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# Developmental profile of NTPDase activity in synaptic plasma membranes isolated from rat cerebral cortex

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**Abstract**

In the present study the developmental profile of ATP-hydrolyzing activity promoted by NTPDase 1, its kinetic properties and the enzyme protein abundance associated with synaptic plasma membrane from rat cerebral cortex were characterized. NTPDase 1 activity increased from birth to day 30; afterwards it decreased and remained unchanged from adulthood (90 days) to senescence (365 days). Kinetic analysis revealed that enzyme exhibited the highest specific activity at day 30 and highest apparent affinity for ATP at day 365; however, $V_{max}/K_m$ values remained unchanged for each age studied. Immunoblot analysis demonstrated that relative abundance of NTPDase 1 is highest at day 15 during ontogeny. The discrepancy between maximum enzyme activity and maximum enzyme protein abundance indicates that NTPDase 1 may have an additional role during development.



*Keywords:* Cerebral cortex; NTPDase 1; Developmental profile; Synaptic plasma membranes

The importance of extracellular adenine nucleotides in development has recently begun to be appreciated (Abbracchio et al., 1989; Weaver, 1996; Braun et al., 2003), along with its role in central nervous system (CNS) plasticity (Abbracchio et al., 1995; Bonan et al., 2000a; Bronte et al., 1996) and responses to disease or injury (Neary et al., 1996; Rathbone et al., 1999; Bonan et al., 2000b,c). In CNS, adenosine triphosphate (ATP) is released from neurons, glia, microglia, endothelial cells and blood (for review Rathbone et al., 1999) and it acts at two specific P2 receptor subtypes (Abbracchio and Burnstock, 1994). Extracellular ATP is rapidly inactivated by the conjugated action of surface-located enzyme chain of ectonucleotidases. The first step in the sequential hydrolysis of extracellular ATP is executed by nucleoside triphosphate diphosphohydrolase 1 (NTPDase 1, ecto-apyrase, EC 3.6.1.5) or NTPDase 2 (ecto-ATPase, EC 3.6.1.3). These enzyme have recently been purified and characterized (Kukulski and Komoszynski, 2003) and it was shown that the two enzymes differ in their preference for nucleoside 5′-diphosphates (ADP). NTPDase 1 degrades ATP and ADP equally well directly to AMP, whereas NTPDase 2 hydrolyzes ATP to ADP (Heine et al., 1999). Subsequently, 5′-ectonucleotidase (EC 3.1.3.5) hydrolyzes AMP to adenosine as a principal metabolite (Todorov et al., 1997). Adenosine, which can also be released through its bi-directional transporter, is an even more potent neurotransmitter and neuromodulator than ATP, exerting various trophic roles during development and aging by acting on cognate P1 receptor subtypes (Fredholm et al., 1993, 1994). Thus, ectonucleotidases have the role of removing a signal (ATP) and generating one (adenosine), consequently controlling the levels of adenine nucleotides, and duration and extent of their respective receptor activation.

Extracellular metabolism of adenine nucleotides is susceptible to age-related changes. Thus, in the hippocam-

*Abbreviations:* ADP, adenosine-diphosphate; AMP, adenosine-monophosphate; ATP, adenosine-triphosphate; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; CNS, central nervous system; Ecto-PK, ecto protein kinase; NBT, nitro blue tetrazolium chloride; NTPDase, nucleoside triphosphate diphosphohydrolase

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pus of aged rats increased formation of adenosine from extracellular ATP (Cuhna et al., 2001) and parallel reduction of adenosine release through its bi-directional transporter (Cass et al., 1998) were observed, indicating the importance of ectonucleotidase pathway as a source of extracellular adenosine in senescence. For example, a 40% increase of NTPDase 1 activity (Banjac et al., 2001) and an almost five-fold increase of 5′-nucleotidase activity (Fuchs, 1991; Cuhna et al., 2001) were observed in the hippocampus of aged compared to young rats. Age-related alterations of extracellular ATP-hydrolyzing activity were also observed in whole brain (Muller et al., 1993), cerebral cortex (Gorini et al., 2002) and hippocampal and caudate nucleus plasma membranes (Banjac et al., 2001), in spinal cord synaptosomes (Tores et al., 2003) and in rat hippocampal slices (Bruno et al., 2002). However, the exact mechanisms responsible for age-related changes of extracellular ATP metabolism and its functional consequences in aging brain remain unclear.

In the present study we addressed the following issues. First, we assessed the developmental profile of NTPDase 1 activity in cerebral cortex synaptic plasma membranes, and second, we elucidated if changes in NTPDase 1 activity at different ages derived from changes in the enzyme kinetic properties or from altered enzyme protein expression patterns. Thus, we characterized NTPDase 1 activity, its kinetic properties and protein abundance in cerebral cortex synaptic plasma membranes obtained from young (15- and 30-day-old), adult (90- and 180-day-old) and old (365-day-old) male rats.

## 1. Experimental procedures

### 1.1. Materials

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

### 1.2. Animals

Male rats of the Wistar Albino strain were used in the study. On the day of birth, litters were culled to six pups. The animals were maintained on a 12 h light/ 12 h dark cycle in constant temperature and humidity colony room and had free access to food and water. Animals from each group were sacrificed at day 15, 30, 90, 180 and 365 after the birth.

Before sacrifice by a guillotine (Harvard Apparatus), animal body weight was measured. Brains were quickly removed on ice for brain weight measurements and for immediate dissection of cortex. Cortical tissue was homogenized in 10 times volume of isoosmotic medium containing 0.32 mol/l sucrose, 10 mmol/l Tris–HCl, pH 7.4. After homogenization, protein yields in samples at different ages were determined.

### 1.3. Methods

#### 1.3.1. Synaptic membrane preparation

Synaptic plasma membrane fraction (SPM) was prepared essentially following the procedure of Gray and Whittaker (1962), as previously described (Nedeljkovic et al., 1998). Cortices from each group ($n$ = 6) were pooled for the isolation of SPM. Protein content was determined by the method of Markwell et al. (1978) and samples were kept on −80 °C until use.

#### 1.3.2. Enzyme assay

NTPDase 1 activity was determined by the method previously described (Nedeljkovic et al., 1998). ATP-hydrolyzing activity was assessed in the reaction medium containing (in mmol/l): 50 Tris–HCl, pH 7.4, 1 EGTA, 5 $MgCl_2$, 1 ATP (unless otherwise indicated), 5 $NaN_3$ and 20 μg of membrane preparation in a final volume of 200 μl. Reaction mixture was preincubated for 10 min at 37 °C and the reaction was started by addition of ATP 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 method of Pennial (1966), using $KH_2PO_4$ as the reference standard. The purity of SPM was evaluated with the use of several ATPase inhibitors (data not shown) and the results obtained indicated no significant cross-contamination with other subcellular fractions (≤10%). Activation of NTPDase 1 by increasing concentrations of ATP was estimated under the same conditions as previously was described in the presence of 0.2, 0.4, 0.5, 0.7, 0.8, 1.0, 1.2, 1.5, 1.7, 1.8, 2.0 and 2.5 mmol/l ATP. All samples were run in triplicate in $n$ independent enzyme preparation. Enzyme activities were expressed as μmol Pi/mg of protein/min.

#### 1.3.3. Immunodetection analysis and quantification

Immunodetection of NTPDase 1 was performed as described previously (Nedeljkovic et al., 2003). Briefly, SPM samples obtained from rats at different ages were diluted with SDS sample buffer, vortexed and boiled for 5 min. Ten micrograms of each sample was resolved by 5–12% SDS–PAGE gradient gels according to Leamlli (1970). Proteins were transferred to PVDF membrane by transblotting in mini trans-blot transfer cell system (Bio-Rad). After blocking with 2% BSA in Tris buffer saline, Tween 20 (TBST) the PVDF membrane was incubated overnight 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 NTPDase 1 (Stout et al., 1994). Visualization procedure was performed with the use NBT/BCIP colorimetric system (Bio-Rad) after incubating PVDF membrane in antirabbit IgG alkaline phosphatase conjugated secondary antibody (Sigma Chem. Co., St. Louis). Bands obtained on the blots