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ATP diphosphohydrolase (NTPDase 1) in rat hippocampal slices and effect of glutamate on the enzyme activity in different phases of development

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Abstract

In the present report we describe an NTPDase 1 (ATP diphosphohydrolase; ecto-apyrase; EC 3.6.1.5) in rat hippocampal slices. The effect of glutamate on the ATPase and ADPase activities in rat hippocampal slices of different ages was also studied since adenosine, the final product of an enzymatic chain that includes NTPDase 1 and 5′ -nucleotidase, can act upon A1 receptors in turn decreasing the release of glutamate. Hippocampal slices from 7, 14, 20-23 and 60 day-old rats were prepared and ATPase and ADPase activities were measured. The parallelism of ATPase and ADPase activities in all parameters tested indicated the presence of an ATP diphosphohydrolase. In addition, a Chevillard plot indicated that ATP and ADP are hydrolyzed at the same active site on the enzyme. ATPase activity was significantly activated by glutamate in 20-23 and 60 day-old rats, but ADPase activity was not activated. These results could indicate distinct behavior of the ATPase and ADPase activities of NTPDase 1 in relation to glutamate or the simultaneous action of the ecto-ATPase . Activation of ATPase activity by glutamate may constitute an important role in this developmental period, possibly protecting against the neurotoxicity induced by ATP, as well as producing high levels of ADP, by increasing adenosine production, a neuroprotective compound.

Keywords: ATP diphosphohydrolase; NTPDase 1; Glutamate; Hippocampal slices; Adenosine

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Introduction

The hippocampus is well documented as being involved in memory and learning and as being particularly sensitive to ischemic insults [1]. Levels of glutamate, the major excitatory neurotransmitter in the hippocampus, increase intensely during neuronal damage conditions and this occurrence has been implicated in neuronal death due to pathological situations such as ischemia, hypoxia and seizures [2,3]. Excessive glutamate receptor stimulation, in particular of NMDA receptors, has been involved in neuronal damage in a variety of pathologies [3,4].

Glutamate has been demonstrated to release adenyl compounds in the brain, including adenosine [5]. This response has been observed in vivo [6], as well in rat cortical synaptosomes [7] and brain slices [8].

The increase in adenosine levels, a main inhibitory neuromodulator in the central nervous system, may have significant consequences. For example, adenosine released in response to glutamate can act upon the specific adenosine A_1 receptor and thus decreases the release of a number of neurotransmitters, including glutamate itself [9]. Thus, adenosine may exert its known neuroprotective function preserving the brain from excitoxicity damage [9].

Adenosine can be released as such through bi-directional adenosine transporters or can be originated from the extracellular catabolism of released ATP by the ecto-enzymes present in the central nervous system [10]. Ecto-ATPases (NTPDase 2; EC 3.6.1.15) are enzymes that hydrolyze preferentially ATP, whereas the NTPDase 1 (ecto-ATP diphosphohydrolase; ecto-apyrase; EC 3.6.1.5) hydrolyzes ATP equally well as ADP to AMP. Both enzymes are expressed in the central nervous system [11]. The AMP formed is metabolized to adenosine by an ecto-5′ -nucleotidase (EC 3.1.3.5), which has also been described in mammalian brain, in neuronal and glial cells in proportions depending on the developmental phase [12–14] and was observed at the surface of hippocampal neuronal membranes [15]. Thus, the action of this ‘‘enzyme chain’’ may regulate the concentrations of ATP, ADP and AMP by increasing/decreasing their hydrolysis with a consequent increase/decrease in adenosine levels, a natural protective metabolite.

The catabolism of adenine nucleotides and, consequently, the production of adenosine has great importance in neural development. The neuritic differentiation of cerebellar granule cells is dramatically reduced when the synthesis of 5′ -nucleotidase is inhibited, but the addition of adenosine to the culture medium results in normal neurite formation [16]. Other studies have also shown that 5′ -nucleotidase is transiently expressed at the surface of developing nerve cells [17].

Considering that during the postnatal development of the rat, the central nervous system exhibits pronounced changes in a variety of biological processes including biochemical events, the enzymes involved in neurotransmitter metabolism and in neural function undergo an increase in their activities during this period [18]. The ontogeny of ATP and ADP hydrolysis by cerebral cortex synaptosomes from rats was examined [19] and results demonstrated a maximum increase in ATP and ADP hydrolysis coincident with the maximum brain growth. Moreover, the parallel development of both activities (ATPase and ADPase) suggested the involvement of an NTPDase 1 with an important function during development [19].

Therefore, in the present report we investigated the kinetic characteristics of ATPase and ADPase activities in hippocampal slices of rats. At the same time, we evaluate the possible involvement of glutamate in the activation of ATP-ADP hydrolysis in this preparation in different brain developmental stages. These results should contribute to the understanding of the mechanisms of the extracellular breakdown of adenine nucleotides by an intact tissue.

Material and methods

Materials

ATP, ADP, L-glutamic acid, Malachite Green Base and Coomassie Brilliant Blue G were purchased from Sigma Chemical (St. Louis, MO, U.S.A). All other reagents were of the highest grade available.

Hippocampal slices

Female Wistar rats of different ages (7, 14, 20-23 and 60 days-old) were killed by decapitation and the brains were rapidly removed into a bicarbonate-buffered salt solution with the following composition: 115 mM NaCl, 3.0 mM KCl, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 10 mM glucose, 2.0 mM $CaCl_2$, pH 7.4, and gassed with 95% O_2 and 5% CO_2 mixture (incubation medium). The brains were cut longitudinally, their hippocampi dissected and slices transversely cut to 400 μm thick on a Mcllwain tissue chopper. The animals were not anaesthetized before killing.

Determination of ATPase and ADPase activities

Two slices per tube (approx. 0.16 mg protein) were preincubated for 10 minutes at 37 °C with 500 μl of the incubation medium (described above) and gassed directly with 95% O_2 and 5% CO_2. The reaction was started by adding ATP, or ADP, to a final concentration of 2.0 mM, except in experiments in which the substrate concentration dependence was evaluated. An incubation time of 20 min was chosen in order to ensure the linearity of the reaction. The protein concentration used was chosen from experiments in which all the conditions were maintained constant and the number of slices was varied from 2 to 10 (0.12 mg – 0.58 mg of protein). The reaction was stopped by the addition of 100μl of 10% trichloroacetic acid. Non-enzymatic Pi released from nucleotides into assay medium without slices and Pi released from slices without nucleotide were subtracted from total Pi released during incubation. All assays were performed in duplicate or triplicate. Pi was measured by the method of Chan et al., 1986 [20] and the enzymatic activity was expressed as nmol of Pi released per minute per milligram of protein.

Effect of glutamate

To test the effect of glutamate on the ATP and ADP hydrolysis, hippocampal slices were incubated for 20 min in the absence or presence of 0.1, 1.0 and 5.0 mM glutamate (20-23 day-old rats) or 1.0 mM (rats of other ages, as it is indicated in the results) under the same incubation conditions described above.

Protein determination

Slices were homogenized in 400 μl of the incubation medium, aliquots of 50 μl were removed and the protein concentration was determined colorimetrically by the Coomassie Blue method [21] using bovine serum albumin as a standard.

Statistical analysis

The data obtained are expressed as means ± S.D. values of at least four experiments with slices from different animals. Each experiment was performed in duplicate or triplicate in slices from the same animal. The results were analyzed statistically by Student's *t* test. Values of $p<0.05$ were considered significant.

Results

ATP and ADP hydrolysis

In the present work, we first investigated the ATP and ADP hydrolysis by hippocampal slices from 20-23 day-old rats in order to determine the best assay conditions and identify the presence of this enzyme in this fraction. Slices were incubated in the conditions described in Materials and Methods with ATP or ADP as substrates and the inorganic phosphate determined. The time course of ATP and ADP hydrolysis was linear for at least 20 minutes for both activities (Fig. 1A). Similarly the influence of protein concentration on ATP and ADP hydrolysis was linear for at least 0.5 mg of protein per tube for both substrates (Fig 1B). In order to verify the effect of Ca^{2+} upon ATP and ADP hydrolysis, $CaCl_2$ was added to varying final concentrations of between 0 and 5.0 mM, whilst ATP and ADP concentrations were fixed at 2.0 mM. The results (Fig. 2) indicated that the enzymatic activity increased with increasing concentration of calcium added up to 2.0 mM. It is important to note that the pattern for time, protein concentration and ion dependence was similar for both tested substrates.

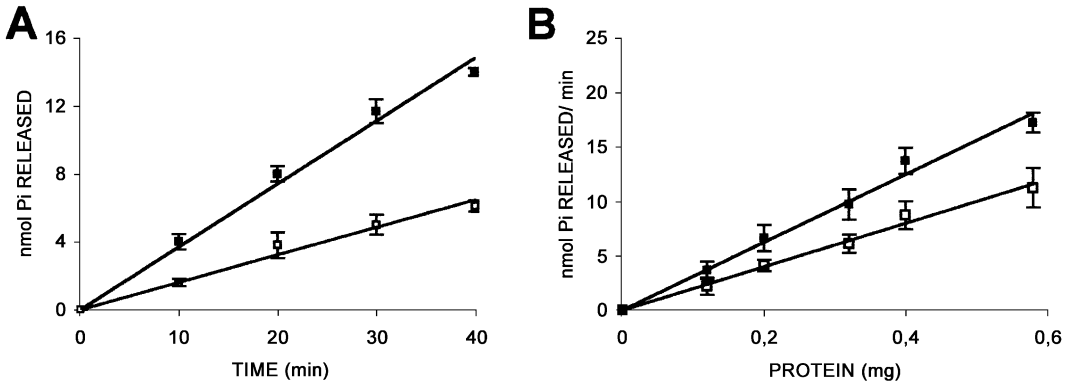

Fig. 1. Progress curve for ATP (■) and ADP (□) hydrolysis as a function of time (A) and slice protein concentration (B). For the time curve the protein concentration was approximately 0.16 mg/ tube and for the protein curve the incubation time was 20 minutes. The enzyme assays are described in Materials and Methods. The data are means ± SD of four determinations with different slice preparations.

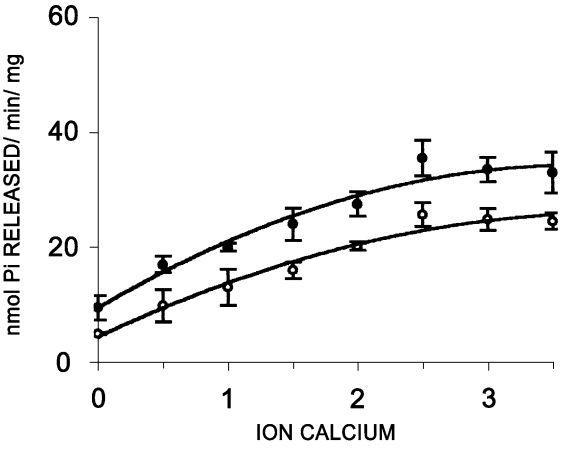

Fig. 2. Dependence of ATP (■) and ADP (□) hydrolysis upon calcium ion. Hippocampal slices (0.16 mg) were incubated for 20 minutes at 37 °C. The reaction medium and the enzyme assays are described in Materials and Methods. The data are means ± SD of four determinations with different slice preparations.

Kinetic parameters

The relationship between the substrate concentration and the enzymatic activity was evaluated using concentrations ranging between 0.25-2.5 mM for ATP and ADP, with calcium fixed at 2.0 mM. The results (insets in Fig. 3) show that the enzyme activity increased with increasing concentrations of both nucleotides up to 2.0 mM. This nucleotide concentration is sufficient to saturate the enzyme and a parallel profile was obtained for both substrates. The Woolf-Augustinson-Hofstee plot of the results obtained in the range of 0.25-2.5 mM is shown in Fig. 3. The apparent Michaelis-Menten constants (Km,app) calculated from this plot were 141 ± 11 μM and 167 ± 10 μM with a calculated maximum velocity (Vmax,app) of 70 and 45 nmol Pi/min/mg for Ca^{2+}-ATP and Ca^{2+}-ADP, respectively (mean ± SD, n = 4 for each substrate). It is important to note that similar Km values for both substrates are characteristic for other ATP diphosphohydrolases described in the literature [22–24].

A single active site

To show that ATP and ADP hydrolysis occur due to an ATP diphosphohydrolase activity and that only one active site is able to hydrolyze the two substrates, we used the competition plot described by Chevillard et al., 1993 [25]. A series of mixtures containing ATP and ADP were prepared. To assay the combination of substrate concentration in a Chevillard plot we chose a concentration at which the rate of hydrolysis was the same when either ATP or ADP was used as substrate (from Fig. 3, insets). The P-values ranged from 1 to 0. In the Fig. 4, the horizontal straight line obtained in the competition plot indicates a constant hydrolysis rate at all substrate combinations tested. The interpretation is that the ATP and ADP hydrolysis correspond to the same active site of a single enzyme. For each point (two substrates) we chose incubation times to ensure linearity of the reaction.

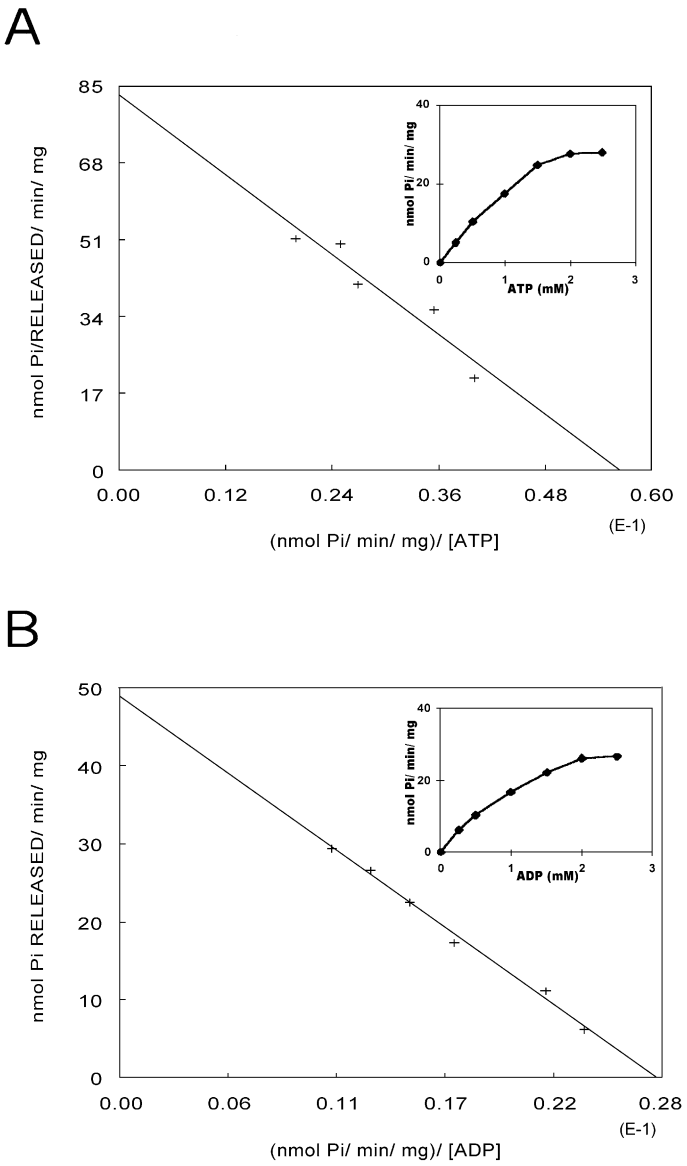

Fig. 3. Woolf-Augustinson-Hofstee plot of NTPDase 1 activity. Hippocampal slices (0.16 mg) were incubated for 20 minutes at 37 °C. The reaction medium and the enzyme assays are described in Materials and Methods. Results for plot were obtained with concentrations of ATP (A) or ADP (B) in the range of 0.25-2.5 mM. The inset shows activation of the enzyme by ATP or ADP at constant 2.0 mM Ca^{2+} concentration. Best-fit analysis indicated a linear relationship.

Effect of glutamate

Firstly, the effect of glutamate on the ATPase and ADPase activities was tested in 20–23 day-old rats. Glutamate was tested at concentrations of 0.1, 1.0 and 5.0 mM. The ATPase activity was significantly

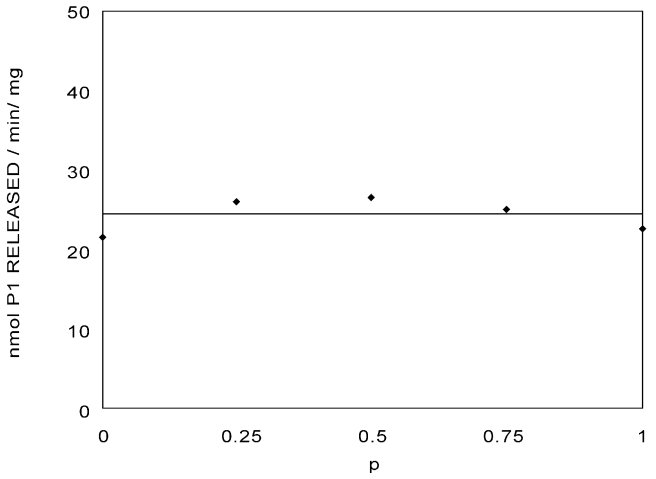

Fig. 4. Competition plot. Incubation time was 20 minutes; substrate A (ATP) concentration at P = 0 was 0.45 mM and substrate B (ADP) concentration at P = 1 was 1.0 mM. The reaction medium and the enzyme assays are described in Materials and Methods. Data represent a typical experiment that was run in triplicate.

activated by 0.1 and 1.0 mM glutamate but not by 5.0 mM glutamate (Fig. 5). However, the ADPase activity was not stimulated by glutamate at these concentrations (Fig. 5). Conversely, ATPase (Fig. 6A) and ADPase (Fig. 6B) activities were not changed by 1.0 mM glutamate in slices from 7 and 14 day-old rats. However, in slices from 20-23 and 60 day-old rats, 1.0 mM glutamate activated the ATPase activity (Fig. 6A) but not the ADPase activity (Fig. 6B). The in vitro effect of 1.0 mM glutamate upon the

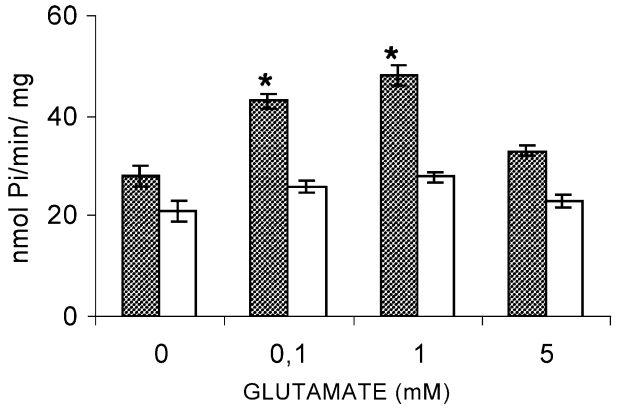

Fig. 5. Effect of glutamate on ATP (closed columns) and ADP (open columns) hydrolysis. Hippocampal slices (0.16 mg) were incubated for 20 minutes at 37 °C. The reaction medium and the enzyme assays are described in Materials and Methods. The data are means ± SD of four determinations with different slice preparations. * $P < 0.05$, compared with the control.

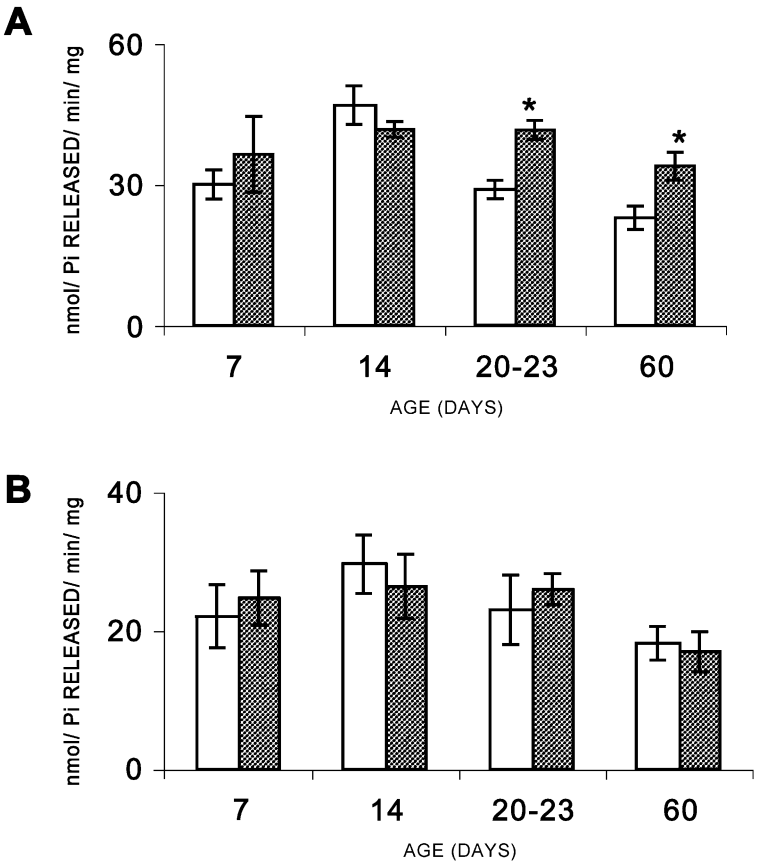

Fig. 6. Effect of glutamate on ATP (A) and ADP (B) hydrolysis. Hippocampal slices (0.16 mg) were incubated for 20 minutes at 37 °C. The reaction medium and the enzyme assays are described in Materials and Methods. The data are means ± SD of four determinations with different slice preparations. Open columns represent the controls (without glutamate) and the closed columns, glutamate 1 mM. * $P < 0.05$, compared with the control.

ATPase activity appearing in hippocampal slices from 20-23 day-old rats, and in slices obtained from 60 days old-rats, was also observed.

Discussion

The work reported here demonstrates, for the first time, the presence of an NTPDase 1 activity in hippocampal slices of rats. The enzyme described has the following general properties which characterize NTPDase 1: (a) parallelism for ATP and ADP hydrolysis in relation to incubation time, protein concentration and calcium activation (Fig. 1A, 1B and Fig. 2), (b) similar apparent Km values for both enzyme substrates (ATP and ADP) (Fig. 3), (c) a linear straight line in the Chevillard competition plot (Fig. 4). The parallelism in kinetic behavior (similar profile for both substrates) is also characteristic of other ATP diphosphohydrolases described in the literature [22–24]. Furthermore, we used the Chevillard

plot, which is a decisive method to determine if two substrates bind at the same active site of an enzyme [25]. The constant velocity demonstrated in the competition plot indicates that ATP and ADP are hydrolyzed at just one active site of the enzyme (Fig. 4).

Some previous studies have described ATP and AMP hydrolysis by slices from the central nervous systems of rats. Results in synaptosomes and slices from hippocampus of rats showed that ATP and its analogs are converted into adenosine to exert their inhibitory effects on synaptic transmission in the hippocampus [26]. Whilst it is clear that the AMP hydrolysis occurs via the action of a 5′ -nucleotidase [27,28], the enzyme or enzymes involved in the ATP hydrolysis mediated by slices are, as yet, unidentified. We previously described the presence of the ATP diphosphohydrolase enzyme in central and peripheral nervous systems [22,29]. Furthermore, recent findings demonstrated that the most of NTPDase 1 activity in brain should be related to microglia [30]. We have proposed that the most obvious physiological role for this enzyme is to participate in an "enzymatic chain" together with a 5′ -nucleotidase for the complete hydrolysis of the neurotransmitter ATP to adenosine, inactivating the action of this neurotransmitter. This proposed physiological role for NTPDase 1 may be significant, in view of the fact that the product of the complete hydrolysis of ATP, adenosine, is a neuromodulator [31]. Another investigation has described the co-existence of an ATP diphosphohydrolase and ecto-ATPase [11]. Although no clear functional role has been established for the presence of different ectonucleotidases, the broad substrate specificity may reflect a requirement to modulate the levels of the broad range of ligands in the purinergic system [32].

It has been shown that adenosine plays a crucial role in cell survival and differentiation [16] and, thus, adenosine production is important during growth and development when the central nervous system of rats demonstrates pronounced changes in morphological, physiological and biochemical processes.

Since the degradation of adenine nucleotides, via ecto-nucleotidases, can be a source of extracellular adenosine, we investigated the effect of glutamate on the ATPase and ADPase activities in rat hippocampal slices in different development stages. The hippocampus was chosen because it is involved in functions such as memory and learning [1]. Thus, the neuromodulation and neuroprotection mediated by adenosine in the hippocampus may be of significant importance during the postnatal period.

In the results presented here, 1 mM glutamate significantly stimulated ATPase activity in rats of 20-23 days and in rats of 60 days (Fig. 6A). However, the ADPase activity was not stimulated by glutamate (Fig. 6B). The presence of glial and neuronal cells in slice preparations may contribute to these apparently controversial results. The activation of ATPase activity by glutamate in rats of 20-23 and 60 days old may constitute an important role in adenosine production during this development period to counteract the neurotoxicity induced by glutamate. Furthermore, the activation of ATP hydrolysis has a beneficial effect since the ATP, which acts as a neurotransmitter [33] must be rapidly metabolized to adenosine [31]. This increase in ATPase activity may result in a feed-forward activation.

Recently, the effect of glutamate on these ecto-nucleotidases activities was studied in cultured neuronal cells and the hydrolysis of ADP and AMP was stimulated by 1 mM glutamate after 8 days culture in vitro [34]. The activation of ADP hydrolysis in cultured neuronal cells in presence of glutamate [34] and not in hippocampal slices, showed here, can be due to presence of the other cells such as astrocytes in this preparation that could be responding differently to glutamate in relation to balance of the extracellular ATP, ADP and adenosine levels.

The increase only in ATP hydrolysis in the presence of 1 mM glutamate may be explained by the action of an ecto-ATPase, which is co-expressed with ATP diphosphohydrolase in the central nervous

system [11] or could also indicate a distinct behavior of ATP diphosphohydrolase, described here, in relation to glutamate.

In conclusion, we describe herein the presence of an NTPDase 1 activity in hippocampal slices of rats, which in association with 5′-nucleotidase, carries out the complete hydrolysis of ATP to adenosine. Moreover, the activation of ATP hydrolysis by glutamate provides the rapid elimination of ATP and, consequently, a source of extracellular adenosine as a neuroprotective agent, contributing to the prevention of a variety of pathologies that involve excessive glutamate release.

Acknowledgements

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