Effect of the Pyridoindole Antioxidant Stobadine on the Cardiac Na^+,K^+-ATPase in Rats with Streptozotocin-Induced Diabetes

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Abstract. In the present study we examined the effect of dietary supplementation with the pyridoindole antioxidant stobadine on functional properties of the cardiac Na^+,K^+-ATPase in diabetic rats. Diabetes lasting sixteen weeks which was induced by a single i.v. dose of streptozotocin (55 $mg \cdot kg^{-1}$) was followed by decrease in the enzyme activity. Evaluation of kinetic parameters revealed a statistically significant decrease in the maximum velocity (V_{max}) (32 % for ATP-activation, 33 % for Na^+-activation), indicating a diabetes-induced diminution of the number of active enzyme molecules in cardiac sarcolemma. The ATP-binding properties of the enzyme were not affected by diabetes as suggested by statistically insignificant changes in the value of Michaelis-Menten constant, $K_{M(ATP)}$. On the other hand, the affinity to sodium decreased as suggested by 54 % increase in the $K_{M(Na^+)}$ value. This impairment in the affinity of the Na^+-binding site together with decreased number of active Na^+,K^+-ATPase molecules are probably responsible for the deteriorated enzyme function in hearts of diabetic animals. Administration of stobadine to diabetic rats dramatically improved the function of cardiac Na^+,K^+-ATPase with regard to Na^+-handling, as documented by statistically significant elevation of V_{max} by 66 and 47 % decrease in $K_{M(Na^+)}$. Our data suggest that stobadine may prevent the diabetes-induced deterioration of cardiac Na^+,K^+-ATPase, thus enabling to preserve its normal function in regulation of intracellular homeostasis of Na^+ and K^+ ions.

Key words: Sodium pump — Heart — Diabetes — Stobadine

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Introduction

Myocardial disease is one of the leading causes of mortality in diabetes. Previous studies have indicated that the bioenergetic depression is accompanied by alterations of cardiac transport systems, including Na^+,K^+-ATPase (Ku and Sellers 1982; Pierce and Dhalla 1983; Michea et al. 2001; Ziegelhöffer et al. 2003), sarcolemmal Na^+,Ca^{2+}-exchanger (Chattou et al. 1999), and Ca^{2+} transport activities (Heyliger et al. 1987; Makino et al. 1987) during the development of diabetes. The magnitude and direction of the changes depend on the duration of diabetes and the organ involved. In experimental diabetes, changes in Na^+,K^+-ATPase activity have been reported in the heart (Kjeldsen et al. 1987; Ng et al. 1993; Gerbi et al. 1997), peripheral nerve (Gerbi et al. 1998), kidney (Wald et al. 1993; Cole et al. 1995; Vrbjar et al. 2004), intestine (Barada et al. 1994) and pancreas (Busik et al. 1997). The Na^+,K^+-exchanging ATPase also known as the sodium pump (EC 3.6.3.9) sited in the plasma membrane of most eukaryotic cells is responsible for the extrusion of three Na^+ ions from the intracellular space in exchange for two external K^+ ions at the expense of free energy derived from ATP hydrolysis. This is the major mechanism for maintenance of the membrane potential required for a multitude of cellular functions (Lingrel and Kuntzweiler 1994). The insulin-dependent diabetes is characterised by hyperglycemia resulting from a lack of insulin secretion by pancreatic β-cells. Moreover, insulin has been shown to modulate vascular reactivity inducing vasodilatation (Zemel et al. 1992). This effect is partially linked to the activation of the Na^+,K^+-ATPase. Sodium pump is one of the targets of insulin action in different tissues acting by variations in turnover number of the α_2 subunit, enhancing thus availability of the intracellular cation or substrate concentration (Lingrel et al. 1990; Ohara et al. 1991). Hyperglycaemia is accompanied with increased generation of reactive oxygen species (ROS) (Larkins and Dunlop 1992; Gross et al. 2003). Studies in the streptozotocin (STZ)-induced diabetic rat model showed oxidative damage of Na^+,K^+-ATPase in several tissues including brain (Öner et al. 1997; Tugrul and Bekpynar 1997), heart (Ziegelhöffer et al. 2003) and kidney (Thomas and Reed 1990). Beneficial effects of various antioxidants were described (Baynes and Thorpe 1999). The protective effect of pyridoindole stobadine was suggested to arise from its antioxidant properties (Horáková and Štolc 1998) and ability to scavenge deleterious ROS such as hydroxyl, peroxyl and alkoxyl radicals both in aqueous and lipid phases (Račková et al. 2002). The cardioprotective effect of stobadine in STZ-induced diabetic rats was documented by suppressed concentration of conjugate dienes as well as by attenuated angiopathic and atherogenic processes as assessed by electron microscopic examination (Štefek et al. 2000). The present study was designed to investigate the influence of stobadine on properties of Na^+,K^+-ATPase in isolated plasmalemmal membranes from hearts of rats suffering from chronic (16 weeks) STZ-induced diabetes. On studying the function of the enzyme we focused on the response of ATP- and Na^+-binding sites to diabetes and to the treatment by stobadine.

Materials and Methods

Experimental groups

All procedures used in this study were approved by the Veterinary Council of the Slovakia (Decree 289, part 139, July 9, 2003) and conformed to the Helsinki declaration of 1975. Experimental diabetes was induced in 8 weeks old male Wistar rats, weighing 200–230 g, by a single i.v. dose of STZ (55 $mg \cdot kg^{-1}$). For these injections, STZ was dissolved in 0.1 $mol \cdot l^{-1}$ citrate buffer (pH 4.5). Ten days after STZ administration, the plasma glucose level was checked. Rats with level higher than 15 $mmol \cdot l^{-1}$ were considered diabetic and were included in the study.

Age-matched rats receiving a single dose of 0.1 $mol \cdot l^{-1}$ citrate buffer served as controls ($n = 8$), the second group consisted of STZ-diabetic rats ($n = 8$). A third group ($n = 8$) of STZ-diabetic rats was fed with stobadine dipalmitate enriched diet. The drug was added to standard diet prior to its pelletisation. The final concentration of stobadine in pellets was 0.05 % (w/w). In accordance to the food consumption determined in previous experiments (Štefek et al. 2000), the above indicated concentration of the drug in the diet was adjusted to reach final intended effective dose in diabetic animals 21 mg of stobadine base *per* kg b.w. Based on previous results (Gajdošíková et al. 1995), this therapeutical dosage was selected as a medium non-toxic dose. The animal room was air-conditioned with 12-h light/dark cycle and the environment was continuously monitored for the temperature of 23 ± 1 ℃.

At the end of 16 weeks lasting experiment, the animals were killed by cervical dislocation under thiopental anesthesia (65 $mg \cdot kg^{-1}$ b.w.). Hearts were quickly removed, rapidly rinsed with ice-cold physiological saline, weighed, frozen in liquid nitrogen and stored 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).

Chemicals

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

Membrane preparation

Cardiac plasmalemmal membrane fraction was isolated from the hearts by the hypotonic shock-NaI treatment method according to Vrbjar et al. (1984). Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Determination of Na^+,K^+-ATPase activity

50 μg of proteins from isolated membrane fractions were transferred to the Na^+,K^+-ATPase assay medium containing (in mmol·l^{-1}): 100 imidazole (pH 7.4), 4 $MgCl_2$, 10 KCl, 100 NaCl and preincubated for 20 min at 37℃. The reaction was performed in the presence of increasing amount of ATP in the range of 0.08–4.00 mmol·l^{-1} and was terminated after another 20 min by adding of 0.3 ml ice-cold 12% trichloracetic acid and by rapid cooling in ice. The enzyme kinetic for sodium activation in concentration range of 2–100 mmol·l^{-1} of NaCl was determined by the same approach and the amount of ATP was constant (4 mmol·l^{-1}). The inorganic phosphate (P_i) released from ATP was measured according to the method of Taussky and Shorr (1953). Na^+,K^+-ATPase activity was calculated as the difference between the hydrolysis measured in the presence of all three ionic cofactors (Na^+, K^+, Mg^{2+}) and samples assayed in a medium devoid of Na^+ and K^+.

Statistical analysis

The kinetic parameters were evaluated from the data obtained by direct non-linear curve fitting method based on the Marquardt–Levenberg's algorithm according to the Michaelis–Menten equation. All values of experiments were expressed as means ± SEM. Statistical analysis was performed by use of ANOVA and the Bonferroni's test. A probability value less than 0.05 was considered to reflect statistically significant differences.

Results

Experimental diabetes in rats

STZ-induced diabetes was characterized by the presence of hyperglycemia. Blood glucose level determined at the end of the experiment was markedly elevated by 204% in diabetic rats in comparison with controls. Stobadine treatment did not affect noticeably the glycaemic status in diabetic rats. Diabetic animals showed lower gain in body weight as compared to controls. The decrease represented 56% as compared to control group (Tab. 1). Hereby, the total cardiac mass measured immediately after the dissection of the heart was lowered in diabetic rats by 33% but the heart weight/body weight ratio was considerably higher by 54% as compared to control rats. Additional treatment of diabetic animals with stobadine did not influence considerably any of the parameters mentioned above (Tab. 1).

Kinetic properties of Na^+,K^+-ATPase

Diabetes reduced the cardiac Na^+,K^+-ATPase activity throughout the investigated concentration range of substrate as compared with controls. With increasing concentration of ATP, the decrease in activity ranged from 38 to 32% (Fig. 1). These changes in activities were reflected in statistically significant decrease in the V_{max} by 32% with slight, statistically insignificant elevation of $K_{M(ATP)}$ in diabetic

Table 1. Effects of 16-weeks lasting diabetes on blood glucose, body weight, heart weight and heart weight/body weight ratio in non-diabetic control Wistar rats (C); diabetic rats (D) and diabetic rats fed with stobadine (DS)

	C ($n = 8$)	D ($n = 8$)	DS ($n = 8$)
Blood glucose ($mmol \cdot l^{-1}$)	7.40 ± 0.21	22.50 ± 0.81*	23.10 ± 1.63
Body weight (g)	443 ± 9	193 ± 10*	205 ± 7
Heart weight (mg)	1322 ± 42	882 ± 39*	927 ± 36
Heart/body weight (mg/g)	2.984 ± 0.078	4.604 ± 0.182*	4.547 ± 199

Values are means ± SEM; n, number of animals in each group. Statistical significance was * $p < 0.001$, D *vs.* C.

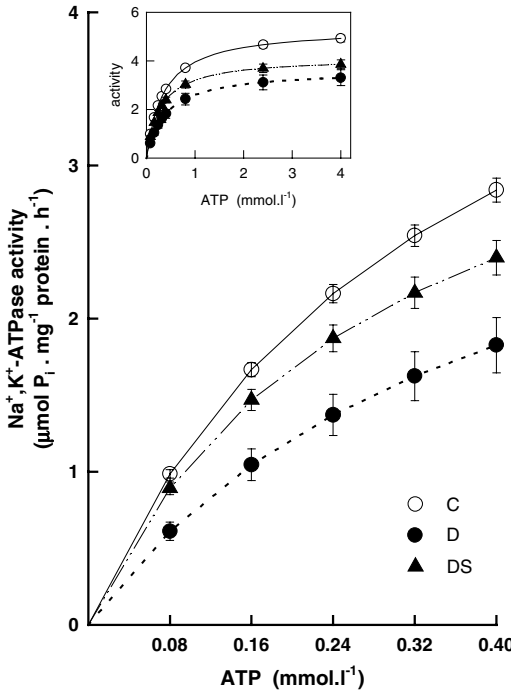

Figure 1. Activation of cardiac Na^+,K^+-ATPase by substrate ATP. Effect of increasing concentrations of ATP in control rats fed with standard diet (C), in rats with 16-weeks lasting STZ-induced diabetes (D) and in diabetic rats treated with stobadine (DS) is shown. Detailed projection of activities in the presence of low concentrations of ATP. Insert: activation of the enzyme in the whole ATP concentration range investigated.

rats (Fig. 2). Comparing the response of Na^+,K^+-ATPase to increasing concentrations of the cofactor Na^+ we observed in the diabetic group considerable impair-

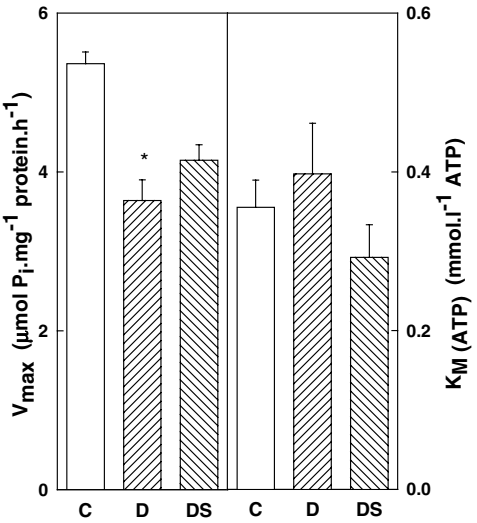

Figure 2. Kinetic parameters of Na^+,K^+-ATPase activation by substrate ATP in hearts from control rats fed with standard diet (C), in rats with STZ-induced diabetes (D) and in diabetic rats treated with stobadine (DS). The left panel shows the maximal velocities (V_{max}) of the enzyme and the right panel the $K_{M(ATP)}$ values representing the concentrations of ATP necessary for half-maximal activation of the enzyme. Data represent means ± SEM, * $p < 0.05$, comparison of D *vs.* C.

ment of the enzymatic activity. At the lowest concentration of NaCl investigated (2 mmol·l^{-1}), the decrease represented 52%. The effect gradually decreased with increasing concentration of NaCl. In the presence of the highest investigated concentration (100 mmol·l^{-1}), this diminution amounted to 34% (Fig. 3). The above activity changes were also manifested in values in kinetic parameters. Diabetes induced statistically significant decrease in the V_{max} by 33% in STZ-treated animals, with concomitant statistically significant enlargement of the $K_{M(Na^+)}$ value (54%) comparing with the controls (Fig. 4).

In the diabetic rats, administration of stobadine induced only a mild increase in the enzyme activity in the hearts when activated the Na^+,K^+-ATPase with increasing concentrations of ATP (Fig. 1). Evaluation of the data showed that stobadine administration to diabetic rats resulted in statistically insignificant changes of both investigated parameters (V_{max} and $K_{M(ATP)}$) (Fig. 2).

Administration of stobadine to diabetic animals induced augmentation in the response of Na^+,K^+-ATPase to increasing concentrations of NaCl. In the presence of the lowest concentration (2 mmol·l^{-1}) NaCl, the stimulation was 172% as compared to diabetic group. With increasing concentrations of the cofactor, the stimulatory effect gradually decreased to 73% as observed in the presence of 100 mmol·l^{-1} NaCl (Fig. 3). Evaluation of the above data by the method of non-linear

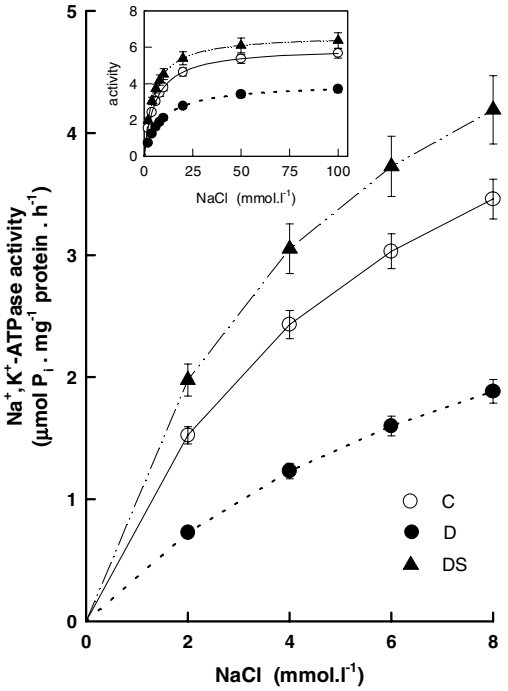

Figure 3. Activation of Na^+,K^+-ATPase in cardiac sarcolemma by cofactor Na^+ in control rats fed with standard diet (C), in rats with 16-weeks lasting STZ-induced diabetes (D) and in diabetic rats treated with stobadine (DS). Detailed projection of activities in the presence of low concentrations of Na^+. Insert: activation of the enzyme in the whole Na^+ concentration range investigated.

regression showed that administration of stobadine to diabetic rats resulted in 66 % increase in V_{max} and 47 % decrease in the $K_{M(Na^+)}$ value. The above mentioned changes were statistically significant (Fig. 4).

Discussion

The present study demonstrated that diabetic rats exhibited a marked depression of the Na^+,K^+-ATPase activity in cardiac tissue. This finding is in agreement with previous observations (Ku and Sellers 1982; Pierce and Dhalla 1983; Ng et al. 1993; Kato et al. 1999; Michea et al. 2001). The extent of diabetes-induced desactivation of sodium pump, representing 30–50 % in our study, fits well with those published by Kato et al. (1999) and Ramasamy et al. (1999). In previous measurements, the enzyme activity was estimated at certain conditions in which the concentration of NaCl exceeded the intracellular level of Na^+ (Pierce and Dhalla 1983; Kato et al. 1999; Ramasamy et al. 1999). Therefore, for further elucidation of

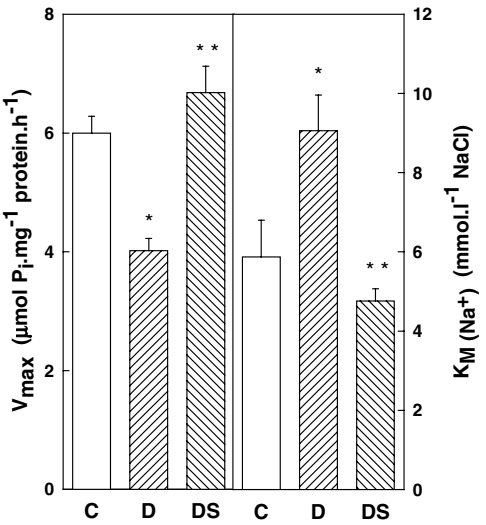

Figure 4. Kinetic parameters of Na^+,K^+-ATPase activation by cofactor Na^+ in hearts from control rats (C), in rats with STZ-induced diabetes (D) and in diabetic rats treated with stobadine (DS). The left panel shows the maximal velocities (V_{max}) of the enzyme and the right panel the $K_{M(Na^+)}$values representing the concentrations of Na^+ necessary for half-maximal activation of the enzyme. Each value represents the mean ± SEM with probability * $p < 0.05$, comparison of D *vs.* C; ** $p < 0.05$, comparison of DS *vs.* D.

the molecular principle of the above process we used kinetic measurements as a tool. Variations of substrate ATP and cofactor Na^+ concentrations provide information about properties of their respective binding sites in the Na^+,K^+-ATPase molecule.

Presented data give evidence that under the diabetic state the activity of Na^+,K^+-ATPase was strongly decreased over a wide range of Na^+ concentrations, even in the presence of lower amounts of Na^+ which are physiologically relevant. Concerning the properties of Na^+-binding site, the increased $K_{M(Na^+)}$ and simultaneously depressed V_{max} values point to a possibility of a mixed type of inhibition by an endogenous inhibitor which can bind to the free enzyme and also to the enzyme-Na^+ complex as a consequence of diabetes.

Concerning the properties of ATP-binding site, the decreased V_{max} together with unchanged $K_{M(ATP)}$ value suggest a possibility of non-competitive inhibition of the sodium pump during experimental diabetes in rats.

These eventual inhibitions may be caused by several factors. One of them might be interaction of an inhibitory compound with the Na^+,K^+-ATPase molecule somewhere apart from its substrate binding site. 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 (Chen et al. 1993a,b). Digitalis-glycosides bind

to the extracellular domain of Na^+,K^+-ATPase in its phosphorylated state and prevent also the dephosphorylation of the enzyme by decreasing its affinity for potassium, disturbing thus the reaction cycle (for review, see Feraille and Doucet 2001). The kinetic behavior of the enzyme after chronic diabetes might be satisfactorily described by the above mechanism. However, 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). The third possible explanation, supported by the long duration of our experiment during which the expression of the Na^+,K^+-ATPase molecule could have been altered, is that the number of enzyme molecules is decreased, as suggested by markedly lowered V_{max} values. Our observation of decrease in the number of active ATPase molecules (by 32–33 %) agrees with previous findings that STZ-induced diabetes selectively reduces cardiac Na^+,K^+-ATPase concentration by around 1/4 (Ziegelhöffer et al. 2003). This proposal is supported also by previous studies documenting loss of activity as well as decreased expression of the enzyme due to chronic diabetes (Kato et al. 1999). Moreover, increased level of ROS accelerated the degradation of damaged Na^+,K^+-ATPase (Zolotarjova et al. 1994). The other argument for involvement of ROS in the deactivation of the Na^+,K^+-ATPase may be the fact that increased *in vitro* production of $^{\bullet}OH$ radicals caused strong decrease in Na^+ affinity of the enzyme in cardiac tissue (Ravingerová et al. 1994). This negative role of $^{\bullet}OH$ radicals may be the explanation for our observation of diabetes-induced deterioration of Na^+-binding properties in the molecule of cardiac Na^+,K^+-ATPase. Recent studies documenting a 4.5-fold increase in $^{\bullet}OH$ radicals in diabetic myocardium support the above hypothesis (Fiordaliso et al. 2006). In smooth muscles as well as in myocardium hyperglycemia increases superoxide concentration (Gupta et al. 2002; Fiordaliso et al. 2006). Moreover, increased amount of superoxide radicals resulted in impairment of Na^+,K^+-ATPase activity in smooth muscles (Gupta et al. 2002). 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- as well as Na^+-concentration range resulting thus in deteriorated utilization of energy and deteriorated binding properties for sodium, both necessary requirements for transport of excessive sodium out from the cell.

The second aim of present study was to investigate the effect of supplementation of pyridoindole antioxidant stobadine on the myocardial Na^+,K^+-ATPase. Stobadine was found to be able to protect lipoprotein membranes against oxidative damage by preventing lipid peroxidation and protein oxidation (for review, see Horáková and Štolc 1998). As has been published recently, stobadine treatment of STZ-diabetic rats reduced oxidative damage of myocardial tissue (Štefek et al. 2000) and normalized calcium homeostasis in diabetic heart (Pekiner 2002).

Previously it was shown that increased level of ROS induced deterioration of the Na^+,K^+-ATPase function. Especially the negative role of singlet oxygen (Vinnikova et al. 1992; Kaplán et al. 2005), hydroxyl radical (Ravingerová et al. 1994), hydrogen peroxide (Clough and Bünger 1995), alkoxyl radical (Maulik et al. 1998), superoxide radicals (Gupta et al. 2002) were documented. *In vitro* experiments showed the ability of stobadine to prevent partially the negative effect of ROS on the Na^+,K^+-ATPase function in brain (Lehotský et al. 1999). In our experiment, *in vivo* administration of stobadine to diabetic rats improved the function of cardiac Na^+,K^+-ATPase in respect to Na^+-handling. Evaluation of Na^+-kinetic measurements resulting in profound increase in the V_{max} suggested a suppression of diabetes-induced damage in heart by stobadine, which might be caused by increase in the amount of active Na^+,K^+-ATPase molecules. The affinity of the enzyme to Na^+ was also improved as documented by statistically significant decrease in the $K_{M(Na^+)}$ value. Besides amended ability of the enzyme to bind Na^+ in its lower intracellular concentration, stobadine improved the capability to bind and transport the excessive Na^+ out of the cell also in the presence of higher concentrations of sodium. The above our conclusion is supported by observation of other authors using antioxidants in preventing diabetes induced complications and oxidative damage (Clough and Bünger 1995; Tsimaratos et al. 2001). In addition, restoration of Na^+,K^+-ATPase function by application of γ-linolenic acid to diabetic rats was accompanied with increased expression of enzyme molecules (Tsimaratos et al. 2001).

Moreover, concerning the mechanism of stobadine-induced protection, this compound is known to act as an effective scavenger of hydroxyl radicals (Štefek and Beneš 1991), alkoxyl radicals (Horáková et al. 1995) and an effective quencher of singlet oxygen (Steenken et al. 1992). Thus, the protection of Na^+,K^+-ATPase function in diabetic rats treated with stobadine suggests that the diabetes-induced deterioration of the enzyme in the vicinity of Na^+-binding site was caused probably by hydroxyl radicals, alkoxyl radicals and singlet oxygen. Concerning the disability of stobadine to restore the diabetes-induced changes in the ATP-binding site, it may be hypothesized that malfunction of the enzyme in this area is probably caused by superoxide radicals as suggested by the incapability of stobadine to protect the enzyme activity. This hypothesis is supported by the fact, that stobadine did not show an efficient scavenging effect against superoxide radicals (Kagan et al. 1993). In conclusion, our findings show that administration of antioxidant stobadine to diabetic rats antagonizes the negative effects of diabetes on the cardiac Na^+,K^+-ATPase, especially in its Na^+-binding site. This way, stobadine enables to preserve normal enzyme function in regulation of intracellular homeostasis of Na^+ and K^+ ions.

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