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# Phenylalanine and phenylpyruvate inhibit ATP diphosphohydrolase from rat brain cortex

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**Abstract**

The main objective of the present study was to characterize the inhibition by phenylalanine and phenylpyruvate of ATP diphosphohydrolase activity in synaptosomes from the brain cortex of rats. This enzyme participates together with a 5′-nucleotidase in adenosine formation from the neurotransmitter, ATP, in the synaptic cleft. The inhibition of ATP diphosphohydrolase was competitive for nucleotide hydrolysis but 5′-nucleotidase was not affected by these metabolites. Furthermore, the two substances inhibited enzyme activity by acting at the same binding site. If the enzyme inhibition observed in vitro also occurs in the brain of PKU patients, it may promote an increase in ATP levels in the synaptic cleft. In this case, the neurotoxicity of ATP could possibly be one of the mechanisms leading to the characteristic brain damage of phenylketonuria. © 2001 ISDN. Published by Elsevier Science Ltd. All rights reserved.

*Keywords:* ATP diphosphohydrolase; Apyrase; 5′-nucleotidase; Phenylketonuria; Phenylalanine; Phenylpyruvate

## 1. Introduction

Phenylketonuria (PKU) is an inherited deficiency of phenylalanine hydroxylase activity in the liver, which leads to increased plasma and brain levels of phenylalanine (Phe) and its deaminated metabolites (Scriver et al., 1995). The most crucial effect, i.e. permanent brain damage, occurs during the early period of postnatal brain development and may be related to improper neuron differentiation and synaptogenesis (Nigan and Labor, 1979; Bauman and Kemper, 1982; Ushakova et al., 1997). However, the mechanisms underlying brain damage are possibly multiple and not fully understood Hommes, 1991.

ATP is known to exert potent effects on the central nervous system, where it can act as a neurotransmitter or as a modulator regulating the activity of other neurotransmitters (Burnstock, 1972; Richardson and Brown, 1987; Edwards et al., 1992). ATP released from synaptosomes must be metabolized to adenosine, this being the principal form of nucleoside re-uptake and neuromodulation (Edwards et al., 1992). Extracellular adenine nucleotides are subject to degradation by ectonucleotidases and can generate adenosine in the synaptic cleft (Battastini et al., 1991; Garcia et al., 1997). Thus, these enzymes which promote the extracellular hydrolysis of nucleotides may represent a mechanism for the control of nucleotide/nucleoside concentration and consequently for the duration and extent of cell-to-cell receptor activation.

ATP diphosphohydrolase (ATPDase, apyrase, EC 3.6.1.5) is the general designation for enzymes that hydrolyze triphospho and diphosphonucleosides to

*Abbreviations:* ADP, adenosine-5′-diphosphate; AMP, adenosine-5′-monophosphate; ATP, adenosine-5′-triphosphate; EDTA, ethylenediamine tetraacetate; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; HPA, hyperphenylalaninemia; Phe, phenylalanine; PKU, phenyolketonuria; PP, phenylpyruvate; THA, 9-amino-1,2,3,4-tetrahydroacridine.

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their equivalent monophosphonucleosides and inorganic phosphate (Meyerhof, 1945). The physiological role proposed for ATP diphosphohydrolase in the peripheral and central nervous systems is its participation together with 5′-nucleotidase (EC 3.1.3.5) in an 'enzyme chain' which hydrolyzes ATP to adenosine in the synaptic cleft (Battastini et al., 1991; Garcia et al., 1997). The adenosine thus formed can act on P1-purinoceptors and is rapidly taken up via high-affinity uptake systems that are present in neurons and mediate the salvage of physiological purines for incorporation into cellular nucleotides (Burnstock, 1972; Zimmermann, 1996).

We have previously reported that Phe and its deaminated metabolite phenylpyruvate (PP) inhibit in vitro ATP diphosphohydrolase activity in synaptosomes from the cerebral cortex of rats by approximately 30% (Wyse et al., 1994). Now, we have determined the type of inhibition of Phe and PP on ATP diphosphohydrolase and also studied the effect of these substances on 5′-nucleotidase, in order to better understand the pathophysiology of brain damage in PKU.

## 2. Experimental procedures

### 2.1. Materials

Nucleotides (ADP, AMP), HEPES, Trizma Base and EDTA were obtained from Sigma Chemical Co (St. Louis, MO, USA). Sucrose, $CaCl_2$, and KCl were obtained from Merck (Darmsdat, FRG). Percoll was purchased from Pharmacia (Uppsala, Sweden) and was routinely filtered through Millipore AP15 pre-filters to remove aggregated and incompletely coated particles. All other reagents used were of analytical grade.

### 2.2. Animals

Wistar rats from our own breeding stock were maintained on a 12-h light:12-h dark cycle (lights on at 07:00 h) in an air-conditioned constant temperature ($20 \pm 1$ °C) colony room. Pups in litters that averaged eight in number remained with the dam, unless otherwise indicated, until weaning at 21 days of age. The rats were killed between 25 and 30 days of age, a period in which ATP diphosphohydrolase has achieved its maximum activity (Müller et al., 1993) and the brain are still in development.

### 2.3. Synaptosomal preparation

The synaptosomes were isolated as described previously (Battastini et al., 1991). The synaptosome pellet was resuspended to a final protein concentration of approximately 0.5 mg/ml. The material was prepared fresh daily and maintained at 0–4 °C throughout preparation.

### 2.4. Enzyme assays

The reaction medium used to assay ecto ATP diphosphohydrolase activity was essentially as described previously (Battastini et al., 1991) and contained 5.0 mM KCl, 1.5 mM $CaCl_2$, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose, and 45 mM Tris–HCl buffer, pH 8.0, in a final volume of 200 μl. The enzyme preparation (10–20 μg protein) was added to the reaction mixture and preincubated for 10 min at 37 °C. The reaction was started by the addition of ADP (vanadium-free sodium salt) to a final concentration of 1.0 mM and stopped by the addition of 200 μl of 10% trichloroacetic acid. The reaction medium used to assay ecto 5′-nucleotidase activity contained 10 mM $MgCl_2$, 0.1 M Tris–HCl, pH 7.0 and 0.15 M sucrose in a final volume of 200 μl. The reaction was started by the addition of AMP (vanadium-free sodium salt) to a final concentration of 1.0 mM and stopped by the addition of 200 μl of 10% trichloroacetic acid. For both assays the enzyme preparation (10–20 μg protein) was preincubated for 10 min at 37 °C. The concentration of Phe used in the assay was in the 0.5–3.0 mM range and the concentration of PP was in the 2.5–7.5 mM range. The samples were chilled on ice for 10 min and 100 μl samples were taken for the assay of released inorganic phosphate (Pi) (Chan et al., 1986). In both enzyme assays, incubation time and protein concentration were chosen in order to ensure the linearity of the reactions. Controls with the addition of the enzyme preparation after the addition of trichloroacetic acid were used to correct for nonenzymatic hydrolysis of the substrates. All samples were run in duplicate and the means were taken for statistical analyses.

### 2.5. Protein determination

Protein was measured according to Bradford (1976) using bovine serum albumin (BSA) as standard.

### 2.6. Characterization of enzyme inhibition

The inhibition type was characterized by the Lineweaver–Burk plot and the interaction between inhibitors by the Chevillard, Cárdenas and Cornish–Bowden plot Chevillard et al. (1993).

### 2.7. Statistical analysis

Data were analyzed by one-way ANOVA, followed by the Duncan multiple range test when $F$ was significant. All analyses were performed with an IBM compatible computer using the SPSSPC software.

## 3. Results

The activity of ATP diphosphohydrolase in synaptosomes from rat cerebral cortex was 72.2 ± 14.3 nmol Pi per min per mg protein for ADP hydrolysis. These values are similar to those previously obtained (Battastini et al., 1991).

The type of inhibition was determined using a Lineweaver-Burk double-reciprocal plot. The activity for ADP hydrolysis was analyzed over a range of 0.1–0.25 mM substrate concentration in the absence and presence of Phe (0.75–1.5 mM) or PP (4.25–4.75 mM). The results clearly indicate that the inhibition of ADP hydrolysis by Phe or by PP in synaptosomes from the cerebral cortex is competitive (Fig. 1A and B).

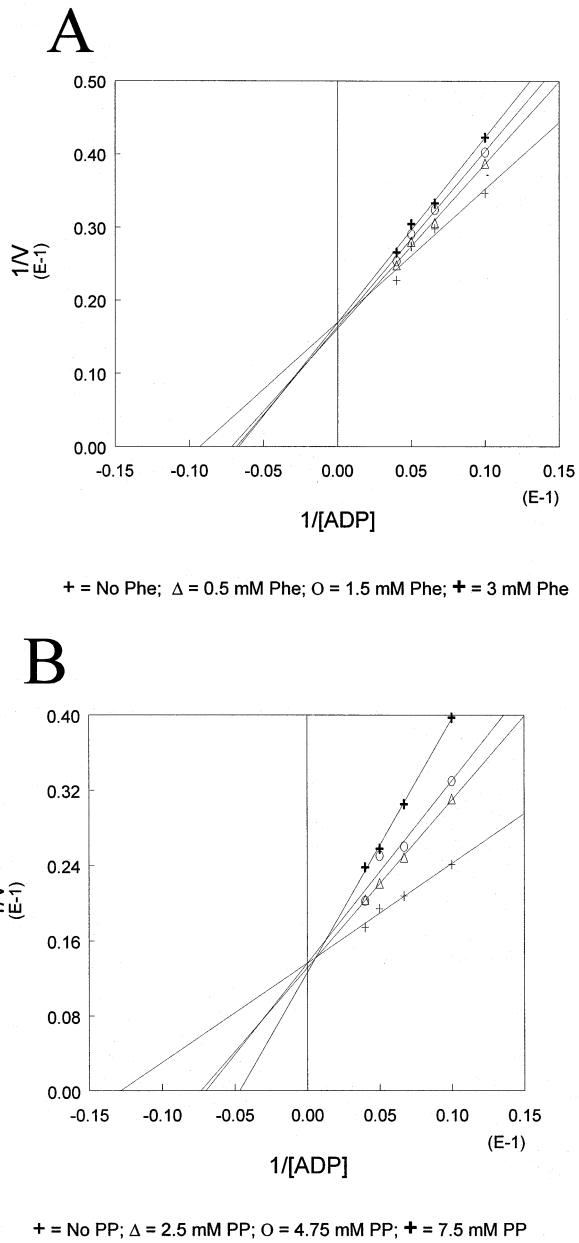


Fig. 1. Kinetic analysis of the inhibition of ATP diphosphohydrolase by Phe (A) and PP (B) in synaptosomes from cerebral cortex of rats. The graphs show double-reciprocal plots of apyrase for ADP concentrations ( 0.1, 0.15, 0.2, 0.25 mM) in the absence and in the presence of Phe (A) or PP(B). All experiments were repeated at least three times and similar results were obtained. Data presented are from individual experiments.

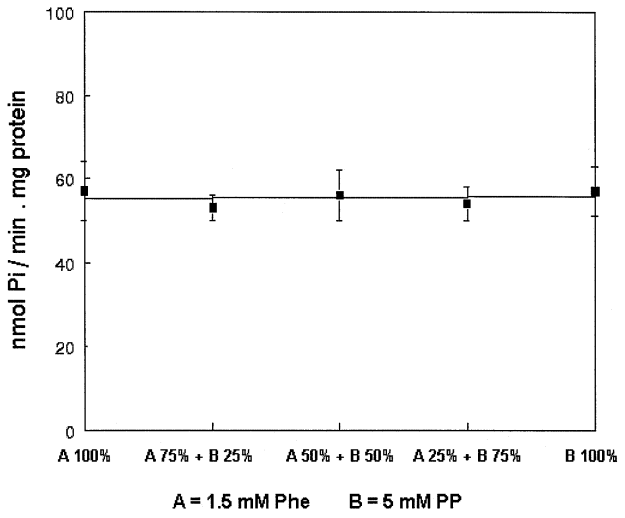


Fig. 2. Competition plot. The concentration of Phe or PP at which the inhibition was the same for ADP hydrolysis was chosen for the Chevillard et al. (1993) plot. The assay conditions are described in Section 2. Incubation time was 20 min. Inhibitor A (Phe) at $P = 0$ was 1.5 mM, and inhibitor B (PP) at $P = 0$ was 5 mM. Data are mean ± S.D. for three independent experiments performed in duplicate.

To determine whether or not the inhibition caused by Phe and PP occur at the same site of the enzyme, we performed kinetic studies on the interaction between the two compounds in terms of their inhibitory action on ATP diphosphohydrolase, according to the adaptation by Wyse et al. (1998) of the model described by Chevillard et al. (1993). The horizontal straight line obtained in the resulting competition plot, i.e. a constant rate independent of the proportion between Phe and PP concentration, indicates that the inhibitory activities occur at a common site of the enzyme (Fig. 2).

The activity of 5′-nucleotidase in synaptosomes from rat cerebral cortex was 9.42 ± 0.92 nmol Pi per min per mg of protein for AMP hydrolysis. These values are similar to those previously obtained (Bonan et al., 1997). The enzyme activity was not significantly affected by Phe ($F_{(6.21)} = 0.048$; $P > 0.05$) or by PP ($F_{(3.12)} = 0.177$; $P > 0.05$) (results not shown).

## 4. Discussion

The extracellular hydrolysis of nucleotides is not only of functional importance during synaptic transmission, where it functions in signal elimination, but also plays a

crucial role in the survival and differentiation of neuronal cells in vitro and presumably during neuronal development in vivo (Zimmermann, 1996).

The rapid breakdown of ATP (and ADP) also acts to reduce the extracellular concentration of these nucleotides within the vicinity of P2 purinergic receptors that mediate the various biological responses to ATP and ADP (Kobayashi et al., 1997). The activity of ATP diphosphohydrolase results in hydrolysis of these nucleotides to AMP, which is further degraded to adenosine by the action of a 5′-nucleotidase (Kobayashi et al., 1997).

An important aspect to be considered is the action of the same enzyme hydrolysing both nucleotideos (ATP and ADP) and modifying the role of the synaptic transmission and, thus, reducing the availability of ligands for the next ectonucleotidase in the enzyme chain, the 5′-nucleotidase.

We have demonstrated that synaptosomal ATP diphosphohydrolase activity from rat brain is inhibited by THA, a drug proposed for the treatment of Alzheimer's disease (Bonan et al., 1997). In the present study we showed, for the first time, this type of inhibition by Phe and PP of the enzyme ATP diphosphohydrolase of synaptosomes obtained from rat cerebral cortex in vitro. Conversely, Phe and PP did not significantly alter the 5′-nucleotidase activity in the same fraction.

We have previously demonstrated that Phe and its deaminated metabolite PP have opposite effects on ATP diphosphohydrolase activity: at low concentrations (1–3 mM) Phe inhibits the enzyme activity whereas PP stimulates it; at higher concentrations (4–6 mM), Phe stimulates and PP inhibits the enzyme activity. We have postulated that the enzyme has two binding sites for Phe and PP, with different affinities for the two metabolites, one stimulating and the other inhibiting enzyme activity (Wyse et al., 1994).

Now we investigated the mechanism of enzyme inhibition by Phe or PP. The results obtained with the Chevillard plot demonstrated that the two substances act at the same site and the Lineweaver–Burk plot suggests that this site is the active one. However, the maximum inhibition achieved was approximately 30% for each one of the substances, and their competition with the substrate (ADP) occurred only at a narrow range of concentrations, being reversed at higher concentrations of each substance (Wyse et al., 1994), a fact difficult to explain by simple competition at the active site. We postulate the existence of a binding site for ADP, Phe and PP, where ADP acts to enhance the enzyme activity and Phe or PP act to decrease it. It is very important to know the type of inhibition of the enzyme ATP diphosphohydrolase by Phe and PP because it is possible that this approach may be used to design drugs to avoid this inhibition.

It may be speculated that the 30% decrease in ATP diphosphohydrolase activity may provide less AMP to 5′-nucleotidase and may thus reduce, at the same time, the ATP cleavage and adenosine formation to some extent. The enhancement of ATP levels in the synaptic cleft may promote a dramatic increase in intracellular calcium levels mediated by P2X receptors which may represent a significant damage similar to that induced by glutamate excess (Lipton and Rosemberg, 1994).

The mechanisms of brain damage in PKU seem to be multiple and are poorly understood. There are many lines of evidence to suggest that phenylalanine is the main factor affecting every phase of brain development, but the role of Phe metabolites remains obscure (Scriver et al., 1995). It has been suggested that Phe interferes with the selective stabilization of developing synapses (Nigan and Labor, 1979; Hommes, 1991). The present data indicate that Phe inhibits the enzyme activity at concentrations usually found in plasma and spinal fluid of PKU patients (1–3 mM). However, plasma and spinal fluid PP levels in PKU patients are much lower (less than 1 mM) than the concentrations necessary to reduce ATP diphosphohydrolase activity (4–6 mM).

In conclusion, phenylalanine and its main deaminated metabolite penylpyruvate inhibit ATP diphosphohydrolase activity, but not 5′-nucleotidase activity in synaptosomes of cerebral cortex of rats. Kinetic studies suggest that the two substances act on the same binding site, possibly together with ADP. If the enzyme inhibition, at least by Phe, also occurs in the brain of PKU patients, it could promote an increase in ATP levels at the synaptic cleft. In this case, the neurotoxicity of ATP could possibly be one of the mechanisms leading to the brain damage characteristic of phenylketonuria.

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