


# Extracellular Interconversion of Nucleotides Reveals an Ecto-Adenylate Kinase Activity in the Rat Hippocampus

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**Abstract** Here, the extracellular interconversion of nucleotides and nucleosides was investigated in rat hippocampal slices and synaptosomes by an HPLC-UV technique. Adenosine 5-triphosphate (ATP) was converted to adenosine 5-diphosphate (ADP), adenosine 5-monophosphate (AMP), adenosine, inosine, and hypoxanthine in the slices, whereas ADP elicited parallel and concentration-dependent formation of ATP and AMP. The specific adenylate kinase inhibitor diadenosine pentaphosphate decreased the rate of decomposition of ADP and inhibited the formation of ATP. No substantial changes in the interconversion of ADP to ATP and AMP were found in extracellular catabolism by various families of ectoenzymes in the presence of dipyridamole, flufenamic acid, the P<sub>2</sub> receptor antagonist pyridoxal-5-phosphate-6-azophenyltetrazine (PPADS), and the alkaline phosphatase substrate *para*-nitrophenylphosphate. Negligible levels of nucleotides were generated when uridine 5-diphosphate (UDP), AMP or adenosine were used as substrates. Ecto-adenylate kinase activity was also observed in purified synaptosomes. In summary, we demonstrate the presence of an ecto-adenylate kinase activity in the hippocampus, which is a previously unrecognized pathway that influences the availability of purines in the central nervous system.

**Keywords** ADP · ATP · Hippocampus  
Ecto-adenylate kinase · Diadenosine pentaphosphate  
NTPDase

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experimental models of stroke [5, 6] and epilepsy [7].

The first step in the extracellular catabolism of ATP is mediated by the family of ectonucleoside triphosphate diphospho-hydrolases (E-NTPDases, EC 3.6.1.5, also known as CD39, ectoATPase or apyrase [4]). E-NTPDases have a molecular mass of 55–60 kDa in their unglycosylated form, one or two transmembrane domains and a highly conserved catalytic region that faces the extracellular space. Until now eight members of this enzyme family (numbered E-NTPDase 1 to E-NTPDase 8) have been identified in molecular terms, but only those that possess two transmembrane domains (i.e., E-NTPDases 1, 2, 3, and

8) are located at the surface. E-NTPDases 1, 2, and 3 are the major ATP catabolizing enzymes of the brain, whereas E-NTPDase 8 is either absent or only present in low amounts in the brain. E-NTPDase 1 hydrolyzes ATP and adenosine 5'-diphosphate (ADP) to adenosine monophosphate (AMP), whereas E-NTPDases 2 and 3 convert nucleoside triphosphates to their respective diphosphates.

In addition to the E-NTPDase family, ATP may also be dephosphorylated by ecto-nucleotide pyrophosphatases (E-NPPs, E.C. 3.6.1.9) and by alkaline phosphatases. Both these phosphatases have a broad substrate specificity and widespread tissue distribution. Activation of E-NPPs leads to the release of pyrophosphate and converts nucleoside di- and triphosphates to nucleoside monophosphates (e.g., ATP to AMP), whereas alkaline phosphatases cleave one phosphoryl group, converting either nucleoside triphosphates to nucleoside diphosphates, nucleoside diphosphates to nucleoside monophosphates, or nucleoside monophosphates to nucleosides. The next step in the ectonucleotidase cascade is the hydrolysis of AMP by the ecto-5-nucleotidase (EC 3.1.3.5) enzyme. This is the rate-limiting step that gives rise to the formation of adenosine monophosphate (AMP) could be also dephosphorylated by alkaline phosphatases. Finally, adenosine may be deaminated in the extracellular space by the adenosine deaminase enzyme and/or may be taken up by specific nucleoside transporters into the nerve terminals or glial cells. Thus, as a result of this rapid and highly effective hydrolysis, endogenous ATP is converted to adenosine resulting in the activation of adenosine A1 receptors within a second in the hippocampus.

Although the enzymatic mechanisms responsible for the inactivation of extracellular ATP that lead to the formation of adenosine are well delineated in the nervous system, less is known about the reverse process which involves the potential rephosphorylation of nucleotides and/or nucleosides to ATP. Adenylate kinases (AKs, EC 2.7.4.3) are ubiquitous enzymes that catalyze the reaction:  $NTP + AMP = NDP + ADP$ , where N represents purine, but not with pyrimidine nucleotides. Four different isoforms of this enzyme family have been identified so far, with AK1 and AK4 localizing to the rat brain. Previous studies have demonstrated catalytic activity in the extracellular space that converts two ADP to ATP and AMP in rat brain synaptosomes with  $K_m$  in the millimolar range. Similarly, ecto-adenylate kinase activity has been reported in the periphery, in NG108-15 cells [21] and in non-neuronal cells [22, 23]. However, the reaction products of such an enzymatic conversion have not been directly demonstrated in intact brain preparations where synaptic organization and receptor distribution are maintained. To fulfill this aim here we examine the interconversion of various nucleotides and nucleosides in rat hippocampal slices and synaptosomes using HPLC-UV.

The following chemicals were all obtained from Sigma (St. Louis, MO, USA): ATP, ADP, AMP, adenosine, diadenosine pentaphosphate (Ap5A), dipyrnidamole,  $\beta$ ufenamic acid, inosine, *para*-nitrophenylphosphate, pyridoxal-5-phosphate-6-azophenyl-2'-disulphonic acid tetrasodium (PPADS), and uridine diphosphate (UDP). All solutions were freshly prepared on the day of use.

Male Wistar rats (140–160 g) bred in the local animal house were used in these studies. All studies were conducted in accordance with the principles and procedures outlined in the *NIH Guide for the Care and use of Laboratory Animals* and were approved by the local Animal Care Committee of the Institute of Experimental Medicine (Budapest, Hungary).

Rats were decapitated under light anesthesia and the brain was quickly put into ice-cold Krebs solution (NaCl 115 mM, KCl 4.7 mM,  $KH_2PO_4$  1.2 mM,  $MgSO_4$  1.2 mM,  $CaCl_2$  2.5 mM, NaHCO<sub>3</sub> 25 mM, glucose 10 mM, oxygenated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, pH 7.4). Both hippocampi were rapidly dissected and slices (400  $\mu$ m thick) were cut transversely with a McIlwain tissue chopper and incubated in 3 ml of Krebs solution at 37°C bubbled with 95% O<sub>2</sub> + 5% CO<sub>2</sub>. A 30 min preincubation period was applied in order to allow the recovery of tissue ATP stores. Subsequently, various ligands, (ATP, ADP, AMP, UDP, adenosine) were added to the bath in different initial concentrations (20, 100, 500, and in some cases 1000  $\mu$ M) in the absence and presence of drugs (diadenosine pentaphosphate (Ap5A), dipyrnidamole,  $\beta$ ufenamic acid, *para*-nitrophenylphosphate, pyridoxal-5-phosphate-6-azophenyl-2',4'-disulphonic acid tetrasodium (PPADS)). Aliquots of 70  $\mu$ l were collected from the bath at different time points (2.5, 5, 10, 15, 20, 25, 30, and 60 min) after the addition of the ligand. The concentrations of ATP, ADP, AMP, adenosine, inosine, and hypoxanthine in the aliquots were measured by high-performance liquid chromatography

combined with ultraviolet detection (HPLC-UV) as described previously [24]. The identification of different purines was based on the retention times of known amount of standards and was carried out by the Agilent ChemStation program (Fig. 1A). The nucleotides and nucleosides were quantified using the standard addition methods for the peak area in the chromatogram. A linear correlation between the peak area and the injected amount of substrate was observed for all nucleotides and nucleosides. The actual concentrations of ATP, ADP, AMP, adenosine, inosine, and hypoxanthine are expressed  $\mu\text{M}$ . Preliminary analyses showed that the ADP ( $20\ \mu\text{M}$ ) solution contained no detectable amount of ATP contamination. The ATP efflux from the slices, measured without addition of ADP, was also negligible within the sensitivity range of this technique (Fig. 1B).

For the determination of the kinetic parameters of nucleotide metabolizing ectoenzymes a linear regression for concentrations of the respective nucleotide (ATP, ADP, AMP) as a function of time was calculated from the concentrations of the first five samples (0, 2.5, 5, 10, and 15 min) after three different initial concentrations of nucleotide (20, 100, and  $500\ \mu\text{M}$ ) and the slopes were determined as initial velocities ( $v_i$ ). These initial velocities were used for calculation of the kinetic parameters from the Lineweaver-Burk plot using linear line regression. Thus,

$$1/v_i - 1 = K_m/v_{\max} \times [S] + 1/v_{\max},$$

where  $v_i$  is the velocity measured when very little substrate has reacted,  $[S]$  is the concentration of the substrate,  $v_{\max}$  (maximal velocity) is the point at which the enzyme is saturated with the substrate,  $K_m$  (Michaelis constant) is the concentration that produces half-maximal velocity.

### Preparation of synaptosomes

A synaptosomal fraction of the hippocampus was prepared as described previously with slight modifications [25]. Four male Wistar rats (140–160 g), were decapitated under ether anesthesia and the brains were quickly put into ice-cold Krebs solution. All hippocampi were rapidly dissected and homogenized in ice-cold 0.32 M sucrose-Krebs solution (containing 1 mM EDTA, 1 mg/ml bovine serum albumin, and 5 mM HEPES, pH 7.4) at  $4^\circ\text{C}$ , and centrifuged at  $5,000g$  for 10 min. The supernatant was centrifuged at  $13,000g$  for 12 min. The pellet was resuspended in ice-cold, 45% (v/v) Percoll in Krebs solution (pH 7.4), and centrifuged at  $13,000g$  for 2 min to eliminate free mitochondria. The top layer was washed twice at  $13,000g$  for 2 min in oxygenated Krebs solution at  $4^\circ\text{C}$ . The adequate composition and morphological integrity of the preparation were verified by electron microscopy.

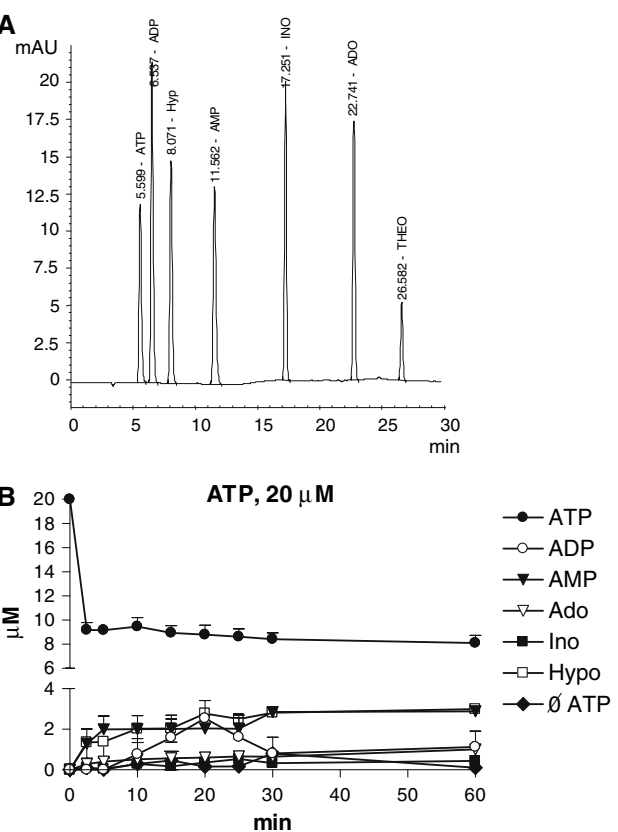


Fig. 1 Extracellular decomposition of ATP in rat hippocampal slices. (A) Representative chromatogram showing the retention times of nucleotides (ATP, ADP, AMP) and nucleosides (ADO, adenosine, INO, inosine, HYP, hypoxanthine). Theophylline (THEO) was used as an internal standard. B. Hippocampal slices were incubated in 3 ml Krebs solution in the presence of ATP ( $20\ \mu\text{M}$ ). Aliquots ( $70\ \mu\text{l}$ ) were collected at different time points (2.5, 5, 10, 15, 20, 25, 30 and 60 min) after the addition of ATP. The amount of nucleotides (ATP, ADP, and AMP) and nucleosides (Ado, adenosine; Ino, inosine; and Hypo, hypoxanthine) in the aliquots was determined by HPLC-UV and expressed  $\mu\text{M}$  as a function of time. The diamonds represent ATP efflux from the hippocampal slices without ATP addition ( $\emptyset$  ATP). Note that there is a break in the x-axis to enhance the resolution. Thus, the rapid decline in ATP concentration is coincident with the appearance of AMP, but not ADP, which is generated after a delay. Data is shown as the Mean  $\pm$  SEM, where 4 independent experiments

100  $\mu\text{l}$  aliquot of the synaptosomes was added to 3 ml of Krebs solution at  $37^\circ\text{C}$ , bubbled with 95%  $\text{O}_2$  + 5%  $\text{CO}_2$ . ADP metabolism studies were performed as described earlier.

After ADP exposition of the hippocampal slices, lactate dehydrogenase (LDH, E.C. 1.1.1.27) activity that had been released into the media was evaluated using the CytoTox96 nonradioactive assay kit (Promega, Madison, WI, USA),

according to the manufacturer's instructions. The LDH activity was quantified by measuring the wavelength coincident formation of ATP and AMP upon the breakdown of ADP was also observed when samples were collected in every minute in the first 5 min after the addition of ADP (Fig. 2D). Morever, this effect was percentage of total LDH activity, which was determined in addition of ADP (Fig. 2D). Moreover, this effect was tissue samples after homogenization in 50% (v/v) Triton-X 100 at the end of the experiment.

## Statistics

All data were expressed as Mean  $\pm$  SEM of  $n$  observations. The statistical analyses were made by one way analysis of variance (ANOVA) followed by Dunnett's post hoc test (multiple comparisons), or Student's  $t$  test (pairwise comparisons).  $P$  values of less than 0.05 were considered to be statistically significant.

## Results

The extracellular decomposition of ATP was examined by adding ATP (20, 100, and 500  $\mu$ M) to hippocampal slices. As shown in Fig. 1, the decomposition of ATP was rapid ( $T_{1/2} < 2.5$  min). As reported previously, we found that in parallel with the breakdown of ATP, the presence of AMP, adenosine, inosine, and hypoxanthine was detected in the extracellular fluid [2], reflecting the activity of ecto-NTPDase/ectoNPPase, ectonucleotidase, adenosine deaminase, and purine nucleoside phosphorylase enzymes (Fig. 3). As shown in Fig. 3A, when the slices were challenged with UDP, UTP formation was not observed and the initial level of UTP only slightly decreased after its addition. Although ATP and AMP formation were observed at very low levels following UDP addition, this was not ADP dependent on the initial amount of UDP added (Fig. 3B). Therefore, under our experimental conditions, the contribution of nucleoside diphosphate kinase to ATP formation in response to ADP seems to be negligible. To test whether the parallel formation of ATP and AMP after exposure of rat hippocampal slices to ADP was due to the activity of an ecto-adenylate kinase, we utilized the specific inhibitor of ecto-adenylate kinase, diadenosine pentaphosphate (Ap5A; 200  $\mu$ M). Ap5A has previously been shown to inhibit ecto-adenylate kinase enzymes in cultured endothelial cells in this concentration [2]. When Ap5A was injected directly onto the HPLC column, it elicited a peak in the chromatogram, with a retention time of  $14.29 \pm 0.43$  min. A signal with an identical retention time was also observed in tissue supernatant that had been exposed to ADP (20, 100, and 500  $\mu$ M) in the presence of Ap5A (200  $\mu$ M). The level of Ap5A was relatively constant and  $89.84 \pm 5.68\%$  of the initial amount was detected at the end of the collection period ( $n = 4$ ). We found that in

Next, the hippocampal slices were incubated with ADP (20, 100, and 500  $\mu$ M; Figs. 2A–C, respectively). The decomposition of ADP was also rapid ( $T_{1/2} < 0.5$  min, Fig. 2D). In parallel with the decline of ADP extracellular levels, the presence of AMP, adenosine, inosine, and hypoxanthine were detected, reflecting the activity of the ectonucleotidase enzyme chain. The  $K_m$  and  $v_{max}$  values of the ADP decomposition were  $321.5 \pm 94.1 \mu$ M and  $22.6 \pm 3.8$  nmol/min/prep., respectively ( $n = 3$ ). In addition, a simultaneous generation of ATP in amounts almost

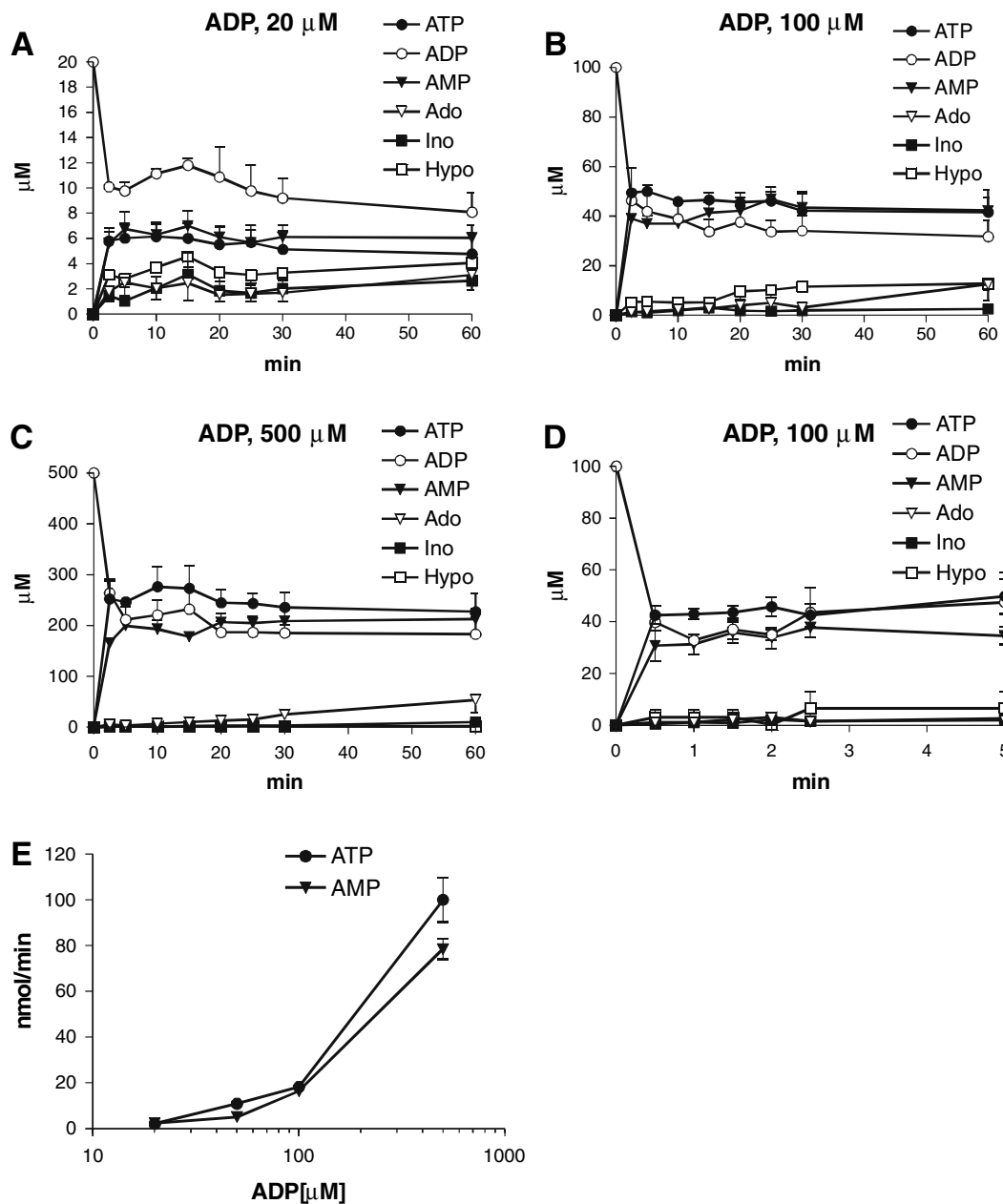


Fig. 2 Extracellular decomposition of ADP in rat hippocampal slices. (A) Hippocampal slices were incubated in 3 ml Krebs solution in the presence of different concentrations of ADP (20, 100, 500 μM). Aliquots (70 μl) were collected at different time points (2.5, 5, 10, 15, 20, 25, 30 and 60 min) after the addition of ADP. (B) The amount of nucleotides (ATP, ADP and AMP) and nucleosides (Ado, adenosine; Ino, inosine; and Hypo, hypoxanthine) in the aliquots were determined by HPLC-UV and expressed in μM. (C) The rate of ATP (circles) and AMP (triangles) formation was calculated from the total amount of nucleotides present in the tissue supernatant during the 60 min period of addition of ADP. The amount of nucleotides (ATP, ADP and AMP) and nucleosides (Ado, adenosine; Ino, inosine; and Hypo, hypoxanthine) in the aliquots were determined by HPLC-UV and expressed in μM. (D) shows the result of a series of similar experiments when aliquots were collected 0.5, 1, 1.5, 2, 2.5 and 5 min after the addition of ADP. (E) the rate of ATP (circles) and AMP (triangles) formation was calculated from the total amount of nucleotides present in the tissue supernatant during the 60 min period of addition of ADP. The amount of nucleotides (ATP, ADP and AMP) and nucleosides (Ado, adenosine; Ino, inosine; and Hypo, hypoxanthine) in the aliquots were determined by HPLC-UV and expressed in μM.

the presence of Ap5A (20 μM), the decomposition of ADP was inhibited at all three concentrations tested (Fig. 4). ADP was significantly slower at all three initial concentrations tested (Fig. 4A and B). In parallel with these changes, the formation of ATP and AMP were also inhibited, with the exception of an initial ADP concentration of 20 μM, when some additional ATP formation was observed (Fig. 4A, C and D). By contrast, AMP formation could be due to leakage of intracellular adenylate kinase

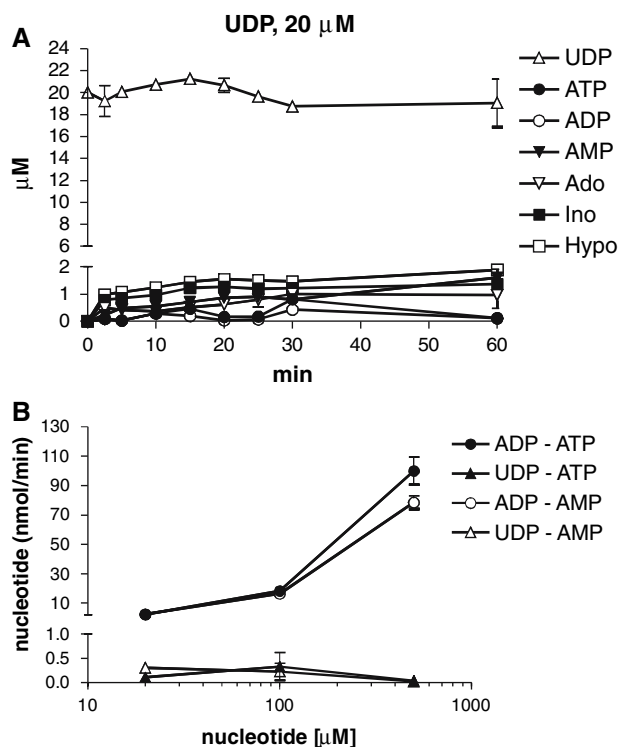


Fig. 3 Extracellular decomposition of UDP (20 μM) in rat hippocampal slices. Hippocampal slices were incubated in 3 ml Krebs solution in the presence of UDP. Aliquots (70 μl) were collected at different time points (2.5, 5, 10, 15, 20, 25, 30 and 60 min) after the addition of UDP. **A**) The amount of nucleotides (UDP, ATP, ADP, and AMP) and nucleosides (Ado, adenosine; Ino, inosine; and Hypo, hypoxanthine) in the aliquots was determined by HPLC-UV and expressed in μM as a function of time. Note that there is a break in the Y axis to enhance the resolution. **B**) (The rate of ATP (filled symbols) and AMP (open symbols) formation following the addition of ADP (circles), and UDP (triangles) was calculated from the total amount of nucleotides present in the tissue supernatant during the 60 min period and is expressed in nmol/min, as a function of initial nucleotide concentration (n = 3 independent experiments)

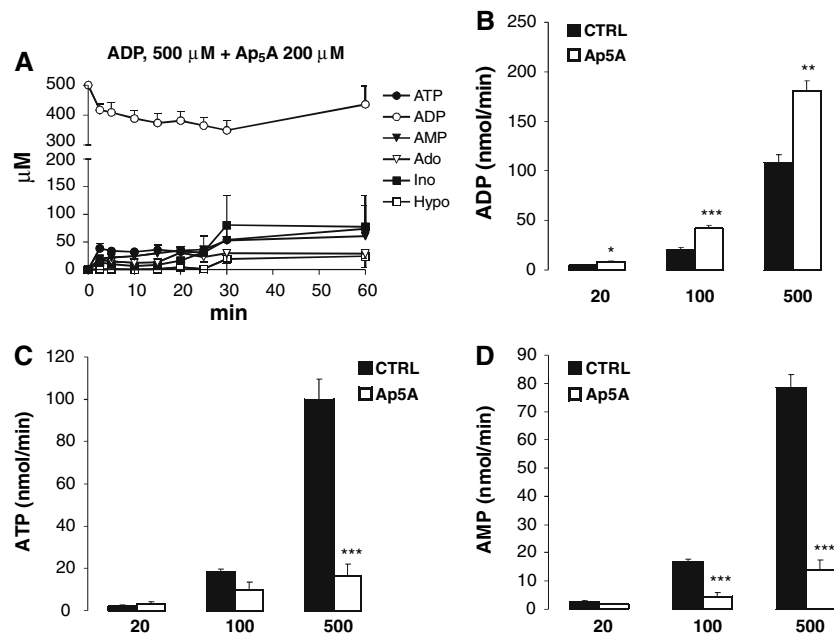
activity through damaged membranes. To exclude this possibility, LDH assay was performed in aliquots of media collected after addition of ADP (100 μM). LDH is a stable intracellular enzyme and a reliable marker of membrane damage. The released LDH activity was low and constant in the samples, and accounted for less than 2.27 ± 0.52% (n = 8) of the total LDH activity found in the slices after disruption by Triton-X 100.

The direct transfer of phosphoryl groups was not controlled in our experiments. Thus, it was essential to exclude the role of any transport mechanism that might influence the interconversion of nucleotides and nucleosides. Bidirectional nucleoside transporters are expressed in the hippocampus and regulate the transmembrane movement of nucleosides under different conditions [26, 27]. To examine the involvement of nucleoside transporters in our system, the interconversion of ADP was evaluated in the

presence of dipyridamole (30 μM), a specific inhibitor of nucleoside transporter. The decomposition of ADP (100 μM) was qualitatively similar to that observed in the absence of dipyridamole (Fig. 5A), suggesting that the activity of nucleoside transporters does not influence the process under our experimental conditions. Moreover, the specific adenylate kinase inhibitor Ap5A (200 μM) inhibited both the decomposition of ADP and the formation of ATP and AMP in dipyridamole-treated slices (Fig. 6A).

Other potential mechanisms that may allow the entry of ATP into the extracellular space in the absence of depolarization include the P2 receptor operated [28] and connexin hemichannel mediated ATP release [29, 30]. PPADS is a P2 receptor antagonist that acts on P2X1, P2X2, P2X3, P2X5, P2X7, as well as P2Y1, P2Y4, P2Y6, and P2Y13 subtypes of P2X and P2Y receptors. Here, it was used at a concentration of 30 μM, which almost maximally inhibits P2 receptors, but has only a negligible effect on ectoATPases [10]. As shown in Fig. 5B, the decomposition of ADP (100 μM) did not significantly change in the presence of PPADS (30 μM). However, a modest inhibitory effect on AMP formation that did not affect ADP degradation was observed when an initial ADP concentration of 20 μM was used (2.482 ± 0.352 and 0.241 ± 0.193 nmol/min in the absence and presence of 30 μM PPADS, respectively, n = 3, P < 0.05). Likewise, in the presence of the connexin hemichannel inhibitor, bufenamic acid (200 μM), neither ADP decomposition, nor ATP and AMP formation significantly changed in response to ADP (100 μM) exposure (Fig. 5B). The role of alkaline phosphatases in the generation of AMP after ADP (100 μM) exposure was examined by co-incubation with para-nitrophenylphosphate (PNP, 1 mM). PNP is a substrate of alkaline phosphatase, and when added in excess it is expected to inhibit the hydrolysis of other substrates of alkaline phosphatases, such as ADP. No significant changes in the levels of ADP, ATP and AMP were observed in the supernatant in the presence of PNP (Fig. 5B), suggesting that alkaline phosphatases were not involved in the interconversion of ADP to AMP.

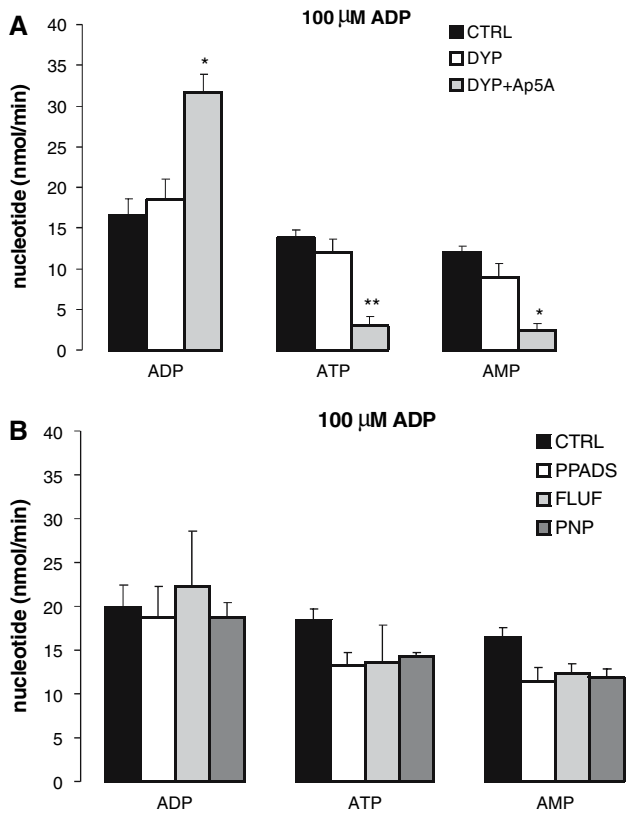
Next, hippocampal slices were exposed to AMP and adenosine to reveal any other ectokinase activity that may phosphorylate adenine nucleotides and nucleosides. When AMP (20 μM) was added to the slices, the formation of adenosine, inosine and hypoxanthine was observed in parallel with the decomposition of AMP, but neither ADP nor ATP were detected in the extracellular fluid (Fig. 6A), and similar decomposition pattern was observed at 100 and 500 μM initial AMP concentration (data not shown). This finding shows that in the absence of ATP, ecto-adenylate kinase does not act in a reverse direction. The half of AMP was 25 min and the  $K_m$  and  $v_{max}$  values of AMP decomposition were 267 ± 52.8 μM and 4.99 ± 0.44 nmol/min/



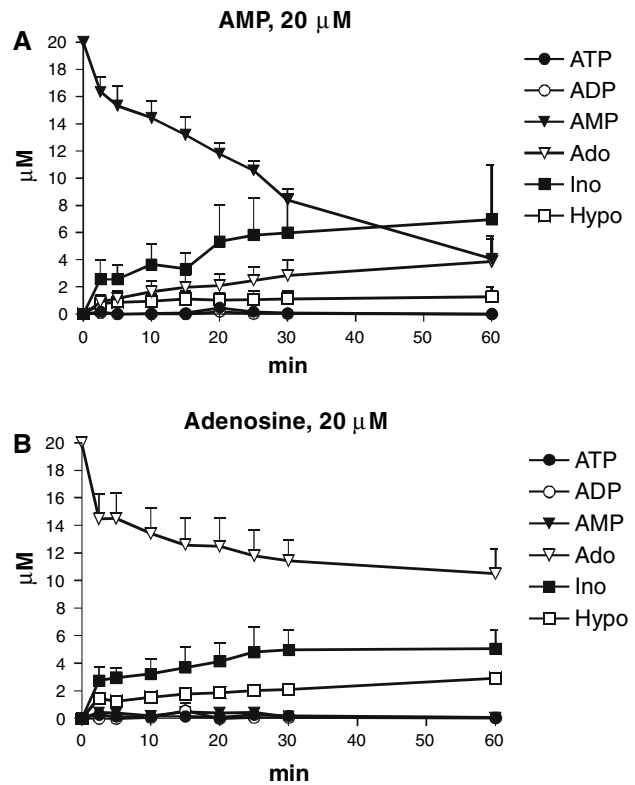
**Fig. 4** The specific adenylylase inhibitor, diadenosine pentaphosphate (Ap5A, 200 μM), inhibits the degradation of ATP and the coincident formation of ATP and AMP in response to ADP application. Hippocampal slices were incubated in 3 ml Krebs solution containing ADP (20, 100 and 500 μM) in the absence (CTRL) and presence of Ap5A (200 μM). Subsequently, aliquots were collected at different time points (2.5, 5, 10, 15, 20, 25, 30 and 60 min) after the addition of ADP. The amount of nucleotides (ATP, ADP, and AMP) and nucleosides (Ado, adenosine; Ino, inosine; and Hypo, hypoxanthine) in the aliquots were determined by HPLC-UV and expressed as a function of time. Note that there is a break in the y-axis to enhance the resolution. The average amount of ADP (B), ATP (C) and AMP (D) present in the tissue supernatant in the 60 min sample collection period following the addition of three initial ADP concentrations (20, 100 and 500 μM) is calculated in the absence (black bars) and presence of Ap5A (white bars) and the results are expressed in nmol/min. Asterisks indicate significant differences between Ap5A treated and control slices ( $P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ ,  $n = 4$  independent experiments)

prep. ( $n = 4$ ), respectively. Similarly, when hippocampal slices were challenged with increasing concentrations of adenosine (20 μM, Fig. 6B, 100 and 500 μM, not shown), only inosine and hypoxanthine were detected in parallel with the breakdown of adenosine. No substantial generation of AMP, ADP or ATP was detected (Fig. 6B).

To determine whether the net formation of ATP in response to ADP application was associated with the activity of various isoforms of E-NTPDases [10, 12, 31]. In addition, the decomposition of ADP was examined in purified synaptosomes derived from the rat hippocampus. The decomposition of ADP was characterized by a delayed and transient, but not coincident production of ADP, which suggested to us that the majority of ADP was generated in the second step of a sequential enzymatic pathway. The  $K_m$  value of ATP hydrolysis established by the present study, broadly lies within the range that has been previously reported in brain slices and isolated tissue [24, 32, 35, 36]. Although the identity of E-NTPDase isoform was not addressed in this study, the weak UDP hydrolyzing activity found in our experiments is indicative for the involvement of E-NTPDase 1 and 2 immunoreactivity is restricted to the resting microglia, blood vessels, and neuronal progenitor cells of the hippocampal dentate gyrus [39]. By contrast,



**Fig. 5** Blockade of the nucleoside transporters (A) and the transmembrane movement of nucleotides (B) do not affect the accumulation of ATP and AMP after ADP addition. Hippocampal slices were incubated with ADP (100 μM) (A) in the absence (CTRL) and presence of the nucleoside transport inhibitor dipyridamole (3 μM, DYP) or DYP + Ap5A (200 μM), (B) in the absence (CTRL) and presence of the P2 receptor antagonist PPADS (100 μM), the connexin hemichannel inhibitor βufenamic acid (FLUF, 50 μM) and the alkaline phosphatase inhibitor para-nitrophenylphosphate (PNP, 1 mM). Experiments were performed according to the protocol shown in Fig. 2B. The average amount of ADP, ATP and AMP present in the tissue supernatant in the 60 min sample collection period following the addition of ADP is calculated in the absence and presence of different drugs and the results are expressed in nmol/min. Asterisks indicate significant differences from control slices (\*P < 0.05, \*\*P < 0.01, n = 3 × 4 independent experiments)



**Fig. 6** Extracellular decomposition of AMP (20 μM) and adenosine (20 μM) in rat hippocampal slices. Hippocampal slices were incubated in 3 ml Krebs-Ringer solution in the presence of AMP or adenosine (20 μM). Aliquots (70 μl) were collected at different time points (2.5, 5, 10, 15, 20, 25, 30 and 60 min) after the addition of adenyl compounds. The amount of nucleotides (ATP, ADP, AMP) and nucleosides (Ado, adenosine; Ino, inosine; and Hypo, hypoxanthine) in the aliquots were determined by HPLC-UV and expressed as a function of time (i = 3 × 4 independent experiments)

E-NTPDase 3 immunoreactivity was associated with axonal processes and presynaptic terminals in the rodent brain and are in accordance with previous studies in whole brain although it was only scarcely detectable in the hippocampus synaptosomes [17]. Moreover, the activity found in our [40]. Thus, the ATP diphospho-hydrolyzing activity study probably represents the activity of AK4, which has observed in the present study could represent either neuronal or non-neuronal activity. The decomposition pattern of ATP level in the pyramidal cell layer of the hippocampus would be also consistent with the activity of NPPase and alkaline phosphatases. However, these latter two enzymes display low abundance in the central nervous system. In addition, alkaline phosphatase has a low affinity for ATP at a physiological pH [38].

The principal new finding of the present study is that in the presence of Mg<sup>2+</sup>, which is required for ectonucleotidase activity addition to the known ectonucleotidase hydrolyzing [18].

activities, there is also catalytic activity in the tissue supernatant of hippocampal slices that converts ADP to ATP and AMP. This activity probably corresponds to the ecto-adenylate kinase activity demonstrated in cortical synaptosomes [16, 19], neuroblastoma cells [21] and at the frog neuromuscular junction [20]. The kinetic parameters of the ecto-adenylate kinase activity we observed in the synaptosomal preparation are in the low millimolar range and are in accordance with previous studies in whole brain synaptosomes [17]. Moreover, the activity found in our [40]. Thus, the ATP diphospho-hydrolyzing activity study probably represents the activity of AK4, which has observed in the present study could represent either neuronal or non-neuronal activity. The decomposition pattern of ATP level in the pyramidal cell layer of the hippocampus would be also consistent with the activity of NPPase and alkaline phosphatases. However, these latter two enzymes display low abundance in the central nervous system. In addition, alkaline phosphatase has a low affinity for ATP at a physiological pH [38].

Conversely, our results differ from those of Kukulski et al. who failed to detect ecto-adenylate kinase activity in porcine brain synaptosomes [41]. Apart from species heterogeneity, these discrepancies may be due to the fact that in the latter study the incubation media did not contain Mg<sup>2+</sup>, which is required for ecto-adenylate kinase activity

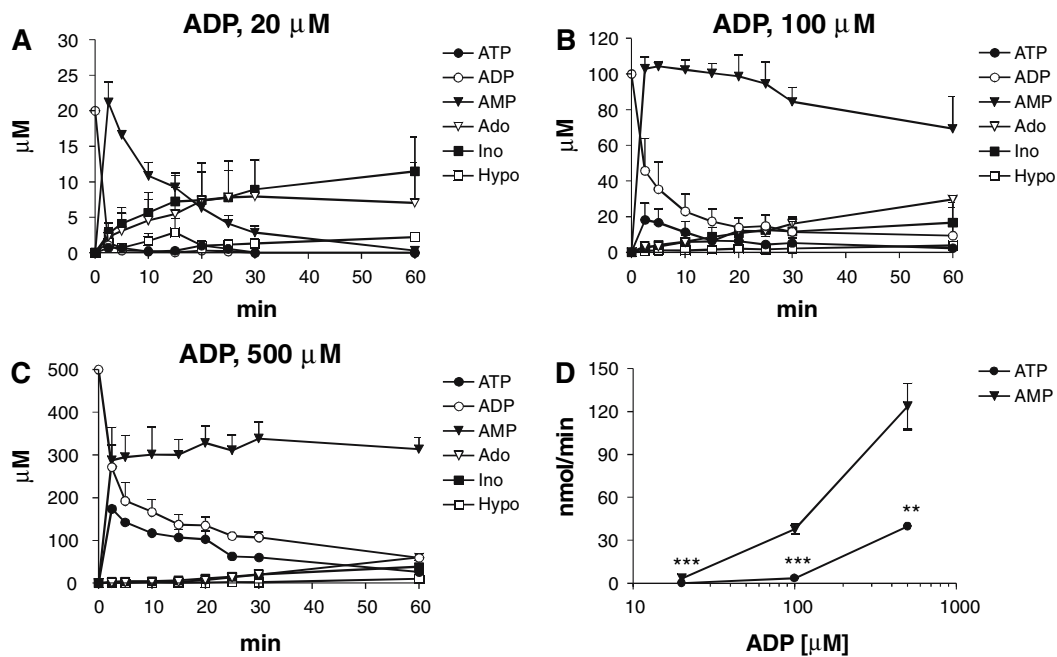


Fig. 7 Extracellular decomposition of ADP in purified synaptosomes and Hypo, hypoxanthine) in the aliquots were determined by HPLC-prepared from rat hippocampal slices. Synaptosomes were prepared and expressed in  $\mu\text{M}$  as a function of time. **A**) The rate of ATP according to the procedure described in Materials and Methods (circles) and AMP (triangles) formation was calculated from the total Aliquots (100  $\mu\text{l}$ ) of the synaptosomes were added to 3 ml of Krebs amount of nucleotides present in the tissue supernatant during the solution and incubated in the presence of different concentrations of 60 min period and is expressed in nmol/min, as a function of initial ADP (A, 20  $\mu\text{M}$ ; B, 100  $\mu\text{M}$ ; C, 500  $\mu\text{M}$ ). Aliquots (70  $\mu\text{l}$ ) were ATP concentration. Asterisks indicate significant differences between collected at different time points (2.5, 5, 10, 15, 20, 25, 30 and ATP and AMP concentrations at identical points  $P < 0.01$ , 60 min) after the addition of ATP. **D**) The amount of nucleotides (ATP, ADP and AMP) and nucleosides (Ado, adenosine; Ino, inosine;  $*** P < 0.001, n = 3$  independent experiments)

The conversion of ADP to ATP may also be related to metabolism of nucleotides in the hippocampus requires the activity of nucleotide nucleoside diphosphate kinase further investigation.

which has been identified on the surface of endothelial and Adenylate kinase can function in both directions: lymphoid cells [22, 23]. However, the substrate specificity according to conventions, the forward direction where of nucleoside diphosphate kinase is different from adenylate kinase since it can also phosphorylate pyrimidinedirection where ATP gives rise to the formation of ADP in dinucleotides [21]. In our experiments, the application of the presence of AMP. Thus, the delayed production of ADP UDP did not result in the appearance of uridine triphosph-in response to addition of ATP may be due to the reverse phates, suggesting that in our system nucleoside activity of adenylate kinase. In this case ATP would be diphosphate kinase is not involved in the conversion of partly converted to AMP by E-NTPDase in the first step, ADP to ATP.

Moreover, a definitive proof for the involvement of ADP by the ecto-adenylate kinase in the second step. In adenylate kinase was obtained by the use of the highly support of this assumption, we show that when only AMP specific adenylate kinase inhibitor Ap5A, which significantly is added to the hippocampal slices, ADP production is not cantly inhibited the decomposition of ADP and the parallel observed, indicating that adenylate kinase cannot act in the formation of ATP and AMP, respectively. Although, we reverse direction in the absence of ATP. could detect a small amount of residual ATP formation Since adenylate kinase is an intracellular enzyme, the even in the presence of Ap5A, this could be due to the ATP activity detected in the tissue supernatant could also be due to release from the preparation by P2 receptor activation leakage and/or transport from the cytoplasm to the extra- other mechanisms, as discussed below. Interestingly, it has been reported recently that the F<sub>1</sub>F<sub>0</sub> ATPase could also possibility of minor contamination by intracellular enzymes, reach the cell surface where it exhibits both ATP synthase our data indicate that the majority of enzyme activity found sizing and hydrolyzing activities in non-neuronal cells [43]. However, its involvement in the extracellular we found that the activity of LDH, a stable intracellular

enzyme and a reliable marker of membrane damage, was low in microvessels. In an attempt to localize the ecto-adenylate kinase activity detected in the hippocampal slices, we also examined the extracellular interconversion of ATP in other cytosolic enzyme activity in the extracellular fluid, purified hippocampal synaptosomes. We found that ATP including nucleoside diphosphate kinase or adenosine kinase, which would have also been released into the medium, although in response to predominantly higher initial ATP concentrations. These findings indicate that the ecto-adenylate activity observed in the hippocampal slices is generation was not detected in the hippocampal slices under basal conditions, in the absence of ADP.

Other mechanisms should also be considered when examining the production of ATP in response to addition of ADP to hippocampal slices. For example, ATP accumulation might be the result of a primary or secondary release of ATP from cellular compartments. It has been shown that exposure of hippocampal slices to high concentrations of nucleotides or nucleosides, results in their uptake followed by initiation of further release of nucleotides and nucleosides through the homo- or heteroexchange by nucleoside transporters [7]. However, dipyrindamole, an inhibitor of heteroexchange, did not affect ATP accumulation in the present study, ruling out the involvement of this particular pathway. Nevertheless, the two mechanisms can operate independently from each other. Lack of ecto-adenylate kinase detection in our previous study was probably due to the fast removal of released compounds by the superfusion system [7], whereas in this study the lack of a continuous supply of nucleotides to the transporter prevented the detection of heteroexchange under these steady state conditions.

P2 receptors and connexin hemichannels are also known to mediate ATP release in the nervous system under certain circumstances [8, 30]. However, neither PPADS (a P2 receptor antagonist) nor  $\beta$ ufenamic acid (the connexin hemichannel inhibitor) affected ATP generation in response to ADP application, except at the lowest initial ADP concentration, where PPADS significantly attenuated AMP generation. These findings suggest that although P2-receptor mediated ATP release might be responsible for the Ap5A-insensitive ATP production, the majority of ATP production is independent from the activity of PPADS-sensitive P2 receptors and connexin hemichannels. Finally, AMP, but not ATP formation from ADP may also be mediated by alkaline phosphatases. However, co-application of PNP with ADP did not modify subsequent AMP production by substrate-inhibition, indicating that this was not the case in our system.

Until now, ecto-adenylate kinase activity has been only demonstrated in cultured cells and nerve terminals [23]. However, our findings in the present study indicate that ecto-adenylate kinase activity may be associated with nerve terminals, astrocytes or other cellular elements in the hippocampal slices such as microglia, endothelia and

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