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Selective Inhibition of Brain Na,K-ATPase by Drugs

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Summary

The effect of drugs from the class of cardiac (methyldigoxin, verapamil, propranolol), antiepileptic (carbamazepine), sedative (diazepam) and antihistaminic (promethazine) drugs on Na,K-ATPase activity of plasma membranes was studied in rat brain synaptosomes. Methyldigoxin in a concentration of 0.1 mmol/l inhibits enzyme activity by 80 %. Verapamil, propranolol and promethazine in concentrations of 20, 20 and 2 mmol/l respectively, entirely inhibit the ATPase activity. Carbamazepine and diazepam in concentrations of 0.02-60 mmol/l have no effect on the activity of this enzyme. According to the drug concentrations that inhibit 50 % of enzyme activity (IC_{50}), the potency can be listed in the following order: methyldigoxin >> promethazine > verapamil ≥ propranolol. From the inhibition of commercially available purified Na,K-ATPase isolated from porcine cerebral cortex in the presence of chosen drugs, as well as from kinetic studies on synaptosomal plasma membranes, it may be concluded that the drugs inhibit enzyme activity, partly by acting directly on the enzyme proteins. Propranolol, verapamil and promethazine inhibitions acted in an uncompetitive manner. The results suggest that these three drugs may contribute to neurological dysfunctions and indicate the necessity to take into consideration the side effects of the investigated drugs during the treatment of various pathological conditions.

Key words
Na,K-ATPase • Synaptosomes • Verapamil • Propranolol • Promethazine

Introduction

Sodium, potassium-adenosine 5'-triphosphatase (Na,K-ATPase) is an integral membrane enzyme that actively transports K^+ and Na^+ ions against the respective cellular concentration differences. The Na,K-ATPase (EC 3.6.1.37) uses energy derived from hydrolysis of ATP to pump Na^+ out of and K^+ into the cell. The gradient produced by this enzyme is coupled to physiological functions such as cell proliferation, volume regulation, maintenance of the electrogenic potential required for the function of excitable tissues, i.e. muscle and nerves, and secondary active transport (Post *et al.* 1960, Kaplan 1978, Jørgensen 1992, Boldyrev 1993, Vasilets and Schwartz 1993, Basavappa *et al.* 1998). By regulating sodium and potassium ion concentrations, this enzyme also participates in the control of plasma membrane and mitochondrial Na/Ca exchange, the endoplasmic reticulum and plasma membrane Ca-ATPase activity, as well as Ca^{2+}-channel activity. All these events control the cellular Ca^{2+} level and influence heart and vascular muscle contractility and neuronal excitability (Jørgensen

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1992, Qadri and Ferrandi 1998, Nikezić and Metlaš 1985, Stahl and Harris 1986, Bernardi 1999). This enzyme is a glyco-protein composed of two subunits, a catalytic α subunit involved in splitting of ATP and a β subunit. The catalytic subunit of Na,K-ATPase is expressed in various forms (α1, α2 and α3), the proportions of which may differ in various tissues. The α3 isoform seems to show a lower affinity for intracellular Na^+ and the intracellular concentration of Na^+ seems to be higher in cells containing mainly this version of the Na,K-ATPase, such as neuronal cells (Munzer *et al.* 1994). This enzyme is also known as ouabain-sensitive Na,K-ATPase because, with regard to the pharmacological aspects, this enzyme is considered to act as an ouabain (cardiac glycoside) receptor. Ouabain binds to the extracellular part of α subunit of the enzyme and inhibits its transport and enzymatic activity (Lingrel *et al.* 1998, Jortani and Valders 1997, Repke *et al.* 1995).

Na,K-ATPase, supporting the ionic homeostasis of the cell, is under control of Na^+, K^+, Mg^{2+} and ATP. Due to the great importance of Na,K-ATPase in the maintenance of neuronal resting membrane potentials and propagation of neuronal impulses, the malfunction of this enzyme has been associated with neuronal hyper-excitability, cellular depolarization and swelling (Lees 1991). In numerous tissues, the activities of Na,K-ATPase may be influenced by different endogenous modulators (Rodrigez de Lores Arnaiz and Pena 1995, Balzan *et al.* 2000, Ewart and Klip 1995). Na,K-ATPase activity is decreased by toxic actions of normal neurotransmitters such as glutamate (Brines *et al.* 1995), which is the cause of cell injury and death of neurons, the basic events of cerebral ischemia in epilepsy and in various neurodegenerative disorders (Wyse *et al.* 2000, Grisar 1984, Lees 1993). Catecholamines induce marked stimulation of Na,K-ATPase activity by stimulation of β_2-adrenoceptors, leading to hyperpolarization of the cell membrane (Clausen and Flatman 1977).

Additionally, this enzyme may be under the influence of various exogenous factors including certain divalent metals and organic compounds of toxicological interest (Horvat *et al.* 1997, Nikezić *et al.* 1998, Vasić *et al.* 1999, 2002) as well as some drugs. It was demonstrated that antiallergic (Gentile *et al.* 1993), antiepileptic (Friel 1990, Stahl and Harris 1986) and opioid drugs (Samsonova *et al.* 1979, Rekhtman *et al.* 1980, Brase 1990) may exert opposite effects on Na,K-ATPase activity, while some beta-blocking agents and cardiac glycosides inhibit enzyme activity (Whikehart *et al.* 1991, Pelin 1998, Clausen 1998, Quadri and Ferrandi 1998) in various tissues.

With regard to the importance of this enzyme for the proper functioning of cells and tissues and in the induction of cytotoxicity, especially in nerve cells, the present study was undertaken in order to examine the effects of particular drugs on Na,K-ATPase. The effect of drugs from the class of cardiac (methyldigoxin, verapamil, propranolol), antiepileptic (carbamazepine), sedative (diazepam) and antihistaminic (promethazine) drugs on Na,K-ATPase in synaptic plasma membranes prepared from the whole rat brain were investigated. The information on pharmacological effects of these drugs on synaptosomal Na,K-ATPase activity is still lacking. Methyldigoxin was known to inhibit Na,K-ATPase in various tissues, but it was included in our experiments with the purpose to compare its efects with effects of other two antiarrhythmic drugs. There is little evidence for propranolol, while for verapamil and promethazine there is no information about their effects on brain Na,K-ATPase. Carbamazepine and diazepam were examined because their effect on synaptosomal Ca-ATPase, sodium channels and ATPDase were previously observed, but there is no information about their Na,K-ATPase activity modulation. Also, the effects of the chosen dugs on commercial porcine brain Na,K-ATPase were examined with the aim to evaluate if the effects of these drugs may act directly on the enzyme protein. In addition, extensive kinetic studies were undertaken to determine the nature of the drug action.

Methods

Chemicals

Methyldigoxin (β-methyldigitoxin) was obtained from ICN Pharmaceuticals, Inc., USA. Adenosine 5`-triphosphatase (sodium- and potassium-activated, ouabain-sensitive and vanadate-inhibited; EC 3.6.1.3.) purified from porcine cerebral cortex (ouabain-sensitive activity 0.4 U/mg protein), propranolol chloride (1-[iso-propylamino]-3-[1-naphthyloxy]-2-propanol), verapamil chloride, carbamazepine (5H-dibenz[b,f]azepine-5-carboxamide), diazepam (7-chloro-1-methyl-5-phenyl-3H-1,4 benzodiazepine-2(1H)-one) and promethazine and all other chemicals were purchased from Sigma-Aldrich (Germany).

Synaptosomal plasma membrane preparation

Experiments were performed on 3-month-old

male Wistar rats obtained from the local colony. Animals were kept under controlled illumination (lights on: 7:00 – 19:00 h) and temperature (23±2 °C), and had free access to commercial rat pellets and water. All experiments with animals were performed in accordance to the current European Convention. After decapitation with a small animal guillotine, the brains from 6 animals were rapidly excised for immediate synaptosomal plasma membrane (SPM) isolation. The SPMs were prepared according to the method of Towle and Sze (1983). The procedure of SPM preparation was described previously (Horvat *et al.* 1995). The purity of membrane preparation was analyzed in our preliminary experiments by electron microscopy and by activity of membrane specific enzyme. From micrography of SPM we observed that the preparation consists mainly of membrane vesicles, without significant contamination by other organelles including mitochondria, nuclei, lysosomes or endoplasmatic reticulum (Peković 1986). The inhibition of ATP hydrolysis by about 80 % with ouabain, a specific inhibitor of Na,K-ATPase, as well as the presence of adenylate cyclase activity also confirmed high levels of SPM in our preparation (Horvat and Metlaš 1984, Peković *et al.* 1986, 1997). The contribution of other ATP hydrolyzing enzymes in the SPM preparation were less than 15 %. From the inhibition of ATP hydrolysis by 5 mmol/l NaN_3 and 2 μg/ml of oligomycine which are specific for mitochondrial ATPase we concluded that the level of mitochondrial contamination was less than 7 %. Judging from the inhibition of Na,K-ATPase activity by various inhibitors of other ATPases (1 mmol/l theophylline, 1 mmol/l NaF as inhibitors of phosphodiesterase, membrane protein phosphatase, acid phosphatase) we concluded that no significant cross-contamination of these ATPases is present in our SPM preparation. Total lipids were 9 mg/ml of SPM samples and the proportion of lipid/protein was 1.56, which further confirms that our preparation consists mainly of plasma membranes. The protein content was determined by the method of Markwell *et al.* (1978) and total lipid were determined by a method introduced by Folch *et al.* (1957) and further developed by Bligh and Dyer (1959).

ATPase assay

Na,K-ATPase activities in SPMs were measured by colorimetric determinations of inorganic phosphate (Pi) liberated from ATP (ATP-Tris salt, vanadium-free) as previously described (Peković *et al.* 1997). Typical incubation mixture for the Na,K-ATPase activity measurement contained (in mmol/l): 50 Tris-HCl, pH 7.4, 1 EDTA, 100 NaCl, 20 KCl, 5 $MgCl_2$, 20 μg of SPM proteins and 2 ATP in a final volume of 200 μl. The reaction mixtures in the absence of ATP were preincubated for 10 min at 37 °C and incubated in the presence or absence of drugs for additional 30 min. The concentration range of the drugs applied in the enzyme assay is: 0.1 μmol/l - 0.1 mmol/l of methyldigoxin (MDO), 1 μmol/l - 20 mmol/l of propranolol (PPNL), verapamil (VP) and promethazine (PMZ) and 20 μmol/l - 60 mmol/l of carbamazepine (CMZ) and diazepam (DZ). After incubation, the enzyme reaction was started by the addition of ATP, allowed to proceed for 15 min and stopped by the addition of 3 mol/l trichloracetic acid. Samples were chilled on ice for 15 min and used for the assay of released inorganic phosphate. The activity of Na,K-ATPase was obtained by subtracting the activity in the absence of Na^+ and K^+ and presence of 2 mmol/l ouabain (specific inhibitor of Na,K-ATPase) from the activity obtained in the presence of Na^+, K^+ and Mg^{2+}. Activity of purified Na,K-ATPase from porcine cerebral cortex was performed as described previously in the presence of purified enzyme instead of SPM. Purified enzyme concentration in the assay was calculated according to the specific activity (liberated μmol Pi from ATP/mg protein/min). An appropriate protein concentration (0.0195 mg protein) was added to all mixtures. It liberates the same quantity of Pi from ATP as SPM under control conditions (7.8 nmol Pi/min). The results are expressed as the mean percentage of enzyme activity compared to the corresponding control (mean ± S.E.M.) of at least three independent experiments done in triplicate. Data were analyzed using Student's t-test and $p<0.05$ values were considered as statistically significant.

Kinetic analysis

Kinetic analysis was undertaken to determine the nature of the enzyme inhibition induced by drugs. The membranes were incubated for 30 min at 37 °C with or without the drugs concentrations that is calculated to inhibit 50 % of enzyme activity (IC_{50}) (1 mmol/l of PMZ, 2 mmol/l of VP, 3 mmol/l of PPNL per 200 μl of incubation mixture) in the presence of increasing concentrations of ATP, while maintaining the concentrations of other ions (Na^+, K^+, Mg^{2+}) and SPM protein concentrations (20 μg) constant. The kinetic constants (K_m, V_{max}) of Na,K-ATPase were determined in the presence or absence of specific drugs employing EZ-FIT program for PC. The apparent V_{max} was

expressed as μmol Pi/mg SPM proteins/min. and K_m as mmol/l of ATP.

Results

Vesicular orientation

The plasma membrane preparation consisted largely of sealed vesicles and lower portion of non-sealed membrane fragments, as observed by electron microscopy. Synaptosomal plasma membranes formed sealed vesicles by hypotonic lysis, which may be right-side out or of inverted orientation. To evaluate membrane sidedness, we investigated ATPase activity in the presence and absence of specific inhibitor ouabain (1 mmol/l) which interacts with the binding site located on the extracellular side of plasma membrane (Forbush 1982, Kinne-Saffran and Kinne 2001). In our SPM preparation, about 55 % of detected ATPase activity was inhibited by ouabain indicating proportion of exposed both ouabain and ATP binding sites: non-sealed, broken SPM vesicles or leaky vesicles. Right-side out vesicles expose their ouabain site but the activity of Na,K-ATPase could not be detected since ATP binding sites are inside of vesicles (Forbush 1982, Lopez *et al.* 2002). To determine vesicle orientation we also applied sodium dodecyl sulfate (SDS) and Tween 20 (Tw20) to permeabilize or open all vesicles. Since in right-side out vesicles ATP site of Na,K-ATPase is inaccessible, the increment in Na,K-ATPase activity in SDS or Tw20 treated SPM preparations would be expected to result from the accessibility gained by the substrate to its binding site of the ATPase. According to Na,K-ATPase activity detected in the presence of 0.2 mg of SDS/mg SPM proteins or 2 % Tw20 for 20 min before enzyme assay, which was increased about twofold (0.518 μmol Pi/mg/min for SDS and Tw20) in comparison with SDS/Tw20 untreated (0.249 μmol Pi/mg/min), we concluded that 52 % of total Na,K-ATPase activity are exposed by SDS and that their values represent a proportion of activity existing in right-side out sealed vesicles (Gill *et al.* 1986). This proportion of inverted and leaky vesicles and membrane fragments was 48 %. The results obtained from both treatments indicate that our membrane preparation mainly consisted of right-side out vesicles (52 %), leaky vesicles and non-sealed membrane fragments (26 %) and lower proportion (22 %) of inside-out oriented vesicles.

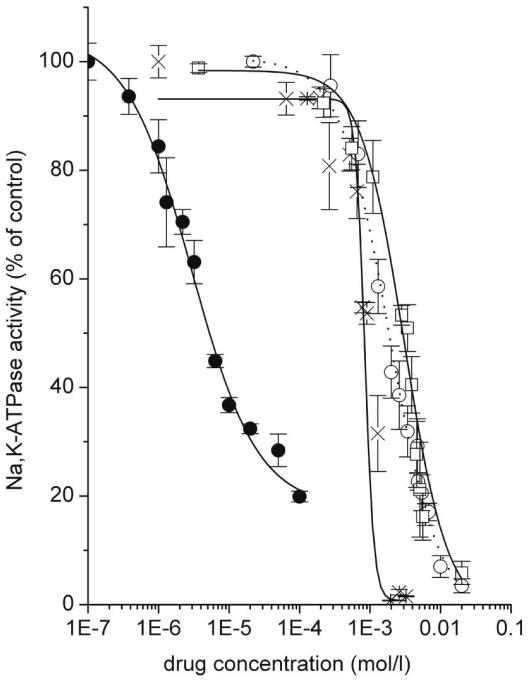

Fig. 1. Inhibition curves of Na,K-ATPase from SPM in the presence of various drugs. SPM (20 μg of proteins) were incubated 30 min in the presence of 0.1-100 μmol/l of methyldigoxin (●), 0.001-20 mmol/l of propranolol (□), verapamil (○) or promethazine (x) without ATP. After incubation, 2 mmol/l ATP (Tris-salt) was added and the enzyme reaction lasted 15 min. Results represent mean percentage of enzyme activity in respect to control velocity, without drugs (0.420 μmol Pi/mg/min) ± S.E.M., as determined from five separate experiments, each assayed in triplicate.

Effects of drugs on Na,K-ATPase activity

Effects of cardiac, antiepileptic, sedative and antiallergic drugs on the ATP hydrolytic enzyme activity were examined in isolated synaptic plasma membranes from whole rat brains. *In vitro* incubation of SPM with MDO, PPNL, VP, and PMZ for 30 min produced a dose-dependent inhibition of Na,K-ATPase activity. Anti-convulsant and sedative drugs, CMZ and DZ did not affect the activity of brain Na,K-ATPase (data not shown).

Figure 1 represents dose-dependent inhibition of Na,K-ATPase by particular drugs. It is known that digoxin inhibits cardiac Na,K-ATPase interacting with the ouabain-binding site on the α catalytic subunit of the enzyme (Repke *et al.* 1995, Jortani and Valders 1997, Lingrel *et al.* 1998). Comparing the effects of ouabain and methyldigoxin (data not shown), we concluded that brain Na,K-ATPase possessed a higher affinity for ouabain, but these two drugs produced similar shaped

inhibition-curves (IC_{50} were 2.195±0.42 μmol/l for ouabain and 2.97±0.38 μmol/l for MDO). Higher affinity of ouabain binding sites for ouabain than for digoxin were also found in ox and rat brain frontal cortex membranes (Mazzoni *et al.* 1990, Acuna Castroviejo *et al.* 1992). Maximum inhibition of the enzyme was achieved in the presence of 0.1 mmol/l of MDO. Incubation of SPM with antiarrhytmic drugs propranolol and verapamil similarly inhibited Na,K-ATPase activity in a dose-dependent manner. The inhibition was significant (p=0.002) at concentrations greater than 0.5 mmol/l for both drugs. Maximum enzyme activity inhibition of PPNL and VP were achieved at concentrations of 20 mmol/l.

The antihistaminic drug, promethazine, exerts a higher inhibition effect than the former two antiarrhythmic drugs with maximum inhibition at the concentration of 2 mmol/l. Significant enzyme activity inhibition was also detected at concentrations greater than 0.1 mmol/l.

Dixon plots (Dixon and Web 1987) of data for all drugs applied were used to determine whether drug binding was in equilibrium with inhibitory sites on the enzyme by plotting 100/(100 – %inhibition) vs. drug concentration. Linear Dixon plots implying equilibrium binding were obtained in all cases. The half-maximum inhibition (IC_{50}) was calculated from the Hill analysis of the experimental results. The IC_{50} values and Hill coefficient, n, determined from inhibition curves by the Hill analysis are summarized in Table 1. According to IC_{50} values, the potency order of applied drugs was: methyldigoxin >> promethazine > verapamil ≥ propranolol.

Table 1. IC_{50}, percentage of maximum inhibition and Hill coefficient (n) from *in vitro* application of various drugs on SPM (20 μg) and commercial porcine brain cortex (0.0078 U) Na,K-ATPase activity

drug	IC_{50} (mmol/l)		% of inhibition	
	SPM	Commercial	SPM	Commercial
methyldigoxin	0.00297±0.00038		80	
n	0.697±0.066			
propranolol	3.07±0.24	3.15±0.04	94	95
n	1.13±0.14	1.51±0.19		
verapamil	1.9±0.14	2.01±0.51	98	95
n	1.41±0.05	1.86±0.03		
promethazine	0.84±0.0005	0.165±0.02	98	95
n	2.62±0.29	1.80±0.23		
diazepam	no		no	
carbamazepine	no		no	

Table 2. Kinetic parameters of Na,K-ATPase from SPM in the absence (control) and presence of 3, 2 or 1 mmol/l propranolol, verapamil or promethazine, respectively, in incubation mixture (20 μg of SPM proteins).

	K_m mmol/l	V_{max} μmolPi/min/mg	Type of inhibition	Hill coefficient (n) for ATP
control	3.589±0.460	1.188±0.120		0.95±0.07
propranolol	1.272±0.215	0.346±0.023	uncompetitive	1.59±0.19
verapamil	1.409±0.163	0.388±0.019	uncompetitive	1.40±0.12
promethazine	1.079±0.368	0.470±0.058	uncompetitive	1.03±0.08

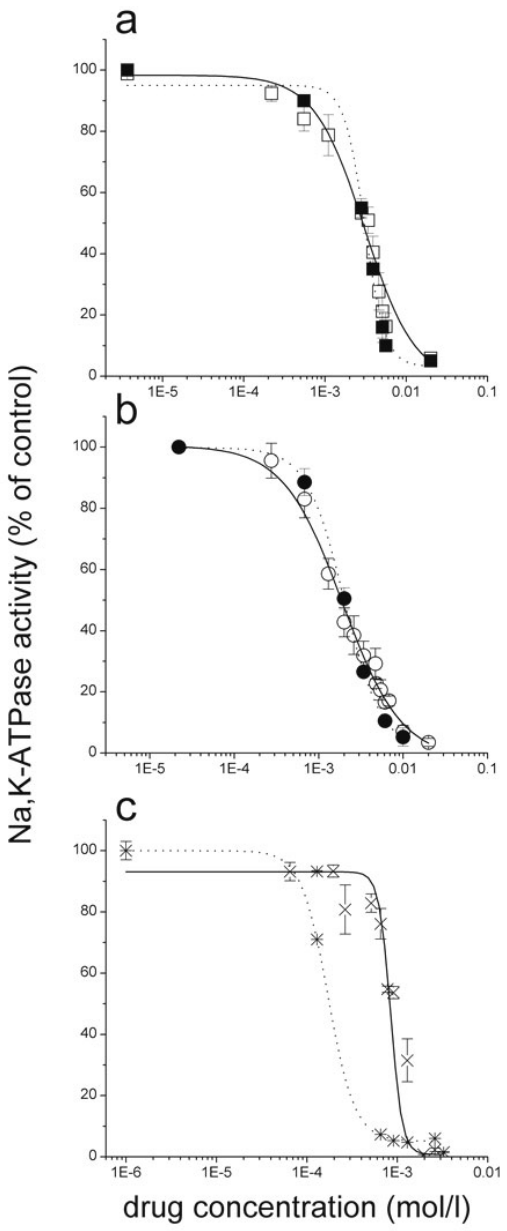

Fig. 2. Inhibition of Na,K-ATPase activity from SPM and purified-commercial enzyme. SPM (20 μg) and commercial Na,K-ATPase isolated from pig brain cortex (0.0078 U) were incubated with a) propranolol (□ for SPM, ■ for commercial enzyme), b) verapamil (○ for SPM, ● for commercial enzyme), or c) promethazine (x for SPM, ∗ for commercial enzyme). Incubations of both enzymes were done as described in the legend of Fig. 1. The results represent mean percentage of enzyme activity in the presence of drugs in respect to control velocity ± S.E.M., as determined from five separate experiments, each assayed in triplicate.

To evaluate if these drugs exert their effects acting directly on the sodium pump, we incubated Na,K-ATPase isolated and purified from porcine cerebral cortex (commercially available) and from SPM preparations (Fig. 2). The effect of MDO was not examined since it is known that this drug binds to the ouabain site on α subunits of the enzyme, as mentioned above. PPNL and VP identically inhibited enzyme

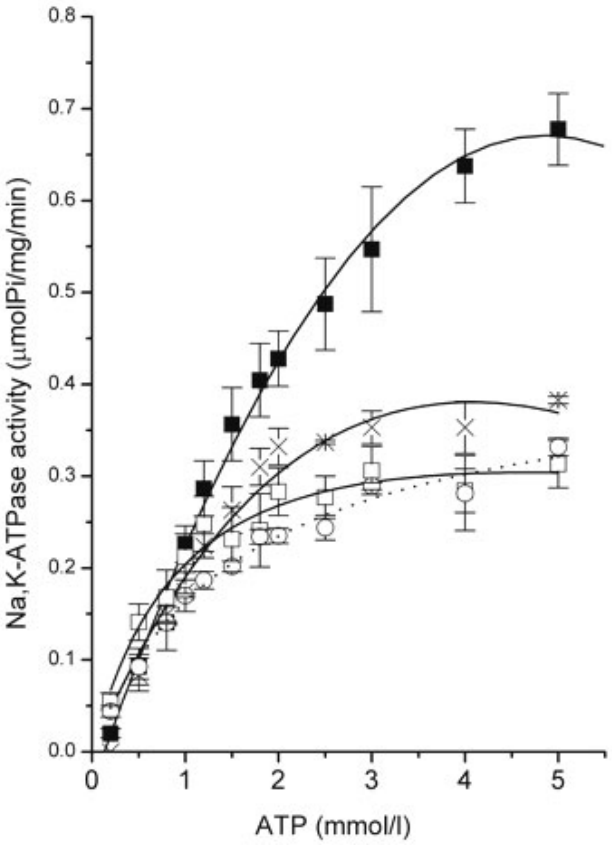

Fig. 3. Concentration-dependent activation of Na,K-ATPase with ATP. SPM (20 μg) was incubated without (■) and with 1 mmol/l of promethazine (x), 2 mmol/l of verapamil (○, dotted line) or 3 mmol/l of propranolol (□) for 30 min. After incubation, the enzyme assay was started by the addition of increasing concentrations of ATP (0.2-5 mmol/l). Results are presented as μmol Pi/mg/min ± S.E.M., as determined from five separate experiments, each assayed in triplicate.

activity from both sources (Fig. 2a,b). PMZ exerted total inhibition of Na,K-ATPase activity from both sources (Fig. 2c); according to IC_{50}, commercial Na,K-ATPase preparation possessed about fourfold higher sensitivity than the SPM preparation. Table 1 summarizes data obtained with *in vitro* incubation of SPM and commercial Na,K-ATPase.

Mechanism of action

To evaluate the nature of enzyme inhibition, the kinetic analyses of the effects of drugs mentioned above on the enzyme activation by substrate were carried out. Kinetic parameters, V_{max} and K_m, were determined by varying the concentration of ATP (0.2-5 mmol/l) in the presence and absence of the mentioned drugs. The effect of MDG on the kinetic properties of Na,K-ATPase was not examined for the reason mentioned previously. The effects of PPNL, VP and PMZ were determined in the presence of concentrations of 3, 2 and 1 mmol/l,

respectively. These particular concentrations were chosen from inhibition curves, as the IC_{50} concentrations.

The dependence of the reaction rate *vs.* ATP concentration for Na,K-ATPase in the presence and absence of chosen drugs exhibited typical Michaelis-Menten kinetics (Fig. 3). Kinetic constants, K_m and V_{max}, were calculated from the Eadie-Hofstee transformation of experimental data and are summarized in Table 2. The types of inhibition were analyzed from double-reciprocal plots of velocity vs ATP concentrations. However, K_m as well as V_{max} values decreased in the presence of all three drugs indicating an uncompetitive type of inhibition.

Discussion

In this paper we have investigated the effects of various drugs, whose pharmacological effects have not yet been studied on the rat brain synaptosomal Na,K-ATPase activity. The present study has shown that antiarrhythmic drugs propranolol (β-adrenergic receptor antagonist) and verapamil (calcium channel blocker) as well as antihistaminic drug promethazine (histaminic H_1 receptor blocker) inhibit rat brain Na,K-ATPase activity in a dose-dependent manner. In contrast, tricyclic antidepressant carbamazepine and anticonvulsant diazepam had no effects on Na,K-ATPase activity.

The results on cardiac sarcolemma showed that various antiarrhythmic drugs, other than PPNL and VP, inhibit Na,K-ATPase activity by interacting with the same or similar receptor site as ouabain (Almotrefi *et al.* 1999). To compare the efficacy of cardiac drugs, PPNL and VP, as a control effect, we measured the inhibition of the enzyme activity in the presence of cardiac glycoside, ouabain and methyldigoxin. It is well known that digoxin is a very potent and specific inhibitor of Na,K-ATPase activity in heart cells (Almotrefi *et al.* 1999), renal and red blood cells (Rodriguez *et al.* 1994). The inhibitory effects of *in vivo* applied digoxin have also been observed on Na,K-ATPase from the liver, muscle, renal medulla and aorta (Li *et al.* 1993). The catalytic α subunit of the enzyme is a site of inhibitory action of cardiac glycoside. In the rat brain, at least three isoforms of the α (α1, α2 and α3) subunit have been predicted from cDNA cloning experiments (Sweadner 1985, Herrera *et al.* 1987, Jewell *et al.* 1992) and they differ in amino acid composition, molecular weight, sensitivity to ions and cardiac glycosides like ouabain. Three ouabain inhibitory sites were detected and named: low-, high- and very high-affinity with IC_{50} in the range of mmol/l, μmol/l and nmol/l respectively. These sites contribute to three α subunit isoforms (α1 21 %, α2 50 % and α3 17 %) of Na,K-ATPase (Peković *et al.* 1997, Berrebi-Bertrand *et al.* 1990). The inhibition of SPM Na,K-ATPase activity by ouabain and MDO was very similar, as expected. Since their concentrations were in the range of μmol/l, it means that these inhibitors occupy very high- and high-affinity binding sites (α2 and α3) on Na,K-ATPase. The percentage inhibition of 80 % by MDO indicates an abundance of these α isoforms in the SPM preparations as had been found out for ouabain in our earlier investigation (Peković *et al.* 1997). Higher IC_{50} for MDO was found, which is in agreement with ouabain and MDO inhibition of [^{3}H]ouabain binding to rat cerebral cortex SPM and ox frontal cortex membrane (Acuna Castroviejo *et al.* 1992, Mazzoni *et al.* 1990). This may be explained by higher affinity of ouabain binding sites for ouabain.

The β-adrenoceptor antagonist, PPNL, and Ca-channel blocker, VP, are less potent antiarrhythmic drugs than MDO with higher IC_{50} values. Our findings may indicate that the acting site of PPNL and VP is not the ouabain site on the enzyme. PPNL and VP inhibit 94-98 % of Na,K-ATPase activity and according to IC_{50}, VP is a slightly more potent drug than PPNL.

It was found that PPNL, above concentrations of 20 μmol/l, affects membrane enzyme activity in cardiac sarcolemmal membranes by increasing membrane fluidity (Chatelain *et al.* 1989). Membrane fluidization with PPNL has also been observed on rat platelet membranes (Nosál *et al.* 1985), rat erythrocyte membranes (Weitman *et al.* 1989) and rat brain membranes at concentrations higher than 10 mmol/l (Ondriáš *et al.* 1987, 1989). Using electron spin resonance techniques, it was found that PPNL fluidizes these membranes in a depth-specific fashion by changing fluidity in the hydrophobic membrane core, influencing thus lipid-protein interaction. On the contrary, in cultured human and murine fibroblasts, it was found that PPNL exerts no effect on membrane fluidity (Eggl *et al.* 1986) and composition of phospholipids (Schroeder *et al.* 1981). The discrepancy in PPNL effects on membrane fluidity may be a consequence of tissue specific characteristics in the membrane content of some phospholipid species and cholesterol.

As for PPNL, the available data indicate that VP exerts a perturbation effect on the lipid part of liposomal membrane prepared from rat platelets (Ondriášová *et al.* 1992) and on rat brain total lipid liposomes (Ondriáš *et al.* 1991, 1992). VP has a bulk hydrophobic mass and its partition into lipid matrix decreases carrier-mediated ion flux at a concentration higher than 10 mmol/l (Shi and

Tien 1986). In our experiments, total inhibition of Na,K-ATPase with VP was found at a concentration above 10 mmol/l. In addition, the similarities which we have seen in IC_{50} for PPNL and VP on Na,K-ATPase and on ecto-ATPase of rat SPM (unpublished data) which activity is insensitive to fluidity changes (Bloj *et al.* 1973), indicate that the effects of these drug is not only due to membrane fluidization. Inhibition of NTPDase in vascular endothelial cells by VP and its metabolites was seen at concentrations higher than 0.1 mmol/l and Ki of these drugs ranged from 0.6-3.9 mmol/l (Gendron *et al.* 2000). Our results with purified Na,K-ATPase indicate similar effects of the investigated drugs on the enzyme affinity and velocity. Since we found that PPNL and VP inhibited SPM Na,K-ATPase activity to the same extent as purified Na,K-ATPase, it may be proposed that the drugs exert their effects by direct action on the brain enzyme protein and not by alterations of membrane fluidity. However, we have no information about lipid content in the commercially available enzyme. Since a phospholipid environment is necessary for the activity of Na,K-ATPase, it may be postulated that the purified Na,K-ATPase contains the enzyme incorporated in phospholipids. PPNL and VP, by inserting in the hydrophobic part of membrane phospholipids close to catalytic subunits, may affect the conformational change of enzyme protein, but the effects on the extramembrane part of ATPase cannot be excluded.

As confirmation of the existence of extramembrane site of action of antiarrhythmic drugs we calculated Hill coefficient from inhibition data, which indicated positive cooperativity in VP action, no cooperativity in PPNL action and negative cooperativity in MDO action. Since the type of inhibition by these cardiac drugs were different, we may conclude that they acted on different sites of the enzyme. According to kinetic parameters, the inhibitions of PPNL and VP were uncompetitive, decreasing maximum velocity and affinity for ATP. Both drugs change the type of ATP binding to Na,K-ATPase, from uncooperative to positive cooperativity type as compared to the enzyme under control conditions.

In our experiments, ATP hydrolyzing activity of the enzyme was detected in 48 % of isolated SPM, the high drug/protein concentrations ratio was a consequence of the high percentage of right-side out vesicles which bind the drugs and thus the effects could not be detected. According to this fact, the real drug/protein ratio is lower. Had it been possible to detect 100 % of ATPase activity, the inhibition curves would shift to the left in respect to those found in our experiments.

It was shown that some beta-blocking agents inhibit Na,K-ATPase in cultured corneal endothelial and epithelial cells up to 78 % (Whikehart *et al.* 1991). The results of Gopalaswamy *et al.* (1997) showed that PPNL inhibited brain Na,K-ATPase, Mg^{2+}-ATPase and Ca^{2+}-ATPase activity. These authors found that IC_{50} for all three enzymes were 1.5-1.8 mmol/l and that inhibition of Na,K-ATPase was of an uncompetitive type with respect to ATP. Our results were similar to those previously reported in respect to Na,K-ATPase. Minor discrepancy in the results may be caused by differences in the enzyme assay. We included PPNL into our study with the aim to determine its IC_{50} order with respect to other studied drugs.

According to our knowledge, the effect of VP on brain Na,K-ATPase has not been studied up to now. It was shown that some calcium channel blocking agents inhibit the activity of Na,K-ATPase in myocardial sarcolemma (Džurba *et al.* 1991) and for VP specific inhibition of the calmodulin-stimulated (Ca^{2+} + Mg^{2+})-ATPase activity was reported in human erythrocyte, rat and guinea pig ventricular sarcolemma and rat brain synaptosomes (Raess and Gerstten 1987, Kim and Raess 1988, Raess and Record 1990, Hoechen 1977, Dong and Xue 1994).

The effect of PMZ on brain Na,K-ATPase has not been examined up to now. It was found that some antiallergic drugs with anti-inflammatory action, but not PMZ, may increase the depressed platelet Na,K-ATPase activity, observed in allergic subjects and it was proposed that this modulation of the enzyme activity could be a possible mechanism of action for these drugs (Gentile *et al.* 1993). Some phenothiazines accumulate in the brain provoking dopamine receptor blockade (Paglini-Oliva and Rivarola 2003) and in rat hepatocytes, promethazine possesses an antioxidant effect (Albano *et al.* 1991). In human erythrocytes, it was proposed that PMZ, as cationic drug, was bound to and/or penetrated the intermembranes and induced hemolysis as a result of drug binding, forming mixed micelles with the membrane and disrupting membrane structure (Yamamoto and Aki 1991). Inhibition of Na,K-ATPase activity by PMZ has been seen in filarial parasite *Setaria cervi* (Agarval *et al.* 1990). According to our present results, PMZ is a potent inhibitor of rat brain SPM Na,K-ATPase activity producing total enzyme inhibition at concentrations of 2 mmol/l and with IC_{50} of 0.84 mmol/l. Commercial, pure porcine brain Na,K-ATPase was more sensitive to PMZ and IC_{50} was calculated to be 0.165 mmol/l. The lower

efficiency of PMZ on SPM Na,K-ATPase activity may be due to the environmental milieu in plasma membrane and partial coverage of binding site for PMZ, which is uncovered in the commercial preparation of the enzyme and by sidedness of SPM vesicles. Another possibility is the different species-dependent sensitivity of synaptosomal Na,K-ATPase. The effect on the pure commercial enzyme indicates that this drug directly affects the enzyme but these effects did not exclude effects *via* damage of the membrane, alteration in membrane fluidity and damage of cytoskeleton. The described effect on ATP dependent Na,K-ATPase activity suggests that this drug affects enzyme activity by decreasing both velocity and affinity for ATP (60 % and 70 %, respectively), indicating uncompetitive inhibition. The Hill coefficient calculated from inhibition data ($n>1$) indicates to the existence of positive cooperativity in the enzyme activity inhibition. PMZ in contrast to PPNL and VP did not change the no-cooperativity type of ATP binding to the enzyme ($n=1$).

It was found that the anesthetic drug, pentobarbital in concentration of 50 μmol/l, causes twofold decrease of the percentage of phosphatidyl-inositol, phosphatidylglycerol and phosphatidylserine which is intimately involved in divalent ion binding to membranes and may partially regulate activities of membrane-bound and ion transporting enzymes (Schroeder *et al.* 1981). Exploring pentobarbital action on ouabain receptor affinities of three isoforms of the catalytic subunit of Na,K-ATPase in rat brain and Na dependence of the enzyme activity, it was found that pentobarbital-induced anesthesia, caused fatty acid modification of brain membranes and significant sensibilization of α2 and α3 isoforms to ouabain. The authors concluded that pentobarbital-induced alterations could be related to a selective modification of the fatty acid composition and/or to the presence of a specific binding site for pentobarbital on these two neuronal digitalis receptors (Gerbi *et al.* 1997). Ondriáš *et al.* (1983) found that local anesthetics may incorporate into the lipid part of synaptosomes from rat brain and induce perturbation of the membrane. In our experiments, tricyclic antidepressant CBZ and anticonvulsant DZ which are anesthetics, had no effect on Na,K-ATPase activity in synaptosomal membranes. The absence of such an effect of CBZ and DZ indicates that these drugs did not induce perturbations of membrane lipid bilayer.

Abnormal functioning of synaptosomal Na,K-ATPase may be the cause of many different types of neurological disorders, since constant depolarization of the cell membrane induce abnormally excessive amounts of certain neurotransmitters to be released. As a consequence of reductions in sodium pump function by the drugs we have explored, destruction of the sodium gradient which drives the uptake of acidic amino acids and a number of other neurotransmitters may occur. This results in both a block of reuptake and a stimulation of the release not only of glutamate but also of other neurotransmitters which modulate the neurotoxicity of glutamate. An exocytotic release of glutamate and other neurotransmitters can also occur because of depolarization of the membrane as a consequence of inhibition of the enzyme (Lees 1991). In addition, increased intracellular concentrations of Na^+ as a consequence of Na,K-ATPase inhibition, will increase its exchange for Ca^{2+} by Na/Ca exchanger which may induce extensive neurotransmission. Retention of sodium may result in osmotic swelling and possible cellular lysis.

It was shown that under *in vivo* conditions the investigated drugs may pass the blood brain barrier and may even be accumulated in the brain of rodents or humans and released after membrane depolarization of nerve endings (Myers *et al.* 1975, Srivastava and Katyare 1983, Street *et al.* 1984, Bright *et al.* 1985, Mariyama *et al.* 1993, Hendrikse *et al.* 1998). Thus, our findings on the inhibition of brain Na,K-ATPase activity by the selected drugs *in vitro*, may be related to *in vivo* effects of these drugs. Under various pathological states, decreased activities of Na,K-ATPase were also found in the aged brain (Murakami and Furvi 1994, Park 1994). Decreased synaptosomal Na,K-ATPase activities were found in the actively spiking regions of epileptic temporal human cortex (Nagy *et al.* 1990, Nagy 1997). Additional inhibition of this enzyme with drugs investigated in this work may lead to further synaptosomal hyperactivity and increased brain tissue excitability in the epileptic brain. In conclusion, our findings point to the necessity of considering the side effects of the investigated drugs when treating various pathological conditions.

Abbreviations

SPM - synaptosomal plasma membranes
EDTA - ethylenediamine-tetraacetic acid
MDO - methyldigoxin
PPNL - propranolol
VP - verapamil
PMZ - promethazine
CMZ - carbamazepine
DZ - diazepam

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