After treatment of diabetic patients, as well as experimental animals with antioxidants, positive effects have been described (Trachtman et

Correspondence to: Norbert Vrbjar, Institute for Heart Research, Department of Biochemistry, Slovak Academy of Sciences, Dúbravská cesta 9, P.O.Box 104, 840 05 Bratislava 45, Slovakia
E-mail: usrdnorb@savba.sk

al.1995; Koya et al. 1997; Ha et al. 1999; Soulis-Liparota et al. 1999; Stefek et al. 2000; Sotnikova et al. 2006; Vlkovicova et al. 2006). Therefore oxidative stress has been considered to be a common pathogenesis of diabetic complications. Oxidative damage of Na,K-ATPase is demonstrated in several tissues including brain (Tugrul and Bekpynar 1997), heart (Ravingerova et al. 1994) and kidney (Thomas and Reed 1990). In the present study we investigated the effect of the pyridoindole antioxidant stobadine (STB) on diabetes-induced changes of Na,K-ATPase, especially those concerning the utilisation of its substrate ATP.

Materials and Methods

Experimental groups

All procedures and experimental protocols used in this study were approved by the Veterinary Council of the Slovak Republic (Decree No. 289, part 139, July 9th 2003) and they conform with Principles of Laboratory Animals Care (NIH publication 83-25, revised 1985). The animals were of monitored conventional quality and came from the Breeding Facility of the Institute of Experimental Pharmacology Dobrá Voda (Slovak Republic). Experimental diabetes type-1 was induced in 8-week-old male Wistar rats, weighing 200–230 g, by a single i.v. injection of streptozotocin (STZ) in a dose 55 mg·kg^{-1}. STZ was dissolved in 0.1 mol·l^{-1} citrate buffer (pH 4.5). The animals were fasted overnight prior to STZ administration. Water and food were available immediately after dosing. Ten days after STZ administration, animals with plasma glucose level higher than 15 mmol·l^{-1} were considered diabetic and were included in the study.

At the beginning of 16-weeks lasting experiment, rats were randomly divided into four groups. Age-matched rats receiving a single dose of 0.1 mol·l^{-1} citrate buffer served as controls (C, $n = 8$), the second group consisted of STZ-diabetic rats (D, $n = 8$). Both aforementioned groups (C and D) were feed with pelleted standard diet. A third (CS, $n = 4$) and fourth (DS, $n = 8$) groups of control and STZ-diabetic rats, respectively, were feed with STB dipalmitate-enriched diet (0.05% (w/w)). The drug was added to standard diet (KKZ-P-M) prior to its pelletisation. Animals had free access to food and drinking water. In accordance to the food consumption determined in previous experiments (Jorgensen et al. 2001) the above indicated concentration of the drug in the diet was adjusted to reach final intended effective dose in diabetic animals 21 mg of STB base/kg b.w. Based on previous results (Gajdosikova et al. 1995), this therapeutical dosage was selected as a medium non-toxic dose.

During the experiment, the animals were housed in groups of two in cages of the type T4 Velaz (Prague, Czech Republic) with bedding composed of wood shaving (changed daily). The animal room was air-conditioned and the environment was continuously monitored for the temperature of 23 ± 1°C and relative humidity of 55–60%.

At the end of the experiment, the animals were killed by cervical dislocation under thiopenthal anesthesia (65 mg/kg b.w.) followed by complete dissection and tissue collection. The samples were immediately frozen and stored in liquid nitrogen until use.

Blood measurements

During the experiment, plasma glucose levels were monitored in 2-weeks intervals using the commercial glucose Trinder kit (Sigma, St. Louis, MO, USA). Blood samples for glucose determination were taken in the mornings after overnight fasting (12–14 h). Heparinized blood samples were collected from the tail vein.

Membrane preparation

The microsomal membrane fraction enriched in plasmalemma was isolated from the whole kidney of individual animals according to the first part of the method described by Jorgensen (1974). Amount of proteins was determined according to Lowry et al. (1951) using bovine serum albumin as a standard.

Na,K-ATPase assay

ATP-kinetics of Na,K-ATPase was estimated at a temperature of 37°C by measuring the hydrolysis of ATP induced by 10 μg plasmalemmal proteins in the presence of the substrate ATP in concentrations increasing within the range of 0.16–8.0 mmol·l^{-1}. The total volume of medium was 0.5 ml containing (in mmol·l^{-1}): 50 imidazole (pH 7.4), 4 $MgCl_2$, 10 KCl and 100 NaCl. After 20 min of preincubation in substrate-free medium, the reaction was started by addition of ATP and after 20 min, the reaction was stopped by addition of 0.3 ml 12% ice-cold solution of trichloroacetic acid. The liberated inorganic phosphorus was determined according to Taussky and Shorr (1953). In order to establish the Na,K-ATPase activity, ATP hydrolysis that occurred in the Na^+ and K^+-free medium was subtracted from the hydrolysis measured in the presence of all three ionic cofactors of the enzyme i.e. Na^+, K^+, Mg^{2+}.

Chemicals

STZ was purchased from Sigma (St. Luis MO, USA). STB, (-)-cis-2,8-dimethyl-2,3,4,4a,5,9b-hexahydro-1H-pyrido[4,3b]indole, was obtained from Slovakofarma (Hlohovec, Slovak Republic) as a dipalmitate salt. Other chemicals were analytical-grade quality from local commercial sources.

Analysis of data

The kinetic parameters were evaluated from the obtained data by direct non-linear curve fitting method based on the Marquardt–Levenberg's algorithm according to the Michaelis–Menten equation using the Sigmaplot package from Jandel Scientific Co. All results were expressed as mean ± SEM. The significance of differences between the individual groups was determined with using ANOVA, Bonferroni test. A value of $p < 0.05$ was regarded significant.

Results

Weight parameters and glycaemic state of rats

After 16 weeks of STZ-induced diabetes type-1 we observed a 56% decrease in body weight gain (D: 193 ± 10 g) as compared to control group (C: 443 ± 9 g). Administration of STB to controls did not alter significantly the body weight (CS: 438 ± 16 g). Similarly, no significant difference was observed between diabetic animals fed with standard diet (group D) and with STB-enriched diet (DS: 205 ± 7 g). Kidney weight was not affected significantly by diabetes either in control animals or in rats fed with STB diet (C: 2.27 ± 0.08 g, D: 2.44 ± 0.10 g, CS: 2.56 ± 0.08 g, DS: 2.66 ± 0.09 g). STB by itself induced a slight but statistically significant 13% increase in kidney weight in control rats. However, the ratio of kidney to body weight, representing the relative kidney weight was significantly increased in consequence of diabetes by 150% in control animals and by 123% in STB-treated rats.

STB treatment did not affect significantly glycaemic state either in controls (C: 7.40 ± 0.21 mmol·l^{-1}, CS: 5.80 ± 0.15 mmol·l^{-1}) or in diabetic rats (D: 22.50 ± 0.81 mmol·l^{-1}, DS: 23.10 ± 1.63 mmol·l^{-1}).

Kinetic properties of renal Na,K-ATPase after 16 weeks of diabetes in animals fed with standard diet

When activating the enzyme with increasing concentrations of ATP, we observed a slight decrease in the enzyme activity in kidneys of diabetic animals (D group) in comparison with controls (C group). At lower ATP concentrations investigated in the range of 0.16–0.4 mmol·l^{-1}, the decrease represented 2–5%. With growing concentration of the substrate, the effect gradually increased to 18%, observed in the presence of 8 mmol·l^{-1} (Figure 1). Evaluation of the above data by the method of nonlinear regression showed that diabetes induced a statistically significant decrease of V_{max} by 20% along with a decrease in K_m value by 20% (Figure 2).

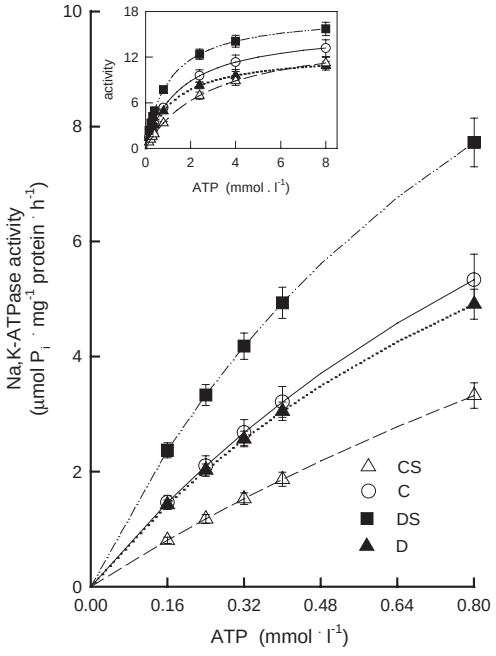

Figure 1. Activation of renal Na,K-ATPase by substrate ATP. Effect of increasing concentrations of ATP in control rats fed with standard diet (C, $n = 8$), in rats with STZ-induced diabetes (D, $n = 8$), in control rats fed with STB-enriched diet (CS, $n = 4$) and diabetic rats fed with STB-enriched diet (DS, $n = 8$). Detailed projection of activities in the presence of low concentrations of ATP. Insert: Activation of the enzyme in the whole ATP concentration range investigated.

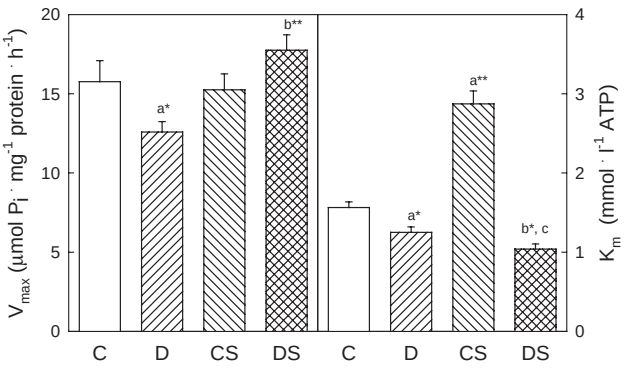

Figure 2. Kinetic parameters of Na,K-ATPase activation by substrate ATP in kidney. Parameters were evaluated at the end of the 16th week after onset of experimental diabetes by the method of nonlinear regression of data presented in Figure 1 from kidneys in control rats fed with standard diet (C, $n = 8$), in rats with STZ-induced diabetes (D, $n = 8$), in control rats fed with STB-enriched diet (CS, $n = 4$) and in diabetic rats fed with STB enriched diet (DS, $n = 8$). The left panel shows the maximal velocities (V_{max}) of the enzyme and the right panel the K_m values representing the concentrations of ATP necessary for half-maximal activation of the enzyme. Data represent means ± SEM. a, comparison with group C (* $p < 0.05$, ** $p < 0.001$); b, comparison with group D (* $p < 0.05$, ** $p < 0.001$); c, comparison with group CS ($p < 0.001$).

Influence of STB on renal Na,K-ATPase in control rats

As shown in Figure 1, CS group showed decreased activity of renal Na,K-ATPase in comparison with C group. At the lowest ATP concentration investigated (0.16 mmol·l^{-1}), the degree of the enzyme deactivation represented 45%.

With increasing concentration of the substrate, the effect gradually decreased to 15%, observed in the presence of 8 mmol·l^{-1} (Figure 1). Evaluation of the above data by the method of nonlinear regression showed that application of STB to control rats resulted in unchanged V_{max} and a statistically significant increase in K_m by 84% (Figure 2).

Influence of STB on the renal Na,K-ATPase in diabetic rats

In the diabetic rats, administration of STB (DS group) induced significant augmentation in the response of Na,K-ATPase to increasing concentrations of ATP (Figure 1). In these rats, STB induced significant alterations in kinetic behaviour of Na,K-ATPase. During activation with ATP, we observed an increase in the enzyme activity, which was most significant in the presence of lower concentrations of ATP, as compared to the diabetic animals fed with standard diet (D group). In the presence of 0.16 mmol·l^{-1} ATP, this increase represented 66%. The elevation of ATP concentration was followed by a gradual diminution of the above effect and, at 8 mmol·l^{-1} ATP, the stimulation was 44%. These changes are reflected also in values of kinetic parameters of the enzyme. Evaluation of the data showed that STB administration to diabetic rats resulted in significant 41% enhancement of the V_{max} value and a smaller but statistically significant 17% diminution of the K_m value (Figure 2).

Discussion

Altered sodium homeostasis is a consistent finding in diabetes. Both insulin-dependent and non-insulin-dependent diabetic patients were reported to have a significant increase in total exchangeable sodium (O´Hare et al. 1985; Weidmann and Ferrari 1991). Considerable attention has been focused on abnormalities of renal sodium handling in diabetes, since the kidney is the main regulator of body salt and water homeostasis. One of the crucial systems involved in maintaining sodium balance is Na,K-ATPase, which utilises energy derived from hydrolysis of ATP for transmembraneous transport of Na^+ and K^+ ions against their respective concentration gradients. Our observations of the deteriorated function of renal Na,K-ATPase after 16 weeks of STZ-induced diabetes are in agreement with previously published data of Tsimaratos et al. (2001b), who reported that after 12 weeks of diabetes the activity of the enzyme was significantly decreased. In the present study we aimed to explain the mechanism of diminution of Na,K-ATPase activity by means of an enzyme kinetic assay using various concentrations of the substrate. The simultaneous decrease in the V_{max} and K_m values suggests a possible uncompetitive inhibition of the enzyme. This inhibition may be caused by several factors. One of them might be interaction of an inhibitory compound on the enzyme-substrate complex, i.e. Na,K-ATPase, with already bound ATP. The role of such a hypothetical inhibitor probably responsible for this process might be ascribed to a digitalis-like substance which was found elevated in STZ-diabetic rats causing a decrease in cardiac Na,K-ATPase activity (Mathews et al. 1991; Chen et al. 1993). An increased level of this substance, characterised as an isomer of the digitalis compound ouabain (Mathews et al. 1991), inhibited also the renal Na,K-ATPase (Pamnani et al. 2000). It is known that digitalis-glycosides bind to the extracellular domain of Na,K-ATPase in its phosphorylated state and besides decreasing the enzyme affinity for potassium, they prevent also the dephosphorylation of the enzyme, disturbing thus the reaction cycle (for review, see Feraille and Doucet 2001). The kinetic behaviour of the enzyme after chronic diabetes in our experiment might be satisfactorily described by the above mechanism. It should be mentioned that the digitalis-like substance is freely circulating in blood and is relatively well soluble. Therefore, in our experiment, the action of such an inhibitor seems to be limited to its presence in tightly membrane bound form. The second plausible explanation for the loss of enzyme activity as a consequence of STZ-induced diabetes may be linked to lack of insulin, which was shown to be a potential stimulator of Na,K-ATPase (Gupta et al. 1996; Tack et al. 1996). In a normally functioning pancreas, C-peptide co-secreted with insulin also improves renal function *via* stimulating Na,K-ATPase activity (Ohtomo et al. 1998). According to the long-lasting time course of our experiment during which the expression of the Na,K-ATPase molecule could have been altered, a third feasible explanation may concern the decrease in the number of enzyme molecules, as suggested by markedly lowered V_{max}. This proposal is supported also by previous studies documenting that chronic diabetes is followed with loss of activity as well as decreased expression of the enzyme in the kidney (Tsimaratos et al. 2001b) as well as in the heart (Ver et al. 1997). Regardless of the mechanism supposed to be responsible for the observed effect, during long-lasting diabetes Na,K-ATPase was found less active throughout the investigated ATP-concentration range resulting thus in deteriorated utilisation of energy necessary for transport of excessive sodium out from the cell.

It has been generally accepted that in the development of chronic diabetic complications ROS play an important role (Coppey et al. 2001). The experimental diabetes type-1 which was applied in our study was shown previously to be accompanied with increased generation of ROS as documented by elevation of 2-thiobarbituric-reactive substances (Pekiner et

al. 2002; Ulusu et al. 2003), as well as by increased content of conjugated dienes (Stefek et al. 2000, 2002a). In the present study we investigated the effect of the pyridoindole antioxidant STB on diabetes-induced changes of Na,K-ATPase, which might be susceptible to radical-induced damage in rat kidney. STB was able to diminish lipid peroxidation and protein impairment under oxidative stress (for review see Horakova and Stolc 1998). In our experiments, administration of STB to control rats was followed by deteriorated utilisation of energy from substrate, as suggested by decreased activity of Na,K-ATPase mainly at lower concentrations of ATP. For explanation of the above effect, at least two different mechanisms may be proposed. One of them, based on significant increase in the K_m value accompanied with unchanged V_{max}, may indicate a competitive inhibition of the enzyme. Due to the rather high lipophility (Kagan et al. 1993) and stability of STB (Horakova and Stolc 1998), it is possible that STB remaining in plasmalemmal membranes also after the isolation procedure inhibits the renal Na,K-ATPase. Due to the fact that the ATP-binding site of Na,K-ATPase is localised in the extramembraneous segment protruding into the intracellular space (for review, see Feraille and Doucet 2001; Jorgensen and Pedersen 2001), a direct interaction of the lipophilic STB with ATP-binding site seems to be less probable. Therefore we deduce that STB induces conformational alterations of the enzyme molecule, which may result in deteriorated ability to bind ATP. The second probable explanation of the STB-induced effect on renal Na,K-ATPase in control rats may be provided by the expression of a new type of Na,K-ATPase with lower affinity to ATP, as suggested by the elevated K_m value. Regardless of the hypothetical mechanism responsible for the observed effect, after long-lasting administration of STB to control rats Na,K-ATPase probably utilises the energy substrate to lesser extent, and that especially in the presence of lower concentrations of ATP which are of physiological relevance. Hence, the transmembraneous transport of Na^+ and K^+ in renal cells might be limited due to administration of STB to controls.

On the other hand, administration of STB to diabetic rats improved the function of renal Na,K-ATPase in respect to utilisation of ATP. Evaluation of ATP-kinetic measurements resulting in profound increase in the V_{max} suggested that suppression of diabetes-induced damage in kidney by STB was followed by increase in the amount of active Na,K-ATPase molecules. The affinity of the enzyme to substrate was also improved as documented by statistically significant decrease in the K_m value as compared to the diabetic group.

Direct comparison of the two groups with STB revealed a significant impairment of Na,K-ATPase function when the antioxidant was administered to controls. This may be due to the suppression of the signalling role of ROS in controls by antioxidant STB. On the other hand, in diabetic group, the antioxidant protected the Na,K-ATPase function against the excessive level of ROS produced as a concequence of the disease.

Besides the direct alterations in the polypeptide chain of the enzyme molecule, recent studies have documented the importance of the membrane lipid composition and their oxidative damage on the function of renal Na,K-ATPase during diabetes (Aragno et al. 2001; Tsimaratos et al. 2001a). In previous papers, STB was shown to reduce oxidative damage of the diabetic heart and kidney tissue as measured by conjugated dienes (Stefek et al. 2000, 2002a) or malondialdehyde (Ulusu et al. 2003). The ability of STB to attenuate lipoxidation reactions in diabetes, and thus the production of toxic aldehydes may account, at least partly, for observed protection of the Na,K-ATPase. So, besides the effects induced by STB directly on the Na,K-ATPase molecule, a reliable explanation of the stobadin-induced protection against diabetic complications may be its antioxidant activity in the annular lipids surrounding the enzyme molecule. The presented STB-induced improvement of ATP-utilisation of the renal Na,K-ATPase during diabetes is in agreement with recently published data documenting that STB antagonises also the negative effect of diabetes on the Na-binding site of the enzyme molecule in kidney (Vrbjar et al. 2004) as well as in the heart (Vlkovicova et al. 2006). STB was documented to protect also the Na,K-ATPase against the ROS-mediated disturbances also in synaptosomal membranes (Kaplan et al. 1997; Lehotsky et al. 1999).

Because of the well known fact that both above mentioned binding sites, for ATP and also for Na^+ are oriented towards the intracellular space, it may be suggested that STB acts directly or indirectly on the enzyme molecule from the intracellular surface of the renal cell

In summary, studies were undertaken to investigate renal Na,K-ATPase activity. Our findings strongly suggest that administration of STB to diabetic rats, besides attenuating albuminuria, enzymuria, kidney lipid peroxidation and matrix collagen crosslinking (Stefek et al. 2002a,b) may play a positive role also in utilisation of energy derived from hydrolysis of ATP, especially in the presence of its lower, physiologically relevant concentrations. Consequently, STB improves the maintenance of ionic homeostasis during diabetes ameliorating thus one of the main functions of kidney.

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