Inhibition of Rat Brain Ecto-ATPase Activity by Various Drugs

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Abstract. The *in vitro* effect of digoxin, verapamil, propranolol, carbamazepine, diazepam and promethazine were investigated on the ecto-ATPase activity of synaptosomal plasma membranes from the rat brain. ATP hydrolyzing activities of the enzyme were not affected by digoxin while the use of all other drugs resulted in significant and dose-dependent ihibition in ATP hydrolysis. According to values of IC_{50} and K_{iapp}, the order of inhibitory potency of the drugs applied was: diazepam > promethazine > verapamil > propranolol >> carbamazepine. Kinetic analysis of the nature of the ATPase inhibition revealed that it resulted from a direct action of drugs on the enzyme protein. The aim of the present study was to determine the potential neuromodulatory side effects of the drugs investigated. The results achieved indicated that all investigated drugs, except digoxin, may modulate neuronal activities *via* the purinergic receptors P2 by increasing extracellular concentrations of ATP as a consequence of inhibition of the ecto-ATPase activity. Our findings indicate that it may be useful to take into consideration the possible side effects of the investigated drugs, when they are used in treatment of different pathologies, particularly in the treatment of epilepsy by carbamazepine and diazepam.

Key words: Ecto-ATPase — Synaptosomes — Drugs — Rat

Introduction

The ecto-adenosine triphosphatase (ecto-ATPase, EC 3.6.1.3) represents an integral membrane protein that, in the presence of divalent cations (Ca^{2+} or Mg^{2+}), hydrolyses extracellular nucleotides because of the outward orientation of its active site (Nagy 1986). By hydrolysing ATP to ADP, ecto-ATPase represents the major inactivating agent in purine-triphosphate signaling. The main product of this inactivation-signaling is ADP representing at the same time also the substrate

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of ecto-ATP diphosphohydrolase (ecto-ATPDase), the product of which, AMP, becomes then the substrate of 5′-nucleotidase. Hence, the final product of these reactions is adenosine. In central nervous system, similarly to other tissues, ecto-ATPase, ecto-ATPDase and 5′-nucleotidase may have multiple roles. They may influence several P1 and P2 receptors-mediated processes by controling extracellular concentrations of ATP and adenosine (Zimmermann et al. 1998; Bruno et al. 2002). ATP itself, as a neurotransmitter and neuromodulator is stored in the vesicles and co-released, together with other neurotransmitters, after depolarisation of the nerve endings (Gordon 1986; Zimmermann 1994). When released into the neural junction, ATP may modulate the release and/or influence other neurotransmitters either by acting through its own receptors or by altering the neurotransmitter receptors (Abbracchio1997; Gendron et al 2002). For instance, the binding of ATP to P2X purinoreceptors directly coupled to ionic channels either mediates fast excitatory neurotransmission or maintains long-term potentiation, by acting in this case as a substrate of ecto-protein kinase (Chen et al. 1996; Pankratov et al. 1998). *Via* activation of P2Y receptors, which are coupled to trimeric G protein, ATP activates the intracellular signaling cascade in order to mobilize the intracellular second messengers: Ca^{2+}, IP_3, diacylglycerol (DAG) (Zimmermann 1996; Gendron et al. 2002).

In central nervous system (CNS), adenosine as the final product of ecto-nucleotidases may inhibit neurotransmission by acting on A1 receptors. However, when acting on A2 receptors, adenosine may also exert a facilitatory action on neurons (Cunha 2001; Zalewska-Kaszubska 2002). In neuronal tissues, the activity and abundance of the ecto-ATPase are not distributed uniformly. This implies a specific control on the levels of extracellular ATP and adenosine in distinct brain areas (Nagy 1986; Nagy et al. 1990; Zinchuk et al. 1999).

No specific inhibitor of the ecto-ATPase has been found up to now. But, in diverse tissues, the activity of ecto-ATPase may be controlled by different endogenous modulators (Zimmermann et al. 1998; Nedeljković et al. 2000; Bruno et al. 2002) as well as by a variety of agents such as detergents, lectins, ATP analoges and drugs (Stout and Kirley 1996; Nagy 1997; Gendron et al. 2002). In respect to drugs, it was demonstrated that antiallergic agents (Agarwal et al. 1990) as well as tricyclic antidepressants and tranquilizers (Hehl et al. 1985; Garcia-Martin and Gutierrez-Merino 1990) may exert opposite effects on the ecto-ATPase activity.

In present study various cardioactive and antiarrhytmic drugs (digoxin, verapamil, propranolol), antiepileptics and anticonvulsants (carbamazepine, diazepam) as well as the antiallergic agent promethazine were investigated, in order to elucidate the links between their neuromodulatory action and the character of accompanying changes in activity of the brain ecto-ATPase. Kinetic studies on modulation of the ecto-ATPase activity were performed on synaptic plasma membranes isolated from whole rat brain.

Materials and Methods

Chemicals

NaN_3 was purchased from Merck (Germany), the other chemicals from Sigma (St. Louis, USA) and all were of analytical grade. Digoxin was obtained from ICN Pharmaceuticals (USA), all other drugs were from Sigma-Aldrich (Germany).

Synaptosomal plasma membrane preparation

Experiments were performed on 3 month's old (300–350 g) male albino 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 food (commercial rat pellets) and water. After decapitation, the brains of 6 animals were rapidly excised for immediate isolation of synaptosomal plasma membranes (SPM) according to Towle and Sze (1983). Details about the procedure and purity of SPM preparation have been described previously (Horvat et al. 1995). The level of mitochondrial contamination estimated on the basis of both morphology and marker enzymes was less than 7%. Protein content was determined by the method of Markwell et al. (1978). All animal experiments were performed in accordance with the current European convention and were also approved by the Ethical Committee of the Institute.

Enzyme assay

Measurements of ecto-ATPase activity were carried out in a reaction medium containing (in $mmol \cdot l^{-1}$): 50 Tris-HCl (pH = 7.4), 1 EGTA (to prevent an activation of the Ca^{2+}-ATPase), 5 $MgCl_2$, 20 μg SPM proteins and 2 ATP in a final volume of 200 μl. The activities of other ATPases representing intrinsic components of the SPM fraction and/or a contamination of the fraction by mitochondrial membranes (Na^+,K^+-ATPase, mitochondrial ATPase and the non-specific phosphatases) were estimated in the presence of their inhibitors (in $mmol \cdot l^{-1}$: 1 ouabain, 5 NaN_3, 1 teophilline, 1 NaF or 100 μg oligomycine$\cdot mg^{-1}$ SPM protein) according to Nedeljković et al. (2000 and 2003). Results revealed that the activity of all other ATP splitting enzymes together did not exceed 15% of the total ATPase activity of the preparation. Reaction mixtures were preincubated at 37°C in the absence of ATP for 10 min and for additional 30 min in the presence or absence of the drugs investigated. After this period, the enzyme reaction was started by the addition of ATP. It was stopped after 15 min by the addition of 22 μl of ice cold trichloroacetic acid (3 $mol \cdot l^{-1}$). The samples were kept on ice for further 15 min, and used for assay of the liberated inorganic phosphate (P_i), by using the method of Pennial (1966) and KH_2PO_4 as the reference standard. Results are given as means ± S.E.M. of at least 3 independent experiments done in triplicate. Specific activities of the enzymes are expressed in $\mu mol\ P_i \cdot mg\ prot^{-1} \cdot min^{-1}$, the inhibition by drugs is indicated in % of the control activity. Data were analysed by means of the Student's *t*-test, the values of $p < 0.05$ were considered as significant.

Enzyme kinetics

Membranes were incubated in absence or presence of drugs (diazepam and promethazine 0.5 mmol·l^{-1}, verapamil and propranolol 1 mmol·l^{-1} and carbamazepine 20 mmol·l^{-1}) at a constant concentration of Mg^{2+} ions (5 mmol·l^{-1}) and the presence of 20 μg SPM protein, with increasing concentrations of ATP (0.2–5.0 mmol·l^{-1}). Kinetic constants (K_m, V_{max}) of the ecto-ATPase were determined by employing the EZ-FIT software for PC. The apparent K_m and V_{max} values were expressed in μmol P_i·mg^{-1} SPM protein·min^{-1} and mmol·l^{-1} of ATP. Results were given as means ± S.E.M.

Drug concentrations in the dose-response studies in vitro

Membrane fraction was incubated (in conditions described in the part Enzyme assay) with investigated drugs in the following range of their concentrations: 0.1–100 μmol·l^{-1} of digoxin (12β-hydroxydigitoxin), 0.003–6 mmol·l^{-1} propranolol chloride (1-[isopropylamino]-3-[1-naphthyloxy]-2-propanol), 0.02–10 nmol·l^{-1} verapamil chloride, 0.004–100 mmol·l^{-1} carbamazepine (5H-dibenz[b,f]azepine-5-carboxamide), 0.003–1.5 mmol·l^{-1} benzodiazepam (7-chloro-1-methyl-5-phenyl-1,4-benzodiazepine-2(1H)-one) and 0.002–2 mmol·l^{-1} promethazine.

Results

Concentration-dependent effects of the drugs

30 min lasting dose-dependent effects of intentionally chosen cardioactive, antiepileptic, sedative and antiallergic drugs on the ecto-ATPase activity were studied *in vitro* by exposing isolated synaptic plasma membranes from whole rat brains (Fig. 1).

Digoxin, within the range of 0.1–100 μmol·l^{-1} did not affect the activity of the brain ecto-ATPase (data not shown). Propranolol and verapamil were inhibitory to the ecto-ATPase activity within the concentration range of 0.1–10 mmol·l^{-1}. At concentrations exceeding 1.5 and 0.2 mmol·l^{-1}, the inhibition by propranolol and verapamil reached the significance of $p < 0.01$. Maximum inhibition with both drugs was achieved at their 6 mmol·l^{-1} concentration and it amounted to 95 and 89.5 % of the control activity, respectively. The tranquilizing drug diazepam inhibited the ecto-ATPase already in concentrations exceeding 0.06 mmol·l^{-1}, with maximum inhibition of 98 % obtained at the concentration of 1.5 mmol·l^{-1}. Antiepileptic drug carbamazepine, an iminostilbene derivative of tricyclic antidepressants, exerted an inhibitory action to the ecto-ATPase within the concentration range of 10–100 mmol·l^{-1}. The maximum inhibition amounted to 91 %. The antihistaminic promethazine was inhibitory to the enzyme in concentrations exceeding 0.07 mmol·l^{-1}. Maximum inhibition amounting 97 % was registered with 2 mmol·l^{-1} concentration of the drug.

In order to estimate the dissociation constants of the enzyme-inhibitor complexes (K_{iapp}) for all studied drugs, we applied the Dixon plots (Dixon and Web

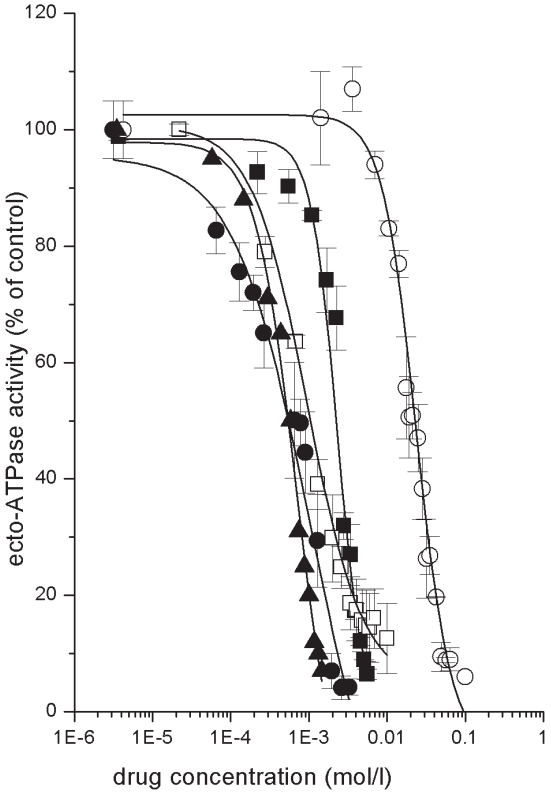

Figure 1. Concentration-dependent inhibition of ecto-ATPase activity by various dugs. Enzyme activities in the presence of various concentrations of diazepam (▲), promethazine (●), verapamil (□), propranolol (■) and carbamazepine (○) were expressed as % in respect to the control activity (no drugs 0.175 ± 0.026 μmol P_i/mg SPM/min). SPM (20 μg) were preincubated at 37°C with various concentrations of drugs for 30 min before adding ATP into the assay mixture. After 15 min of incubation with 2 mmol/l of ATP, liberated P_i was determined. Data represent the mean of triplicate determinations from at least three independent SPM isolations ± S.E.M.

1987). These plots were linear for verapamil only and indicated the presence of one ATP binding site and a non-competitive type of inhibition. However, the non-linear plots were obtained for all other drugs testified for more than one binding site on the enzyme molecule. Nevertheless, by means of this investigation, the possibility could not be excluded that the SPM preparation contained more than one enzyme splitting ATP, either. All values for half maximum inhibition (IC_{50}) and K_{iapp} are presented in the Table 1, and indicate that the investigated substances exhibit the following order of inhibitory potency in respect to the ecto-ATPase: diazepam > promethazine > verapamil > propranolol ≫ carbamazepine.

Effect of drugs on kinetics of activation of the ecto-ATPase by increasing concentrations of ATP

Investigation of ecto-ATPase activation by increasing concentrations of ATP (0.2–5 $mmol \cdot l^{-1}$, Fig. 2) using a PC software package (Vasić et al. 1999) revealed two distinct ecto-ATPase subtypes differing in their affinity to ATP. One subtype present in the SPM preparation, indicated as a high affinity ATPase, was activated by ATP concentrations below 2 $mmol \cdot l^{-1}$ and exhibited Michaelis–Menten kinetics. The

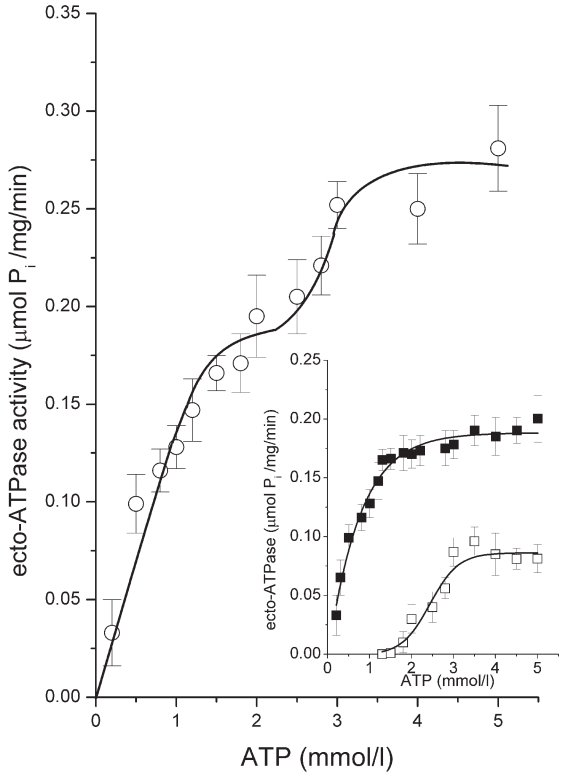

Figure 2. Substrate dependence of SPM ecto-ATPase activity. Activity of enzyme was assayed in the presence of increasing concentrations of ATP (0.2–5 mmol/l) while maintaining the concentrations of SPM (20 μg) constant. Activity of the enzyme was presented as μmol P_i/mg SPM proteins/min (○). Symbols represent mean ± S.E.M. of triplicate determinations from three independent SPM isolations. ATP-dependence of low affinity (□) and high affinity (■) ecto-ATPase activity are calculated by PC software package and presented as inset.

Table 1. IC_{50}, K_{iapp}, percentage of maximum inhibition and Hill coefficient of SPM ecto-ATPase activity in the presence of increasing concetrations of the studied drugs

Drug	IC_{50} (mol/l)	K_{iapp} (mol/l)	Inhibition (%)	Hill coefficient
propranolol	$2.44 \pm 0.63 \times 10^{-3}$	$2.33 \pm 0.32 \times 10^{-3}$	95	2.862 ± 0.384
verapamil	$9.72 \pm 0.20 \times 10^{-4}$	$1.10 \pm 0.05 \times 10^{-3}$	89	1.079 ± 0.432
carbamazepine	$2.1 \pm 0.23 \times 10^{-2}$	$1.19 \pm 0.05 \times 10^{-2}$	91	2.340 ± 0.150
benzodiazepine	$5.75 \pm 0.74 \times 10^{-4}$	$8.25 \pm 0.92 \times 10^{-5}$	98	2.680 ± 0.075
promethazine	$6.82 \pm 1.59 \times 10^{-4}$	$6.34 \pm 0.85 \times 10^{-4}$	97	0.681 ± 0.032

IC_{50} values and Hill coefficient were determined from concentration-dependent inhibition of ecto-ATPase activity, results represented in Fig. 1., by the Hill analysis and K_{iapp} from Dixon analyses of the same data.

other subtype was activated by substrate concentrations over 2 mmol·l^{-1} showed sigmoidal kinetics (Fig. 2 insert) and was indicated as a low affinity

Table 2. Kinetic analysis of high affinity ecto-ATPase activity on SPM in the absence (control) and presence of the studied drugs

Drug	V_{max} (μmol P_i/mg/min)	K_m (mmol/l)	Type of inhibition	Hill coefficient
control	0.224 ± 0.009	0.659 ± 0.026		2.1 ± 0.2
propranolol	0.093 ± 0.008	0.301 ± 0.089	uncompetitive	1.1 ± 0.5
verapamil	0.058 ± 0.001	0.944 ± 0.016	mixed	4.4 ± 0.7
carbamazepine	0.132 ± 0.005	0.476 ± 0.062	uncompetitive	1.6 ± 0.5
benzodiazepine	0.142 ± 0.016	0.964 ± 0.301	mixed	1.8 ± 0.6
promethazine	0.101 ± 0.007	0.857 ± 0.019	mixed	3.8 ± 0.2

Kinetic parameters, K_m and V_{max}, under control conditions and in the presence of: 1 mmol/l of propranolol and verapamil, 20 mmol/l of carbamazepine and 0.5 mmol/l of benzodiazepine and promethazine in 200 μl incubation mixture were calculated by Eadie–Hofstee transformation of the data. Type of inhibitions were judged from Lineweaver–Burk plots.

Table 3. Kinetic analysis of low affinity ecto-ATPase activity on SPM (20 μg) in the absence (control) and presence of the studied drugs

Drug	V_{max} (μmol P_i/mg/min)	K_m (mmol/l)	Type of inhibition	Hill coefficient
control	0.081 ± 0.007	2.650 ± 0.260		4.6 ± 0.3
propranolol	0.065 ± 0.005	3.401 ± 0.137	mixed	2.6 ± 0.3
verapamil	0.059 ± 0.002	2.821 ± 0.086	noncompetitive	2.2 ± 0.8
carbamazepine	0.059 ± 0.007	2.761 ± 0.275	noncompetitive	3.7 ± 0.2
benzodiazepine	0.065 ± 0.002	2.95 ± 0.310	noncompetitive	6.2 ± 1.8
promethazine	0.087 ± 0.001	2.814 ± 0.049	no inhibition	4.8 ± 0.2

Kinetic parameters, K_m and V_{max}, under control conditions and in the presence of: 1 mmol/l of propranolol and verapamil, 20 mmol/l of carbamazepine and 0.5 mmol/l of benzodiazepine and promethazine in 200 μl incubation mixture were calculated by Hill's plot. Type of inhibition was determined from Lineweaver–Burk transformation of the data.

ATPase. Similar types of plots were obtained also with the inhibiting drugs present in their K_{iapp} concentrations. For the high affinity ATPase this is demonstrated on a typical example – on the effect of carbamazepine (Fig. 3). The Eadie–Hofstee transformation of the respective data is demonstrated in the insert in Fig. 3. All data concerning kinetic parameters of the high affinity ATPase, in the presence and absence of the inhibiting drugs, are summarised in Table 2. The activity of the low affinity ATPase was calculated as the portion of the total ATPase activity after subtraction of the estimated high affinity ATPase activity. The effect of inhibiting drugs on kinetic parameters of the low affinity ATPase is also demnostrated on the

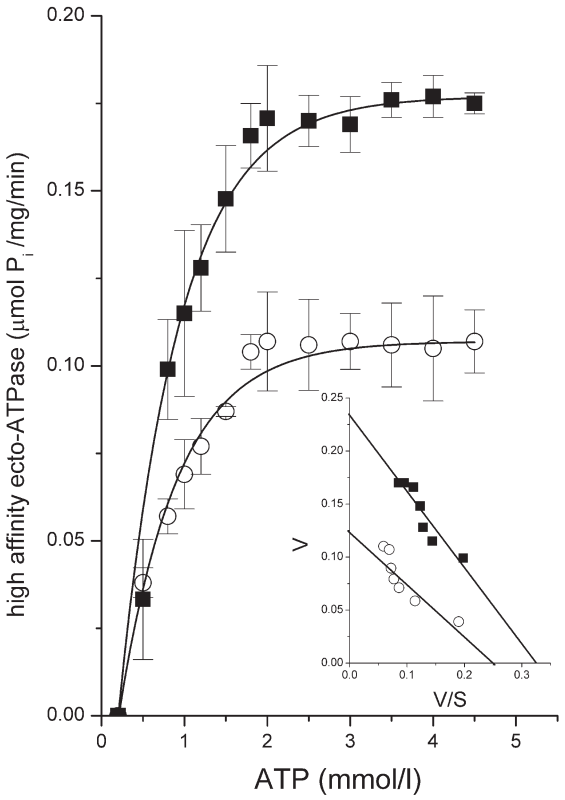

Figure 3. Michaelis–Menten plots of the high affinity ecto-ATPase activity in the absence (■) or presence (○) of carbamazepine in concentration of 20 mmol/l. SPM (20 μg) was preincubated at 37°C for 30 min in the absence or presence carbamazepine, without ATP. After that, additional 15 min of incubation was performed in the presence of increasing (0.2–5 mmol/l) concentrations of ATP. The values given are the mean of triplicate determinations from at least three experiments ± S.E.M. calculated by PC software package. The Eadie–Hofstee transformation of the data is shown in the inset, values of enzyme activity (V) was expressed as μmol P_i/mg/min and for substrate (S) as mmol/l.

typical example of carbamazepine action (Fig. 4). Kinetic parameters itself were obtained by using the Hill plots (see the insert in Fig. 4). Data concerning the kinetic parameters of the low affinity ATPase, in the presence and absence of all inhibiting drugs, are given in Table 3.

Inhibition of the high affinity ATPase activity by carbamazepine and propranolol appeared to be non-competitive in respect to ATP, while promethazine, diazepam and carbamazepine exerted a mixed type of inhibition, as judged from the Lineweaver–Burk plots. Promethazine exerted no inhibitory effect on the low affinity ATPase activity while propranolol and all other drugs behaved as weak inhibitors of the enzyme, exhibitig mixed type and non-competitive type of action, respectively.

Discussion

In the present study we investigated the effects of digoxin, propranolol, verapamil, carbamazepine, diazepam and promethazine on the activity of rat brain synapto-

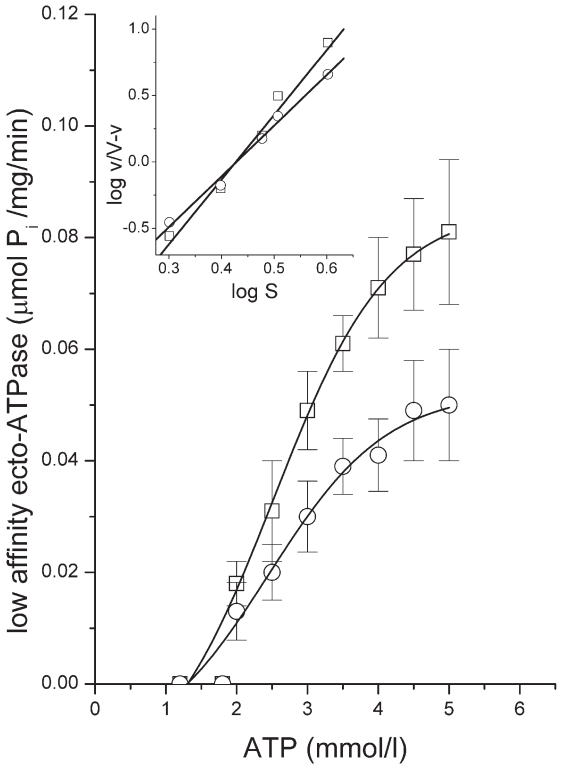

Figure 4. The activity of low affinity ecto-ATPase in the absence (□) and presence (○) of carbamazepine in concentration of 20 mmol/l in incubation mixture and in the presence of increasing concentrations of ATP. Activities were calculated by subtracting calculated high affinity values from total ATPase activities. The values given are the mean of triplicate determinations from at least three experiments ± S.E.M. The Hill transformation of data for the low affinity ecto-ATPase is given in the inset. Maximum enzyme activity detected (V) and current enzyme activity (v) were given as μmol P_i/mg/min and substrate concentrations (S) as mmol/l.

somal plasma membrane ecto-ATPase. The non-linear course of inhibition curves obtained by Dixon analysis testified either for presence of more than one binding site for the tested substances on the enzyme molecule or for more than one ATP-hydrolyzing enzyme present in the membrane preparation. Investigation of ATPase activation by increasing amounts of ATP revealed in SPM preparation the presence of two enzymes, differing in their affinity to ATP: the high and the low affinity ATPase. Similar biphasic activation of ecto-ATPase by increasing concentrations of ATP was also reported in human erythrocyte membrane (Morris et al. 1993). Owing to the already confirmed co-existence of ecto-ATPase and ecto-ATPDase in the same neuronal cells (Kegel et al. 1997; Zimmermann et al. 1998; Bruno et al. 2002 and Nedeljković et al. 2003), it was reasonable to assume that the ATP hydrolysing activity observed in our SPM preparation represented the total of catalytic actions of both the ecto-ATPase and the ecto-ATPDase. According to our earlier findings in SPM of the hippocampus and nucleus caudatus of rat brain, sodium azide (10 $mmol \cdot l^{-1}$) induced a 25 % inhibition of the ADPase activity in both preparations (Nedeljković et al. 2003). In respect to the recent report that sodium azide at higher concentrations inhibits, by interfering with the enzyme-ADP complex, the hydrol-

ysis of ADP by the ecto-ATPDase (Knowles and Nagy 1999), we assumed that the contribution of phosphate originating from ADP hydrolysis by ecto-ATPDase may represent a similar, i.e. 25 % portion of the total phosphate liberated by ATP hydrolysis in our SPM preparation. Since in our present study the V_{max} of the low affinity ATPase amounted to approximately 27 % of enzyme activity representing the total of ATP splitting by ecto-ATPase, ecto-ATPDase as well as the ADP splitting by ecto-ATPDase, we have assumed that the low affinity ATPase may, in fact, represent an ecto-ATPDase hydrolyzing ADP. An other important point is that, the concentration-dependent inhibition of ecto-ATPase and/or ecto-ATPDase by the applied drugs was investigated in the presence of 2 $mmol \cdot l^{-1}$ ATP, i.e. in condition for maximal stimulation of the high affinity ATPase. This allowed us to make two assumptions: i) the substrate-binding sites of both enzymes were, at least, near to fully saturated with ATP (Kukulski and Komoszynski 2003) and ii) in these conditions, the inhibitory effect of applied drugs concerned predominantly the ATP-hydrolyzing activities of the ecto-ATPase and ecto-ATPDase.

All applied drugs exerted dose-dependent inhibition of the enzymes except digoxin. However, the absence of inhibitory effect of digoxin could be expected, since it is well known that it is a specific inhibitor of the Na^+,K^+-ATPase activity. But, it was also welcome, because it confirmed that our assay system excludes any contamination with Na^+,K^+-ATPase-dependent ATP hydrolysis.

All other tested drugs inhibited the ecto-ATPase and ecto-ATPDase activities almost completely, whereby, according to the K_{iapp} and IC_{50} values, diazepam exhibited the strongest and carbamazepine the weakest inhibitory effect. Hill coefficients revealed and the Dixon plots confirmed a positive cooperativity for propranolol, diazepam and carbamazepine and negative cooperativity for promethazine binding to the enzymes. In the case of verapamil binding, the Hill coefficient of 1 and a linear Dixon plot excluded any cooperativity in binding of the drug to ecto-ATPase and ecto-ATPDase.

It was already reported, that propranolol and verapamil may modulate the activities of some enzymes in the brain, particularly by inhibiting the Ca^{2+}-ATPase activity (Garcia-Martin and Gutierrez-Merino 1990; Dong and Xue 1994; Gopalaswamy et al. 1997). The same was reported for propranolol and carbamazepine and the Na^+,K^+-ATPase (Sawas and Gilbert 1981; Wood et al. 1989; Murakami and Furui 1994; Gopalaswamy et al. 1997). Carbamazepine, as a specific adenosine A1 receptor antagonist (Biber et al. 1999), is also known to exhibit a spectrum of anticonvulsant activities, although the mechanism of its action is not yet completely elucidated. Its therapeutic action involves reduction of the brain cell excitability, inhibition of Ca^{2+}-influx into synaptosomes as well as reduction of the voltage-dependent Na^+-current (Backus et al. 1991; Ragsdale and Avoli 1998; Crowder and Bradford 1987). Besides the already mentioned inhibition of the brain Na^+,K^+-ATPase activity in pathological conditions (Wood et al. 1989; Murakami and Furui 1994), the inhibition of ecto-ATPase and ecto-ATPDase, described in this study, may represent an additional way of carbamazepine action on the brain. Our results are in harmony also with the findings of Barcellos et al. (1998) and Schetinger et

al. (2000) describing an inhibition of ATP and ADP hydrolysis by diazepam and the new 1,5 benzodiazepines in rat cererbral cortex. It was also shown that some phenothiazines, but not promethasine, inhibited the activities of both the Mg^{2+}- and Na^+,K^+-ATPase of rat liver plasme membranes (Samuels and Carey 1978), the beef heart soluble Mg^{2+}-ATPase (Palatini 1982) as well as the ecto-ATPase and Ca^{2+}-ATPase of human lymphocyte plasma membranes (Lichman et al. 1982; Hehl et al. 1985). Nevertheless, as concerns promethasine itself there are not available any data about its modulatory effect on brain or mammalian tissue enzymes.

Our kinetic studies revealed that the tested drugs exerted only very mild inhibitory effects on the ADP hydrolyzing activity of the ecto-ATPDase, i.e. on the low affinity ecto-ATPase and promethazine failed completely to influence the activity of this enzyme. The inhibition exerted by propranolol was of mixed type and of the other drugs of non-competitive character. Hill coefficients did not reveal any characteristic changes in substrate binding. Nevertheless, the data about the influence of applied drugs on the ADP splitting will still require further experiments with ADP as substrate.

Verapamil, diazepam and promethazine inhibited the activity of the high affinity ATPase by 74, 37 and 55 % and increased the K_m values by 43, 46 and 30 %, respectively, thus decreasing the affinity of the enzyme to ATP. The results indicated mixed, competitive and non-competitive types of inhibition. Positive cooperativity of substrate binding to the enzyme did no change with these three drugs. Propranolol and carbamazepine exerted an non-competitive type of inhibition, lowering the V_{max} values by 58 and 41 % and the K_m values by 54 and 28 %, respectively. This indicated that the latter two drugs affected the ATP-enzyme complex. In addition, propranolol and carbamazepine also changed the characteristics of substrate binding to the enzymes.

It was speculated that the investigated drugs may interact with membrane phospholipids inducing changes in membrane fluidity (Gopalaswamy et al. 1997; Varga et al. 1999). In our current study we found different types of drug action on the high affinity and low affinity components of the ecto-ATPase/ecto-ATPDase. If the drugs acted *via* disruption of membrane fluidity, the inhibitory effect would be similar for both the ATP and ADP hydrolysis by the ecto-ATPase/ecto-ATPDase, however, this was not the case. Therefore, we could conclude that the drugs propranolol and carbamazepine affected the activity of the high affinity enzyme on the enzyme molecule and at a locus distant from the ATP-binding site. However, in the case of verapamil, diazepam and promethazine, because of the mixed type of inhibition, we could not exclude their action on the ATP-binding site either. In the case of propranolol it could be proposed that the drug may exert a week inhibitory effect by acting in ADP binding site of the ecto-ATPDase.

By inhibiting ecto-ATPase and ecto-ATPDase activities, all chosen drugs may increase the extracellular ATP content and decrease the production of adenosine. This represents a further way by which these drugs may modulate the neuronal activity. ATP, acting *via* its P2Y receptors, may influence a variety of metabolic pathways, channels and neuronal functions (Zimmermann 1996; Insel et al. 2001).

When activating an other type, the P2X receptors which are directly coupled to ionic channels, ATP may mediate the synaptic efficiency *via* the glutaminergic, dopaminergic and nociceptive synapses (Inoue et al. 1996; Chen et al. 1996; Pankratov et al. 1998).

It was shown that some of the investigated drugs may reach *in vivo*, in canine and human plasma, concentrations up to 10^{-5} mol·l^{-1} (Chelly et al. 1987; Fuhr et al. 2002) and that they can pass the blood brain barrier and may even accumulate in the brain of rodents and humans (Myers et al. 1975; Schneck et al. 1977; Srivastava and Katyare 1983; Moriyama et al. 1993; Hendrikse et al. 1998). Hence, our findings on the inhibition of brain ecto-ATPase and ecto-ATPDase by the chosen drugs *in vitro* might be also related to their *in vivo* effects. It was noticed that chronic administration of diazepam decreased both the A1 and A2 receptors in brain regions and inhibited the uptake of adenosine in the cortex as well (Hawkins et al. 1988). These results suggest that adenosine receptors, the extracellular content of adenosine and ATP as well as the inhibition of ecto-ATPase and ecto-ATPDase reported in this paper, may play a role in action of diazepam in the CNS. It was found, that in the human actively spiking regions of the epileptic temporal cortex, the activities of synaptosomal ecto-ATPases are decreased (Nagy et al. 1990; Nagy 1997). Therefore, an additional inhibition of ecto-ATPase and ecto-ATPDase that might be caused by the drugs investigated in this paper, may lead to further synaptosomal hyperactivity and increased tissue excitability in the epileptic brain.

It may be summarised that our findings point to the need to consider the side effects of the investigated drugs, when they are used for treatment of various pathologies, particularly for the treatment of epilepsy by carbamazepine and diazepam.

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