Properties of Mg^{2+}-ATPase in Rat Brain Synaptic Plasma Membranes

N NEDELJKOVIĆ[1], G NIKEZIĆ[1], A HORVAT[1], S PEKOVIĆ[2], M STOJILJKOVIĆ[2] AND J V MARTINOVIĆ[1]

1 *Laboratory of Molecular Biology and Endocrinology, Vinča Institute of Nuclear Sciences, P O B 522, 11001 Belgrade, Yugoslavia*

2 *Department of Neurobiology and Immunology, Institute for Biological Research, 11000 Belgrade, Yugoslavia*

Abstract. In the present study distribution and enzymatic properties of ecto-Mg^{2+}-ATPase were determined in synaptic plasma membrane (SPM) preparations isolated from the hippocampus, caudate nucleus and whole brains of female rats Western blot analysis using anti-ecto-Mg^{2+}-ATPase antibody revealed the association of Mg^{2+}-ATPase with SPM prepared from all the three brain sources, yet the enzyme was most abundant in caudate nucleus membranes, being 30% and 22% more abundant than in the hippocampal and whole brain tissue SPM, respectively The evidence is also presented that kinetic properties of the brain Mg^{2+}-ATPase are not under the control of circulating sex steroids It was confirmed that the enzyme is activated by millimolar concentrations of Mg^{2+} and that it cannot be effectively inhibited by known ATPase inhibitors The most pronounced differences in kinetic properties observed were 2 5 fold higher apparent affinity for ATP and 59% higher specific activity of Mg^{2+}-ATPase of the caudate nucleus as compared with the enzyme from the hippocampus On the other hand, the apparent enzyme affinity for Mg^{2+} was almost equal in all SPM preparations tested Taken together, our results show that ecto-Mg^{2+}-ATPase is not uniformly distributed and differs in respect to affinity for ATP in rat brain regions, thus indicating its substantial role in the process of signal transduction via controlling the levels of extracellular ATP

Key words: Ecto-Mg^{2+}-ATPase — Synaptic plasma membranes — Hippocampus — Caudate nucleus

Correspondence to Dr Nadežda Nedeljković, Laboratory of Molecular Biology and Endocrinology, Vinča Institute of Nuclear Sciences, P O B 522, 11001 Belgrade Tel and Fax (++38-1-11)-455561 E-mail nnedel@rt270 vin bg ac yu

Introduction

The Mg^{2+}-stimulated ATPase belongs to ecto ATPases, a group of enzymes that are localized in the plasma membrane with the active site facing the extracellular compartment The enzyme has been detected in a variety of tissues (Stout and Kirley 1994) and appears most abundant in excitable ones In the brain, Mg^{2+}-stimulated ATPase activity has been found in neuronal and glial membranes (Trams and Lauter 1978, Sorensen and Mahler 1982), especially in neurotransmitter storage vesicles (Toll and Howard 1980) and synaptic plasma membranes (Sorensen and Mahler 1981) A great progress has recently been made in isolation and purification of brain Mg^{2+}-ATPase (Hohmann et al 1993), detection of amino acid sequence (Stout and Kirley 1994) and characterization of basic enzymatic and biochemical properties (Hohmann et al 1993) The enzyme purified from bovine synaptosomal plasma membrane shares the typical biochemical properties reported for ecto-ATPases The enzyme is about equally activated by millimolar concentrations of Ca^{2+} or Mg^{2+}, has optimum pH between 7 5 and 8 5, and cannot be effectively inhibited by known ATPase inhibitors In addition, Mg^{2+}-ATPase displays very broad substrate specificity, hydrolyzing a variety of purine and pyrimidine 5′-nucleotides (Hohmann et al 1993) Despite the above mentioned efforts the physiological role of brain Mg^{+}-ATPase remained unclear, yet it was suggested that the enzyme has an important role in the extracellular metabolism of brain nucleotides (James and Richardson 1993) Furthermore, the role of Mg^{+}-ATPase in pathophysiology of epilepsy and progressive nerve cell degeneration is still subject of controversy (Allen and Seyfried 1994)

One approach to the understanding of the general function of brain Mg^{2+} ATPase is to determine its distribution and enzymatic properties in distinct brain regions The present study was undertaken in order to examine the presence and kinetic properties of Mg^{2+} ATPase in the synaptic plasma membranes prepared from the hippocampus (Hip), the caudate nucleus (NC), and for comparative purposes, the whole brain tissue (WB) In addition, these brain regions were chosen in view of the studies on the possible role of Mg^{2+}-ATPase in dopaminergic transmission (Desaiah and Ho 1977), since the caudate nucleus is relatively rich in dopamine, while the hippocampus has prevailingly a noradrenergic content The regional distribution and enzymatic properties of the enzyme in the brain are expected to reflect the pattern of its physiological activity

Materials and Methods

3-month-old female rats of the Wistar strain (300 350 g) were used After decapitation with a guillotine (Harvard Apparatus), the brains from 13 animals for each experiment were rapidly removed and placed into buffered 0 32 mmol/l sucrose,

pH 7.4 for dissection. Whole brains, hippocampi and caudate nuclei were pooled (3/pool for whole brains and 10/pool for brain regions) for immediate preparation of synaptic plasma membranes.

Synaptic plasma membrane preparation

Synaptic plasma membranes (SPM) were prepared according to the method of Cohen et al. (1977) as modified by Towle and Sze (1983) and as described elsewhere (Horvat et al. 1995). In brief, SPMs were isolated by differential centrifugation in a discontinuous sucrose gradient (0.8 mol/l – 1.2 mol/l). The band containing SPM was removed from the interface of 1.0 mol/l and 1.2 mol/l sucrose, pelleted by centrifugation at 15,000 × g for 20 minutes, resuspended in 5 mmol/l Tris-HCl, pH 7.4, and kept at −70 °C until use. The level of mitochondrial contamination estimated on the basis of both morphological and enzymatic markers was less than 10%. Additional characterization of the SPM preparation is given elsewhere (Peković et al. 1997). Protein content was determined using the method of Lowry et al. (1951), as modified by Markwell et al. (1978).

Western Blot Analysis

Samples (30 μg of SPM protein) were solubilized in Leamlli SDS-reducing (5% β-mercaptoethanol) buffer and heated at 100 °C for 4 min. The samples were then resolved by SDS-PAGE with a 4% stacking gel and a 8% resolving gel according to Leamlli (Leamlli 1970), and electroblotted onto Hybond nitrocellulose membranes at 55 V for 2 hours. After blocking with buffer containing 3% bovine serum albumin (BSA) in Tris buffer saline-Tween 20 (TBST), the blots were incubated overnight in a 1/1000 dilution of anti rabbit T-Tubule ecto-ATPase antiserum (kind gift by Dr. James Stout, University of Cincinnati) in 1% BSA in TBST. The antiserum was raised against a 12 amino acid residue peptide representing N-terminus of rabbit T-Tubule ecto-ATPase (Stout and Kirley 1994) which was shown to be homologous to the N-terminal region of the mammalian ecto-Mg^{2+}-ATPase (Stout et al. 1994). After washing blots in TBST and incubation for 1 hour with rabbit anti-mouse IgG alkaline phosphatase conjugated second antibody (ICN Pharmaceuticals), the presence of ecto- Mg^{2+}-ATPase was detected with the NBT/BCIP colorimetric method. The blots were scanned by a Pharmacia Ultra Scan XL laser densitometer. The quantification was performed by integrating band areas using an Origin 3.5 PC software package.

ATPase assay

Mg^{2+}-ATPase activity was measured by colorimetric determination of inorganic phosphate (Pi) liberated from ATP, as described previously (Peković et al. 1997). Activation of Mg^{2+}-ATPase by increasing concentrations of magnesium ions was estimated by incubating 15 μg SPM protein in a 200 μl incubation medium containing 50 mmol/l Tris-HCl, pH 7.4, 1 mmol/l EDTA, 1 mmol/l ouabain, 1 mmol/l

ATP and $MgCl_2$ within a range of 0.1–10 mmol/l. Ouabain at concentration 1 mmol/l inhibits effectively Na, K-ATPase (Peković et al. 1997). The reaction mixtures were preincubated at 36 °C for 4 minutes. The reaction was started by the addition of ATP, allowed to proceed for 5 minutes, and terminated by the addition of 3 mmol/l perchloracetic acid and immediate cooling on ice.

Activation of Mg^{2+}-ATPase by increasing concentrations of ATP within a range of 0.1–6 mmol/l was estimated under conditions similar to those used for the activation by magnesium. The reaction was allowed to proceed for 5 minutes in the presence of 5 mmol/l $MgCl_2$.

Effects of various ATPase inhibitors on Mg^{2+}-ATPase activity of WB were measured using 5 mmol/l $MgCl_2$ and 1 mmol/l ATP in the presence of 15 μg SPM protein. The data are expressed as the mean percent inhibition relative to the control (no drug added) ± S.E.M. of three independent determinations done in duplicate. The specific activity of the control was always between 0.495 and 0.520 μmol Pi/mg protein/min.

Concentrations of free Mg^{2+} were calculated using the FORTRAN IV program (Storer and Cornish-Bowden 1976). Stability constants were those used by Pershadsingh and Mc Donald (1980). Levels of endogenous Ca^{2+} and Mg^{2+} were determined by atomic absorption, and were found to be 11.5 μmol/l and 7.5 μmol/l, respectively.

Statistics

All measurements were performed in duplicate and are expressed as mean ± S.E.M. of several (n) separate determinations. The results were analyzed by Student's t-test, and considered significant at $p < 0.05$.

Results

Effects of various ATPase inhibitors on Mg^{2+}-ATPase activity

The effects of different inhibitors were investigated using SPM from WB in order to eliminate other ATPase activities that are either intrinsic components of the membrane or are due to contamination. It was found that 1 mmol/l ouabain decreased Mg^{2+}-ATPase activity by 5%, probably due to some residual Na,K-ATPase activity. Sodium azide (5 mmol/l) and oligomycin (2 mg/ml) decreased the enzyme activity reproducibly by 9 ± 1 and 6 ± 2%, respectively. Finally, teophylline and sodium fluoride, inhibitors of non-specific alkaline phosphatases, both at concentrations of 1 mmol/l did not affect Mg^{2+}-ATPase activity of the whole brain SPM.

Immunodetection

The presence of ecto-Mg^{2+}-ATPase was detected in SPM prepared from WB, Hip and NC by using Western blot analysis with an anti-peptide antibody previously

Figure 1. Immunodetection of Mg-ATPase in female rat brain tissues Immunoreactive bands are seen at approximately 70 kDa The following SPM samples (30 μg) were resolved by SDS-PAGE and blotted onto nitrocellulose membranes WB, whole brain tissue, Hip, hippocampus, NC, caudate nucleus Blot was prepared and incubated with the anti rabbit T-tubule ecto-ATPase antibody (1 1000) Antibody-antigen complexes were detected by the NBT/BCIP color development, and intensity of signals was quantified by laser densitometry

shown to recognize the N-terminal region of the rat brain Mg^{2+}-ATPase A representative Western blot of SPM preparations probed with the antiserum is shown in Fig 1 An immunoreactive protein band at approximately 70 kDa was present in SPM from all the 3 sources Relative enzyme abundance was the highest in NC, being 30% higher than in Hip and 22% higher compared to WB SPM preparation

Kinetic constants

Mg^{2+} ion (0 1–10 mmol/l $MgCl_2$ added) stimulated the hydrolysis of ATP in a concentration-dependent manner The enzyme was not stimulated by Mg^{2+} at concentrations lower than 0 1 mmol/l added Since there are large variations in the concentrations of nucleoside phosphates and metal ions in composite mixtures, we measured the dependence of the Mg^{2+}-ATPase rate on free Mg^{2+} concentrations in the presence of 1 mmol/l ATP (Fig 2) Kinetic constants were calculated according to Eadie-Hofstee plot, using 8 concentrations of free Mg^{2+} (0 249 nmol/l 8 02 mmol/l Mg^{2+}) The enzymes from all 3 sources displayed a similar pattern of activation by Mg^{2+}, still Mg^{2+}-ATPase in NC exhibited the highest maximum specific activity (0 667 $\pm$ 0 080 μmol Pi/mg protein/min) The enzyme reached almost equal maximum specific activity in WB and Hip (0 519 μmol Pi/mg protein/min and 0 488 μmol Pi/mg protein/min, respectively) Analysis of the data showed that there were no significant differences in Km values and that the half-maximum activation occurred at about 0 44 μmol/l free Mg^{2+} (0 9 mmol/l $MgCl_2$ added)

In the presence of 5 mmol/l $MgCl_2$ added the enzyme from all three SPM preparations was maximally stimulated by 0 5–1 5 mmol/l ATP, displaying similar behavior for increasing ATP concentrations (Fig 3) However, the kinetic constants differed markedly, indicating the highest apparent affinity for ATP and the highest

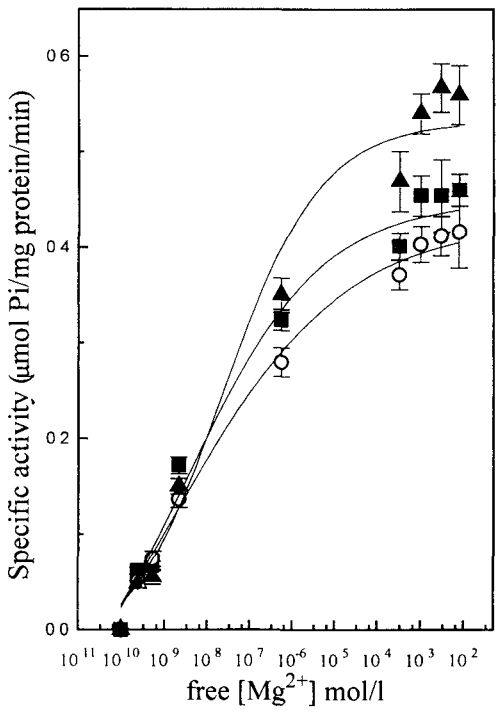

Figure 2. Activation of Mg^{2+}-ATPase by Mg^{2+}, within 0 249 nmol/l – 8 02 mmol/l free Mg^{2+}, in SPM samples isolated from whole brain tissue (■), hippocampus (○) and caudate nucleus (▲) The experiments were carried out as described in Material and Methods in the presence of 15 μg of SPM and 1 mmol/l ATP Symbols represent means ± S E M from four determinations performed in duplicate on three different SPM preparations

maximum specific activity for the enzyme from NC source. The kinetic parameters are summarized in Table 1.

Hormonal status

Since the present work was performed on adult female rats, the possibility that endogenous gonadal hormones interfered with brain Mg^{2+}-ATPase was checked by performing experiments on SPM isolated from the whole brain tissue of randomly selected intact females, chronically ovariectomized (OVX) females and intact males The obtained results showed that endogenous sex hormones do not affect the kinetic properties of Mg^{2+}-ATPase (Fig. 4).

Discussion

The present study confirms the association of Mg^{2+}-ATPase with fractions of synaptic plasma membranes isolated from the rat hippocampus, striatum and the whole brain tissue. The levels of Mg^{2+}-ATPase activities found in this study were similar to those reported for the rat whole brain SPM (Sorensen and Mahler 1981) and caudate nucleus membranes (Lafferty et al. 1985) and were in the same range

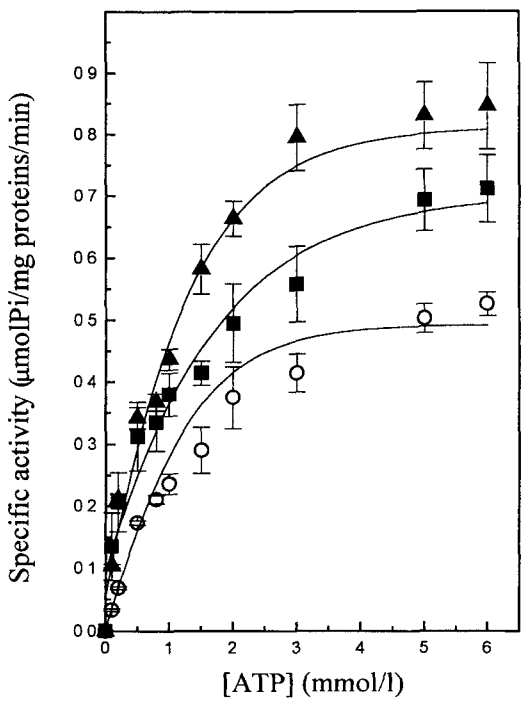

Figure 3. Activation of Mg^{2+}-ATPase by ATP (0 1–6 mmol/l) in SPM samples isolated from whole brain tissue (■), hippocampus (○) and caudate nucleus (▲) The experiments were carried out as described in Materials and Methods in the presence of 5 mmol/l $MgCl_2$ and 15 μg of SPM Symbols represent means ± S E M from five determinations performed in duplicate on three different SPM preparations

Table 1. Kinetic parameters of Mg^{2+}-ATPase in rat brain SPM upon activation by ATP Enzyme activity was assayed as described under Materials and Methods in the presence of 5 mmol/l $MgCl_2$ The kinetic parameters were determined according to Edie-Hofstee model

SPM preparation	K_m (mmol/l)	V_{max} (μmolPi/mg protein/min)
Whole brain	0 756 ± 0 137	0 620 ± 0 117
Caudate nucleus	0 411 ± 0 084**	0 890 ± 0 103*
Hippocampus	1 081 ± 0 141*	0 558 ± 0 044

Results are means ± S E M of at least three independent determinations performed in duplicate * $p < 0\ 05$, ** $p < 0\ 01$ (vs whole brain Mg-ATPase)

as the enzyme activity of smooth muscle membranes (Missiaen et al 1988) and of cardiac sarcolemma (Vrbjar et al 1995) A comparison of the levels of Mg^{2+}-ATPase activity found in this study with those reported for tissues other than brain and muscle (Matsukawa 1990, Van De Put at al 1993, Auland et al 1994)

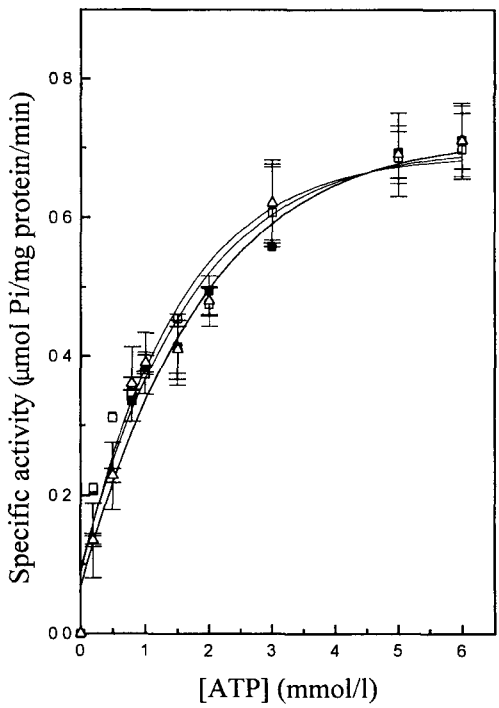

Figure 4. Mg^{2+}-ATPase activity in SPM samples prepared from whole brain tissues of randomly selected intact females (■), chronically ovariectomized females (□) and intact males (▲) The enzyme assay was carried out in the presence of 15 μg of each SPM sample, 1 mmol/l ATP and 5 mmol/l $MgCl_2$ Other details are described under Materials and Methods Symbols represent means ± S E M from at least three independent experiments done in duplicate

lead us to the conclusion that the enzyme has higher activity and possibly higher abundance in excitable than non-excitable tissues. Furthermore, our results indicate that the kinetic properties of Mg^{2+}-ATPase are not under the control of circulating sex steroids This finding, however does not exclude the possibility of sex steroid hormone action on brain Mg^{2+}-ATPase, since it was recently evidenced that mammalian brain is a site of steroid synthesis, independent of the endocrine function of gonads (Negro-Cesi et al. 1996; Poletti et al. 1997).

In our study, Mg^{2+}-ATPase activity was measured in SPM isolated from one brain region relatively rich in dopamine (caudate nucleus) and one brain region having predominantly noradrenergic content (hippocampus). The foremost differences observed with respect to the enzyme between these two sources included significantly higher abundance (30%, $p < 0.05$) and specific activity (59%, $p < 0.01$) and 2.5 fold higher apparent affinity for ATP ($p < 0.01$) of the caudate nucleus Mg^{2+}-ATPase. These findings, along with the previous report that striatal enzyme is not associated with the dopaminergic system (Lafferty et al. 1985), oppose the earlier suggestion that Mg^{2+}-ATPase is involved in the process of storage or release of neurotransmitters such as dopamine (Desaiah and Ho 1977). On the other hand, the evidence is now overwhelming that ATP is an important intercellular messenger,

serving as a co-substrate for surface located protein kinases and acting as a neurotransmitter in the peripheral and central nervous system (James and Richardson 1993; McGeer and McGeer 1993). The physiological activity of a substance acting as a neurotransmitter requires a mechanism(s) of inactivation. In the central nervous system, the activity of Mg^{2+}-ATPase could be involved in the degradation of presynaptically and postsynaptically released ATP, thus controlling the levels of ATP and adenosine in the synaptic cleft (Hohmann et al. 1993). We suggest that the kinetic properties of Mg^{2+}-ATPase are suited to respond to different physiological demands in distinct brain regions. Thus, the high levels of Mg^{2+}-ATPase in the caudate nucleus may potentiate the production of adenosine, which is known to act upon presynaptic A_1 receptors to inhibit the release of dopamine and glutamate (Fredholm and Dunwiddie 1988; Mc Geer and Mc Geer 1993).

In conclusion, our study shows that Mg^{2+}-ATPase is associated with SPM prepared from the rat hippocampus, caudate nucleus and whole brain tissue. The enzyme appears not to have a uniform distribution in the brain If the Mg^{2+}-ATPase is involved in the metabolism of extracellular ATP, then its regional and cellular distribution in the brain reflects the pattern of physiological activity of extracellular ATP in this tissue.

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