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Ontogenetic Profile of Ecto-ATPase Activity in Rat Hippocampal and Caudate Nucleus Synaptic Plasma Membrane Fractions

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Summary

An ontogenetic study of ecto-ATPase activity and the content of enzyme proteins was assessed in the caudate nucleus and hippocampal synaptic plasma membranes isolated from rats at various ages (15, 30, 90, 180 and 365 days). The ontogenetic profile revealed that the enzyme activities in both brain areas were the highest on day 30 and 365, while the ecto-ATPase protein abundance was the highest on day 15 after birth. Possible explanation for obtained ontogenetic profile and the discrepancy between activity and abundance may reside in the fact that ecto-ATPase during development could exert additional roles other than those related to metabolism of ATP. It is likely that ecto-ATPase, regulating the concentration of ATP and adenosine in synaptic cleft, has important role in the processes of brain development and aging.

Key words

Ecto-ATPase • Extracellular purines • Development • Aging • Brain

Introduction

Ecto-ATPase is a membrane-bound enzyme which plays a role in the extracellular metabolism of ATP and other purine nucleotides. In mammalian neural cells, this enzyme activity has been identified in synaptosomes (Nagy *et al.* 1986), nerve endings and synapses (Keller and Zimmerman 1983, James and Richardson 1993, Nedeljkovic *et al.* 1998) and rat cultured astrocytes (Lai and Wong 1991). Cytosolic ATP can be released from intact neuronal cells following activation by different physiological or pathological stimuli. ATP is co-localized in synaptic vesicles with other neurotransmitters (Langercrantz 1976, White 1977, Richardson and Brown 1987) and is released after depolarization (Zhang *et al.* 1988). On the other hand, the most dramatic release of ATP and other purines occurs after cell injury, i.e. following brain trauma, ischemia or in the case of massive necrosis (Rathbone *et al.* 1999). In all cases, ATP released extracellularly undergoes complex metabolic changes exerted by the enzyme chain in which ecto-ATPase, together with 5' nucleotidase, hydrolyzes extracellular ATP to adenosine. In addition to the well-documented functions of ATP and adenosine as neurotransmitters and modulators in the central and peripheral nervous systems (Burnstock 1993), adenosine

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has a direct trophic and neuroprotective role promoting the process of neurogenesis (Weaver 1996), neurite extension (Abbracchio *et al.* 1989), apoptosis (Bronte *et al.* 1996) and repair after hypoxic or ischemic insults (Neary *et al.* 1996, Abbracchio *et al.* 1997). Since ecto-ATPase regulates extracellular concentration of ATP and adenosine which play an important role in the developmental plasticity of the nervous system and its response to disease or injury, we carried out an ontogenetic study of ecto-ATPase in discrete brain areas, the hippocampus and caudate nucleus, both of which are subject to remarkable changes associated with development and aging.

Material and Methods

Animals

Male rats of the Wistar albino strain were used in the study. On the day of birth, litters were culled to 6 pups. The animals were maintained on a 12-h light/12-h dark cycle in constant temperature and humidity of the colony room, and had free access to food and water. Animals from each group were sacrificed on day 15, 30, 60, 90, 180 and 360 after birth.

The rats were sacrificed by a guillotine (Harvard Apparatus) and their brain was quickly removed and placed on ice for immediate dissection of hippocampal (Hip) and caudate nuclei (CN). Brain tissues were homogenized in 10 times volume of iso-osmotic medium containing 0.32 M sucrose, 10 mM Tris-HCl, pH 7.4.

Materials

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and were of analytical grade.

Synaptic membrane preparation

Synaptic membrane fractions were prepared essentially according the procedure of Gray and Whittaker (1962) as previously described (Nedeljković *et al.* 2000). The hippocampal and caudate nuclei from each group were pooled for isolation of the synaptic plasma membrane fraction. The protein content was determined by the method of Markwell *et al.* (1978) and the samples were kept at –80 °C until use.

Enzyme assay

Ecto-ATPase activity was determined as previously described (Nedeljković *et al.* 1998). ATPase activity was carried out in a reaction medium containing (in mM): 50 Tris-HCl. 1 EGTA, 5 $MgCl_2$, 2 ATP, 5 NaN_3, pH 7.4 and 20 μg of membrane preparation in a final volume of 200 μl. The reaction mixture was preincubated for 10 min at 37 °C and the reaction was started by the addition of ATP, allowed to proceed for 15 min and stopped by the addition of 3 M trichloracetic acid. Samples were chilled on ice for 15 min and used for assay of released inorganic phosphate (Pi) by the method of Pennial (1966), using KH_2PO_4 as the reference standard. In order to exclude the presence of other ATP-hydrolyzing activities and to check the purity of the synaptic membrane fraction, several ATPase inhibitors were tested for their ability to interfere with the hydrolysis of ATP: 1 mM ouabain (specific inhibitor of Na,K-ATPase), 5 mM sodium azide (inhibitor of mitochondrial ATPase), 1 mM NaF and 1 mM theophylline (inhibitors of non-specific phosphatases). All samples were run in triplicate in *n* independent enzyme preparations. Enzyme activities were expressed as μmol Pi released/mg of protein/min.

Immunodetection analysis and quantification

Since our study included 10 different membrane preparations, we chose dot blotting for quantification, after qualitative results had been obtained by Western blot. Western blot analysis was performed as previously described (Nedeljković *et al.* 1998), with the use of the antiserum raised against 12 amino acid residue peptides representing N-terminus of rabbit T-tubule ecto-ATPase (Stout and Kirley 1994). After incubation with IgG horse radish peroxidase conjugated second antibody, the presence of ecto-ATPase protein in the samples was detected by the DAB-$NiCl_2$ colorimetric method. An immunoreactive protein band at approximately 70 kD was present in synaptic membrane samples isolated from Hip and CN at different ages (data not shown).

Dot blot analysis was used for a quantification study, according to the same procedure as described for the Western blot, except that the aliquots of samples (20 μg) were spotted directly onto Immobilion membranes using the Bio-Rad dot blot apparatus.

The membranes were incubated in a 1/1000 dilution of anti rabbit T-tubule ecto-ATPase antiserum, which had been shown homologous to the N-terminal region of mammalian ecto-ATPase (Stout *et al.* 1994). After incubation with a second antibody alkaline phosphatase conjugated antibody, the presence of ecto-ATPase protein in the samples was detected with the

NBT-BCIP colorimetric method. Dots were scanned by a computer-based laser densitometer (Pharmacia Ultra Scan XL), while quantification was performed by integrating dot areas using the Origin 3.1 PC software package. Each sample was applied in quadruplicate on the same membrane and the average of *n* separate determinations was taken as the final density of the sample. Data quantified on the blots were then expressed relative to the signal intensity obtained for samples isolated from 90-days old rats, arbitrarily defined as 100 %.

Data analysis

Data were analyzed by the paired *t*-test or by one way ANOVA and, when appropriate, multiple comparison was made using Tukey´s test.

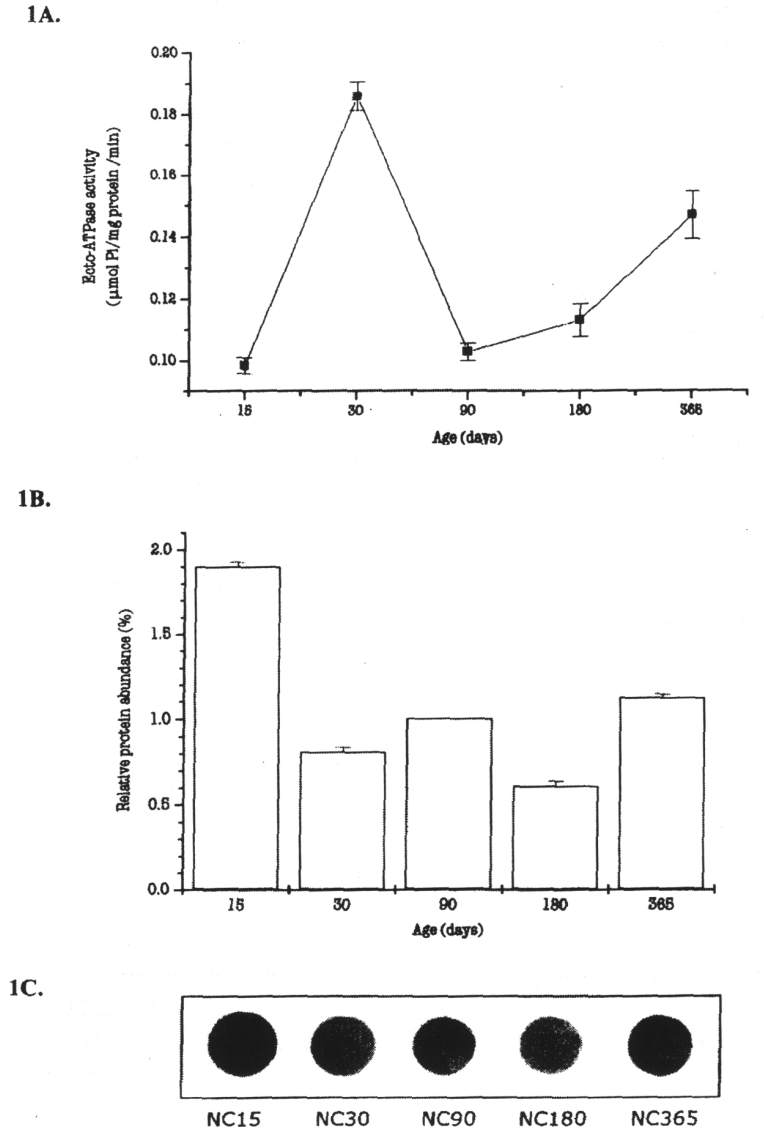

Fig. 1. *Ecto-ATPase activity and its relative abundance in caudal nucleus membrane preparations at different ages. (A) Ontogenetic profile of ecto-ATPase activity. Symbols represent mean activity (µmol Pi/mg protein/min) ± S.E.M., from n=6 independent determinations run in triplicate. (B) Relative ecto-ATPase protein abundance at different ages. Bars represent mean densities ± S.E.M. from n=5 independent determinations performed in quadruplicate, relative to the enzyme protein abundance at the age of 90 days (arbitrarily expressed as 100 %). (C) Representative dot blot of caudal nucleus ecto-ATPase protein abundance analysis.*

Results

Changes in body and brain weight and the yield of protein during ontogenetic development were estimated. The analysis of variance revealed that all parameters studied increased from day 15 after birth throughout the ontogeny.

The effect of different inhibitors was investigated in order to exclude other possible ATP-hydrolyzing activities and to estimate the purity of synaptic plasma membrane preparations. Ecto-ATPase activity was unaltered by ouabain, NaF, theophylline and diethylpyrocarbonate. The mitochondrial ATPase inhibitor sodium azide caused enzyme inhibition of about

20 %, and hence the azide was included in the incubation mixtures. The results obtained indicated that ATP-hydrolyzing activity at all ages were not due to non-specific phosphatase activity and could be attributed to the ecto-ATPase activity.

Figures 1A and 2A show the specific activity of ecto-ATPase in NC and Hip preparations at different ages. The analysis of variance demonstrated that the ecto-ATPase activities in both preparations did not vary significantly throughout ontogeny, except at the age of 30 and 365 days (NC, F=43.09, P<0.0001; Hip, F=14.16, P<0.001). The enzyme activity in both preparations was the highest at the age of 30 days (NC, 0.186±0.005 μmol Pi/mg protein/min; Hip, 0.126±0.004 μmol Pi/mg protein/min) and at the age of 365 days (NC, 0.147±0.008; Hip, 0.146±0.007 μmol Pi/mg protein/min), which was further confirmed by *post hoc* comparison of groups by Tukey´s test.

Figures 1B and 1C represent the quantitative results of ecto-ATPase immunodetection in NC preparations at different ages, assessed by dot bot (Fig. 1B) and representative dot blot support membrane (Fig. 1C). Statistical analysis of data by the *t*-test and multiple comparison by Tukey´s test revealed that the NC ecto-ATPase enzyme protein is the highest at the age of 15 days (190.0±2.9 %, p<0.0001). The analysis of variance revealed a significant effect of age (F=345.8, p<0.0001). In addition, the enzyme abundance at age of 365 days is slightly (111.8±2.4 %), but significantly higher (p=0.0013) than the control NC 90 value.

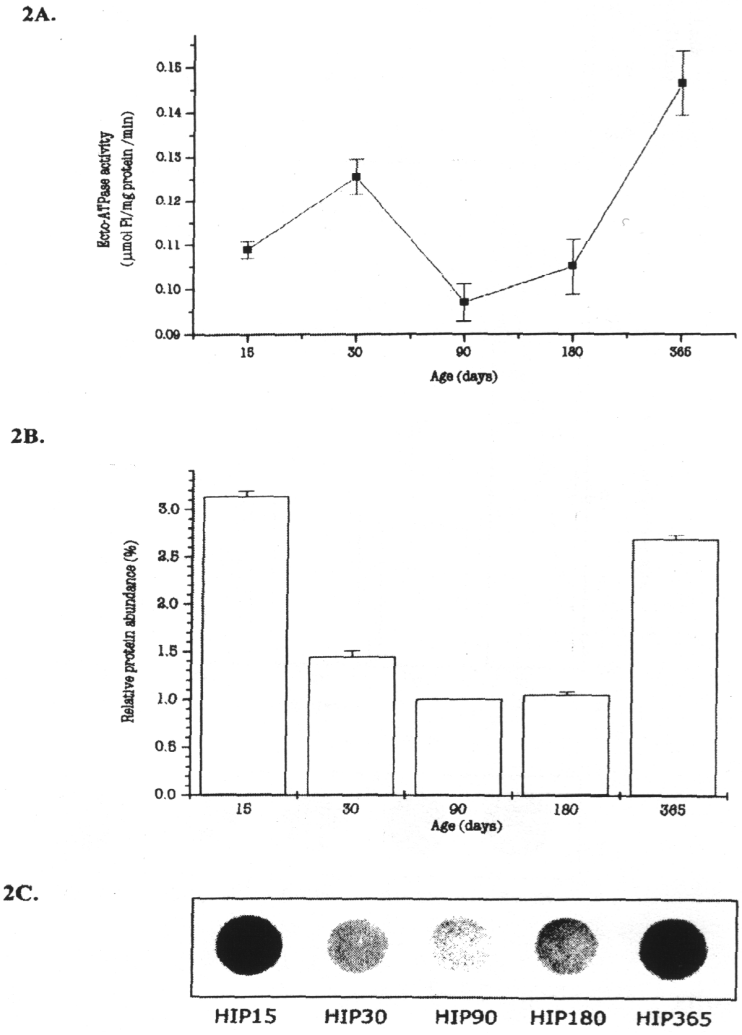

Fig. 2. *Ecto-ATPase activity and its relative abundance in hippocampal membrane preparation at different ages. (A) Ontogenetic profile of ecto-ATPase activity. Symbols represent mean activity (μmol Pi/mg protein/min) ± S.E.M., from n=6 independent determinations run in triplicate. (B) Relative ecto-ATPase protein abundance at different ages. Bars represent mean densities ± S.E.M. from n=5 independent determinations performed in quadruplicate, relative to the enzyme protein abundance at the age of 90 days (arbitrarily expressed as 100 %). (C) Representative dot blot of hippocampal ecto-ATPase protein abundance analysis.*

Figures 2B and 2C demonstrate the quantitative abundance of ecto-ATPase protein assessed by dot blot immunodetection and the representative dot blot of Hip preparations at different ages. The results of dot blot analysis (Fig. 2B) are expressed as the relative enzyme abundance in relation to the enzyme abundance obtained

for the Hip 90 (arbitrarily defined as 100 %). Statistical analysis of data and multiple comparison by Tukey´s test revealed that the ecto-ATPase enzyme protein is most abundant at the age of 15 (313.4±5.8 %, $p<0.0001$) and 365 days (268.3±3.9 %, $p<0.0001$) ($F=500.18$, $p<0.0001$). At other ages the enzyme abundance did not vary significantly with respect to the control Hip 90 value.

Discussion

The ontogenetic profile of ecto-ATPase activity associated with synaptic plasma membrane from hippocampus and caudate nucleus was investigated in the present study. In both brain areas, ecto-ATPase activities rose from day 15, reaching a higher level by day 30, which is agreement with the finding that ecto-ATPase activity from rat cerebral cortex synaptosomes increases 3-5 times from birth until the age of 21 days (Muller *et al.* 1993). One of the most prominent events during this period of postnatal development is the formation of functional synapses (Scheibel and Scheibel 1971). Thus, the increase of ecto-ATPase activity during this period corresponds to the concomitant increase in the activity of various enzymes involved in neurotransmitter metabolism, neuronal function and synaptogenesis (Abdel-Latif *et al.* 1970, Fiedler *et al.* 1987). The main physiological role of ecto-ATPase concerns the extracellular hydrolysis of ATP and precise regulation of adenosine and ATP in the synaptic cleft. Both purines play significant trophic roles during development, interact with other neurotransmitters (Casabona *et al.* 1994, Di Iorio *et al.* 1996) and promote neurite outgrowth, thus affecting the development of neural circuits. However, our results also demonstrate that the ecto-ATPase protein, particularly in the hippocampus, but also in the caudate nucleus, is more abundant on day 15 than on day 30 and later. A possible explanation for this discrepancy between the enzyme activity and its abundance may reside in the fact that ecto-ATPase during development could exert additional functions other than those related to the metabolism of ATP. Brain ecto-ATPase shares a substantial amino sequence homology (Lin and Guidotti 1989) and has a functional relation with the neural cell adhesion molecule (Dzhandzhugazyan and Bock 1993) involved in processes of adhesion, inhibition and recognition between neuronal cells during development (Ronn *et al.* 2000). Thus, ecto-ATPase activity could be catalyzed by the cell adhesion molecule participating in the metabolism of ATP and recognition between cells during early postnatal development. However, this putative bifunctional role of ecto-ATPase awaits to be demonstrated.

Another interesting finding concerns the fact that after a relatively steady level of ecto-ATPase activity from day 30 till adulthood, the enzyme activity and the abundance significantly increased in both brain areas on day 365. One of the most prominent features of the aging brain, especially the hippocampus and caudate nucleus (West *et al.* 1994, Mitchel *et al.* 1999), is programmed neuronal cell loss. Normal aging is often considered as a genetically orchestrated extension of the developmental process, regulated by different classes of signaling molecules. The evidence collected *in vitro* suggests that extracellular adenosine could be involved in the regulation of apoptosis in rat cerebellar neurons (Sei *et al.* 1997). In this context, adenosine-induced cell death could play a role in naturally-occurring apoptosis during brain aging. Thus, higher levels of ecto-ATPase in aged rats could serve to provide adenosine for activating mechanisms involved in aging or for enhancing the response of the brain to cell death associated with the process of aging.

In summary, the ontogenetic pattern of brain ecto-ATPase revealed two peaks of enzyme activity, one coinciding with the termination of synaptogenesis during the postnatal development and the other coinciding with the process of neuronal cell loss during aging. This suggests that ecto-ATPase, together with other signaling networks, could play an important role in brain could have important role in the brain development, as well as in downfall of the brain

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