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Ecto-ATPase and Ecto-ATP-diphosphohydrolase Are Co-localized in Rat Hippocampal and Caudate Nucleus Synaptic Plasma Membranes

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Summary

Enzymes that hydrolyze extracellular ATP, *i.e*. ecto-ATPase and ecto-ATP diphosphohydrolase (ATPDase), can be differentiated by ability of the latter to hydrolyze ADP and by slightly different kinetic properties of the two enzymes. Synaptic plasma membrane fractions isolated from rat hippocampus and caudate nucleus exhibit ADP-hydrolyzing activity, as revealed by the enzyme assay, and the presence of ecto-ATPase protein, as revealed by immunological identification on Western blot. These findings indicate that both enzymes are co-expressed in the synaptic membrane compartment of hippocampal and caudate nucleus neurons. Kinetic analysis was performed to determine the relative contribution of each enzyme to the total ATP-hydrolyzing activity, while an inhibition study was carried out in order to exclude the interference of other nonspecific ATPase and phosphatase activities. Based on the kinetic properties, sensitivity to inhibitors and V_{ATP}/V_{ADP} ratio of about 2, we concluded that a substantial portion of ATP-hydrolyzing activity in both synaptic membrane preparations can be ascribed to the catalytic action of ATPDase. On the other hand, the highest catalytic efficacy when ATP is the substrate and the greater abundance of ecto-ATPase protein in caudate nucleus preparation suggest that the relative contribution of ecto-ATPase to the total ATP-hydrolyzing activity in the caudate nucleus is higher than in the hippocampus.

Key words
Ecto-ATPase • Ecto-ATPhydroxylase • Hippocampus • Caudate nucleus • Rat

Introduction

The importance of extracellular purine nucleotides, such as ATP and adenosine, has recently begun to be appreciated. In the CNS, extracellular nucleotides have been implicated in many important physiological processes, including purinergic signaling (Zimmermann 1994), neuromodulation (Fredholm *et al.* 1993), neurogenesis (Weaver 1996), apoptosis (Abbracchio *et al.* 1995, Bronte *et al.* 1996) and learning (Bonan *et al.* 2000). ATP is released from a variety of cells in several regions (Fredholm *et al.* 1993, Zimmermann 1996) and acts at two receptor types, P2X and P2Y. Extracellular ATP is rapidly inactivated by a

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surface-located enzyme cascade (ectonucleotidases), which sequentially hydrolyze extracellular ATP to adenosine as a principal metabolite (Todorov *et al.* 1997).

Enzymes that hydrolyze extracellular ATP can be divided into two subclasses, both activated by millimolar concentrations of divalent cations: *(1)* ecto-ATPase (E.C. 3.6.1), which also hydrolyzes other nucleoside 5'-triphosphates, and *(2)* ecto-ATPDase (or ecto-apyrase, E.C. 3.6.1.5), which is capable of hydrolyzing either ATP or ADP and other nucleoside 5'-tri- and diphosphates (Zimmermann 1996). Although the two enzymes exhibit a slightly different sensitivity to inhibitors (Plesner 1995, Heine *et al.* 1999), they share a close sequence homology (Handa and Guidotti 1996) which hinders their immunological distinction (Lewis-Carl and Kirley 1997).

In the CNS, the presence of ATP hydrolyzing activity was reported in neuronal and glial cell lines (Stefanovic *et al.* 1976), synaptosomes (Nagy *et al.* 1986, Bonan *et al.* 2000, Schetinger *et al.* 2001), synaptic membrane preparations (Nedeljkovic *et al.* 1998, 2000) and synaptic vesicles (Lin and Way 1984) obtained from different brain areas (Kegel *et al.* 1997). In the previous studies, we analyzed the rate of hydrolysis and kinetic properties of ATP-hydrolyzing activity of synaptic membrane preparations (SPM) isolated from distinct rat brain areas (Nedeljkovic *et al.* 1998, 2000, Banjac *et al.* 2001). However, since both ecto-ATPase and ecto-ATPDase are capable of hydrolyzing ATP, it is not clear to which enzyme the observed hydrolysis of ATP can be attributed. In an attempt to answer this question, we examined the rate of ATP and ADP hydrolysis, kinetic properties and sensitivity to inhibitors of ATP-hydrolyzing activity of synaptic membrane preparations isolated from the rat caudate nucleus and hippocampus. The obtained results and applied criteria for ecto-nucleotidase classification (Dhalla and Zhao 1988, Smith and Kirley 1998) indicate that ecto-ATPase and ecto-ATPDase are colocalized in the synaptic plasma membrane fraction from the rat hippocampus and caudate nucleus.

Material and Methods

Chemicals

NaN_3, orthovanadate and diethylpyrocarbonate were purchased from Merck. Nucleotides, SDS-PAGE, immunoblot reagents and other chemicals were obtained from Sigma Chemical Co. Antirabbit IgG alkaline phosphatase conjugated antibody was purchased from ICN Pharmaceuticals. Beckman rotors were used in all centrifugation preparations.

Animals

Three-month-old rats (320-350 g) of the Wistar strain were used. Five animals per cage were kept at constant temperature, humidity and 12-h light-dark cycle and they had free access to food and water. The animals were decapitated with a guillotine (Harvard Apparatus), the brains were rapidly removed and placed in buffered 0.32 mol/l sucrose, pH 7.4 for dissection. The hippocampi (Hip) and caudate nuclei (CN) were pooled for immediate use of synaptic plasma membrane preparations.

Synaptic plasma membrane preparation

Synaptic plasma membrane preparations (SPM) were prepared essentially according to the procedure of Gray and Whittaker (1962) as previously described (Nikezic *et al.* 1996). The protein content was determined by the method of Markwell *et al.* (1978).

Immunoblotting

SPM isolated from Hip, CN and neuronal cytosol (negative control) were diluted with Laemmli SDS sample buffer to a final protein concentration of 1 mg/ml each. The samples were vortexed and boiled for 5 min. Ten micrograms of each sample were resolved by 5-12 % SDS-PAGE gradient gels according to Laemmli (1970). The separated proteins were transferred onto a PVDF membrane by transblotting overnight at a constant current of 40 mA, in a Mini Trans-blot Transfer Cell System (BIO-RAD). The PVDF membrane was blocked for two hours with 2 % BSA in Tris-buffered saline, Tween 20 (TBST) and incubated overnight at room temperature in 1:1000 dilution of the anti T-tubule polyclonal antibody (Stout and Kirley 1994). The antibody was raised against a 12 amino acid residue peptide representing N-terminus of rabbit T-Tubule ecto-ATPase, which was shown to be homologous to the N-terminal region of the mammalian ecto-ATPase (Stout *et al.* 1994). The PVDF membrane was washed, incubated in antirabbit IgG alkaline phosphatase conjugated secondary antibody for two hours, washed again and developed with NBT/BCIP colorimetric system, as described by the manufacturer.

Ecto-nucleotidase assays

Rates of ATP- and ADP-hydrolysis were measured by colorimetric determination of liberated

inorganic phosphate (Pennial 1966) as previously described (Nedeljkovic *et al.* 1998). All incubations were carried out in a water bath at 37 °C for 10 min. The ATP-hydrolyzing activity was measured in a medium containing (in mmol/l): 50 Tris-HCl, pH 7.8, 1 EGTA, 5 $MgCl_2$, ATP (0.1-5) and 20 μg of membrane preparation in a final volume of 200 μl. ADPase activity was measured in the same incubation medium, with varying concentrations of ADP (0.1-5 mmol/l) instead of ATP. The effect of different inhibitors was investigated in order a) to assess the purity of the SPM preparation, b) to evaluate the interference of other Mg^{2+}-dependent ATPase activities which are the intrinsic components of the membrane, and c) to evaluate their effect on ecto-nucleotidase activity. The inhibitors used were: NaN_3 (5 or 10 mmol/l), NaF (1 mmol/l), teophylline (1 mmol/l), diethylpyrocarbonate (3 mmol/l), oligomycine (2 μg/ml), ethanol (0.5 %), ouabain (1 mmol/l) and orthovanadate (0.1 mmol/l). Since 0.1 mmol/l orthovanadate repeatedly exerted more than 20 % inhibition on the total ATPase activity it was included in all incubation mixtures.

Enzyme kinetic analysis

To determine ecto-nucleotidase kinetics, the activity was measured following incubation with ATP or ADP (concentration range 0.1-5 mmol/l). The relationship between reaction velocities and different substrate concentrations was presented in a Michaelis-Menten plot, whereas kinetic constants V_{max} (apparent maximal velocity) and K_m (apparent Michaelis constant) were calculated by non-linear regression analysis using the Prisma Software Enzyme Kinetics Program. The apparent V_{max} was expressed as μmol Pi /μg SPM/min.

Statistical analysis

The results are presented as means ± S.E.M. from at least three independent determinations performed in triplicate. The data were analyzed using Student's *t*-test and $p<0.05$ values were considered significant.

Results

Immunodetection

Figure 1 presents the representative immunoblot of CN and Hip SPM preparation using the anti T-Tubule ecto-ATPase polyclonal antibody (Stout and Kirley 1994). In both SPM preparations the antibody recognized the 70-kDa band, characteristic for ecto-ATPase. The band is more prominent in NC than in Hip, indicating a higher abundance of ecto-ATPase protein in the caudate nucleus preparation. In addition, in both SPM preparations the antibody recognized another, less abundant immunoreactive band at approximately 80 kDa, and the third immunoreactive band, at about 66 kDa in the CN preparation alone.

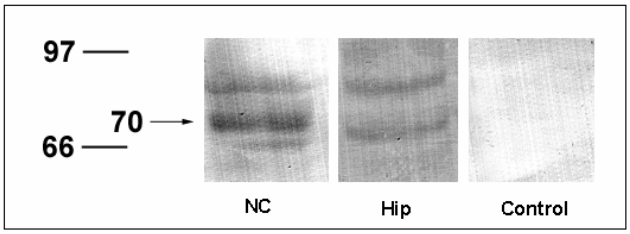

Fig. 1. *Immunoblot analysis of SPM preparations isolated from caudate nucleus (CN) and hippocampus (Hip) and neuronal cytosol (negative control). Samples were resolved on SDS-PAGE, transferred to PVDF membrane and probed with the anti T-tubule antibody. Arrows indicate the position of molecular weight markers.*

Enzyme kinetics

Figure 2 shows the rates of ATP and ADP hydrolysis of SPM isolated from CN, in the presence of higher ATP or ADP concentrations (0.1-5 mmol/l), respectively. Using the Michaelis-Menten plot and an enzyme kinetic analysis program (Prisma Software Enzyme Kinetic Program), ATPase and ADPase kinetic constants, K_m and V_{max}, were determined (Table 1). As shown, the apparent affinity for ADP hydrolysis is significantly higher than for ATP ($p<0.01$), but the ATP-hydrolyzing activity has almost double the maximum velocity ($p<0.001$). Inset to Figure 1 shows linear double reciprocal plots (1/[S] *vs.* 1/V) of ATP and ADP hydrolyzing activities, which both follow hyperbolic enzyme kinetics.

Figure 3 shows the patterns of activation of ATP and ADP hydrolyzing activities by increasing substrate concentrations (ATP or ADP, ranging from 0.1-5 mmol/l), in SPM isolated from hippocampus. The kinetic constants were determined by non-linear regression (Table 1). Analysis of data shows that the apparent affinity for ADP is almost 2.5-fold higher than for ATP ($p<0.01$), however, the ATP hydrolyzing activity reaches almost double the maximum velocity ($p<0.05$). The inset in Figure 2 shows linear double-reciprocal plots of hyperbolic enzyme kinetics.

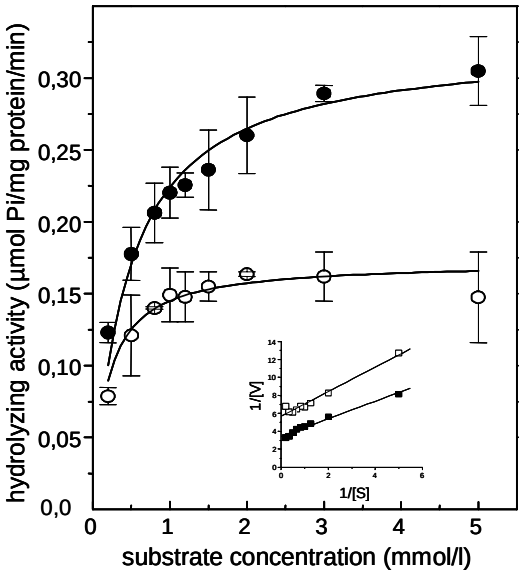

Fig. 2. *Rate of ATP- and ADP hydrolysis of SPM isolated from caudate nucleus in the presence of raising ATP (solid square) or ADP (open square) concentrations (0.1-5 mmol/l). The rate of hydrolysis is expressed as µmol Pi liberated/mg SPM protein/min as evaluated by the spectrophotometric determination. Symbols represent mean activity ± S.E.M. from four independent determinations performed in triplicate. Solid lines represent the best fit obtained by using Prisma Software Enzyme Kinetic Program. Inset to Figure: the linear double reciprocal plot (1/[S] vs. 1/V) of ATP (solid square) and ADP (open square) hydrolyzing activities.*

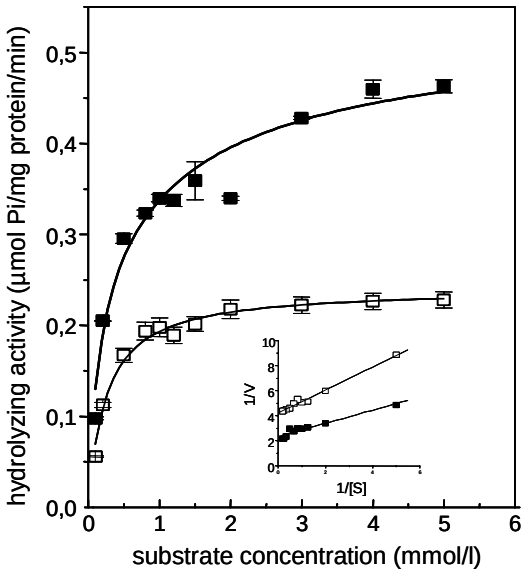

Fig. 3. *Rate of ATP and ADP hydrolysis of in the SPM isolated from hippocampus in the presence of varying ATP (solid circle) or ADP (open circle) concentrations (0.1-5 mmol/l). The rate of hydrolysis is expressed as µmol Pi liberated/mg SPM protein/min. Symbols represent mean activity ± S.E.M. from three independent determinations performed in triplicate. Solid lines represent the best fit obtained by using Prisma Software Enzyme Kinetic Program. Inset to Figure: the linear double reciprocal plot (1/[S] vs. 1/V) of ATP (solid square) and ADP (open square) hydrolyzing activities.*

Table 1. Kinetic parameters of ATP- and ADP-hydrolyzing activities in caudate nucleus and hippocampal SPM preparations.

Preparation	**Caudate nucleus**		**Hippocampus**	
Hydrolyzing activity	**ATP-**	**ADP-**	**ATP-**	**ADP-**
Kinetic parameters				
K_m *(mmol/l)*	0.335 ± 0.066*	0.245 ± 0.024	0.447 ± 0.091*	0.184 ± 0.050
V_{max} *(µmol Pi/mg /min)*	0.463 ± 0.020**$^{\#}$	0.241 ± 0.050	0.324 ± 0.017*	0.172 ± 0.018
V_{ATP} /V_{ADP}	1.92 ± 0.19		1.80 ± 0.14	
V_{max}/K_m	1.38 ± 0.03*$^{\#}$	0.98 ± 0.21	0.72 ± 0.19	0.93 ± 0.16
n_H	0.68 ± 0.17*	1.18 ± 0.11$^{\#}$	0.59 ± 0.06	0.36 ± 0.49

*Kinetic constants V_{max} and K_m were obtained by non-linear regression analysis of the data presented in Figs 2 and 3, using the Prisma Software Enzyme Kinetics Program. Hill coefficient was calculated according to the Hill equation, and V_{ATP} /V_{ADP} and V_{max}/K_m were derived from basic constants. * indicates significant difference between kinetic parameters obtained for ATP- and ADP-hydrolyzing activities in the same SPM preparation, (*p<0.05; ** p<0.001); $^{\#}$ indicates the significance between the respective kinetic parameters obtained in caudate nucleus and the hippocampus ($^{\#}$ p<0.05).*

Table 1 also displays V_{ATP} /V_{ADP} ratios between the rates of ATPase and ADPase activity in the same SPM preparation. The ATPase/ADPase ratio is slightly higher in CN (1.92) compared to Hip (1.80), but the difference is not statistically significant. Table 1 also displays V_{max}/K_m ratios which represent enzymatic physiological efficacy or "kinetic power" of an enzyme reaction (Duggleby and Clarke 1991). The data indicate that despite the differences in kinetic parameters, the physiological efficacy of ADP-hydrolyzing activity is the same in CN and Hip ($p>0.05$). On the other hand, the physiological efficacy of ATP-hydrolyzing activity is almost 2-fold higher in CN compared to Hip ($p<0.001$) and about 40 % higher compared to the ADP-hydrolyzing activity of CN ($p<0.01$). Table 1 presents Hill coefficients (n_H) for ATP- and ADP-hydrolyzing activities, as calculated according to Hill equation. ATP-hydrolyzing activity in both SPM preparation exhibits $n_H < 1$, which indicates negative cooperativity in substrate binding; the same holds for ADP-hydrolyzing activity in Hip. On the other hand, the Hill coefficient for ADP-hydrolyzing activity in CN of $n_H > 1$, suggests a positive cooperativity in ADP binding.

Inhibition study

Table 2 summarizes the effect of different inhibitors on the rate of ATP and ADP hydrolyses in SPM preparations from CN and Hip. As is shown, ATP and ADP hydrolysis were not significantly affected by inhibitors of different membrane and intracellular ATPases (ouabain, 5 mmol/l NaN_3 and oligomycin) or non-specific alkaline phosphatases (NaF, teophylline), indicating that SPM preparations were of satisfactory purity and that other non-specific Mg^{2+}-dependent ATPase activities did not interfere with ATP- or ADP-hydrolyzing activities. Other inhibitors, *i.e.* diethylpyrocarbonate (DEPC) and 10 mmol/l NaN_3, were found to be as potent, but non-specific inhibitors of ecto-ATPase and ecto-ATPDase, respectively. DEPC, an effective ecto-ATPase inhibitor (Saborido *et al.* 1991) did not alter either ATP- or ADP-hydrolysis. On the other hand, despite the fact that inhibition of both nucleotide phosphate activities at 5 mmol/l NaN_3 was insignificant, inhibition of ADP-hydrolyzing activity at 10 mmol/l NaN_3 was enhanced by 25 % ($p<0.05$) with respect to the control.

Table 2. Effect of inhibitors on ATP- and ADP-hydrolysis in SPM preparations isolated from the caudate nucleus and hippocampus

	Residual activity in the presence of inhibitor (% of control)			
Preparation	**Caudate nucleus**		**Hippocampus**	
Inhibitor	**Total ATPase activity**	**ADPase activity**	**Total ATPase activity**	**ADPase activity**
NaN_3 (5 mM)	94 ± 11	92 ± 7	98 ± 1	90 ± 2
NaN_3 (10 mM)	94 ± 2	76 ± 7*	87 ± 7	75 ± 8*
DEPC (3 mM)	96 ± 1	103 ± 1	97 ± 2	95 ± 10
NaF (1 mM)	93 ± 1	94 ± 2	92 ± 3	93 ± 6
teophylline (1 mM)	94 ± 2	95 ± 2	93 ± 1	–
oligomycine (2 μg/ml)	100 ± 3	94 ± 1	102 ± 7	102 ± 1
ethanol (0.5 %)	79 ± 7**	89 ± 8	80 ± 6*	82 ± 12
ouabain (1 mM)	96 ± 11	98 ± 1	90 ± 7	99 ± 6

*Data represent the mean activity ± S.E.M. relative to the control activity obtained without inhibitor (100 %), from three experiments performed in duplicate. Significance level shown inside the table, determined by Student's t-test: * p>0.05; ** p>0.001*

Discussion

Enzymes that hydrolyze extracellular ATP, *i.e.* ecto-ATPase and ecto-ATP diphosphohydrolase were detected in the brain, in different neuronal (Hohmann *et al.* 1993) and glial cell lines (Dalmau *et al.* 1998) and subcellular fractions (Schetinger *et al.* 2001). They were even co-expressed in the same cell (Heine *et al.* 1999) or neuron (Kegel *et al.* 1997), but in mutually exclusive locations (Lewis-Carl and Kirley 1997) indicating that

they perform different physiological functions. In our study, both CN and Hip synaptic membrane preparations exhibited ADP-hydrolyzing activity (Figs 2 and 3), as well as the presence of ecto-ATPase protein, as revealed by immunological detection using an anti-ecto-ATPase antibody (Fig. 1). Thus, ecto-ATPase and ecto-ATPDase are present in synaptic membrane preparations of CN and Hip, indicating that they are co-localized in the same compartment of the plasma membrane. This finding, although surprising because ecto-ATPDase alone would be capable of catalyzing degradation of ATP, suggests that the pattern of sequential ATP hydrolysis in these brain areas requires the presence of both ectonucleotidases.

Besides the 70-kDa band, the immunological identification with anti-tubule antibody also revealed the presence of another band, namely in the 80-kDa region. This band could represent the ecto-ATPDase, which has a molecular weight of about 80 kDa (Lewis-Carl and Kirley 1997). In fact, the sequence similarity between ecto-ATPase and ecto-ATPDase is substantial and in some species two proteins apparently differ in 16 amino acids only (Asai *et al.* 1995). Thus, the presence of the second 80-kD band on the immunoblot could be explained by immunological cross-reactivity, which was also reported in other ectonucleotidase studies and with the use of different antibodies (Lewis-Carl and Kirley 1997, Kirley and Stout 1997). The third band recognized by the anti-ecto-ATPase antibody in the CN preparation may be the truncation product of the mature enzyme or the isoenzyme of ecto-ATPase of lower molecular weight.

Since the data suggest that ecto-ATPase and ecto-ATPDase are co-localized in SPM preparations of CN and Hip, inhibition studies were performed in order to evaluate the purity of SPM preparations and the involvement of other non-specific Mg^{2+}-dependent ATPase or phosphatase activities in the hydrolysis of ATP. The inhibitors were chosen for their ability to inhibit other ATPases (ouabain, NaN_3, oligomycine) and phosphatases (NaF, teophylline) and their inability to affect ecto-ATPase (Nagy *et al.* 1986) or ecto-ATDPase (Schetinger *et al.* 2001). Ouabain, 5 mmol/l NaN_3, oligomycin, NaF and teophylline showed little or no inhibition effect on the hydrolysis of ATP or ADP. On the other hand, 0.1 mmol/l orthovanadate repeatedly inhibited total ATPase activity for 21-24%. It was previously shown that orthovanadate does not inhibit ecto-ATPase (Sarkis and Salto 1991, Cote *et al.* 1992). Thus, we have concluded that orthovanadate inhibited some other Mg^{2+}-dependent ATPase activity and that it was also included in all incubation mixtures.

Since the observed ATP hydrolyzing activity represents the sum of the catalytic action of both ecto-ATPase and ecto-ATPDase, we have further applied biochemical criteria for distinguishing them, in order to get insight into the relative contribution of each enzyme to the total ATPase activity. An inhibition study revealed that DEPC, a potent inhibitor of ecto-ATPase, in a concentration of 3 mmol/l and 5 mmol/l azide were ineffective. On the other hand, 10 mmol/l azide inhibited ADPase activity of both SPM preparations by about 25 %. It was recently reported that azide at higher concentrations inhibits ADP hydrolysis of ATPDase, by interfering with the enzyme-ADP complex (Knowles and Nagy 1999).

Comparison of substrate-dependence properties revealed a similar pattern of activation by ATP of CN and Hip, but lower specific activity on the plateau was detected in Hip. The same holds when only ADP was available as a substrate. In each preparation, the apparent affinity for ADP was higher than for ATP, however, the V_{ATP}/V_{ADP} ratios were about 2. The $V_{ATP}/V_{ADP} > 1$ is a characteristic property of the ecto-ATPDase. Thus, the ratio of purified human brain ecto-apyrase was 2.75 (Smith and Kirley 1998), and those reported for rat synaptosomes (Nagy *et al.* 1986) and bovine synaptic membranes (Hohmann *et al.* 1993) were about 2. Furthermore, in each preparation the catalytic efficiency (V_{max}/K_m) was greater with ATP as a substrate, which is consistent with previous findings for purified chicken oviduct ecto-ATPDase (Knowles and Nagy 1999).

It was reported that ecto-ATPase displays many non-standard enzymatic properties, such as nonlinear double reciprocal plots and negative cooperativity in ATP binding (Moulton *et al.* 1986). In our study, both nucleoside phosphate hydrolyzing activities in each SPM preparation exhibited linear double reciprocal plots (insets in Figs 2 and 3), which is indicative of hyperbolic enzyme kinetics reported for ecto-ATPDase (Kirley *et al.* 1999). However, ATPase in both SPM preparations exhibited negative cooperativity in ATP binding, as deduced from Hill´s coefficient of $n_H < 1$.

In summary, the immunological identification of ecto-ATPase and the presence of ADP-hydrolyzing activity, suggest that ecto-ATPase and ecto-ATPDase are colocalized in the presynaptic membrane compartment. This finding further implies that observed ATP-hydrolyzing activity in SPM preparations is the sum action of both enzymes capable of hydrolyzing ATP.

Based on the kinetic properties, sensitivity to azide and lack of inhibition by DEPC and V_{ATP}/V_{ADP} ratio of about 2, we concluded that a substantial portion of ATP hydrolyzing activity can be ascribed to the catalytic action of ATPDase. On the other hand, the similar rate of ADPase activity, but higher ATPase activity of CN compared to the Hip preparation, the highest catalytic efficacy of CN preparation when ATP was substrate and greater abundance of ecto-ATPase protein in the CN, led us to suggest that the contribution of ecto-ATPase to the total ATP hydrolyzing activity in CN is higher than in Hip, confirming the importance of the ecto-ATPase in the mechanism of ATP signaling in the caudate nucleus.

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