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Catabolism of Ap_4A and Ap_5A by rat brain synaptosomes

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Abstract

Adenosine 5',5'''-P^1,P^4-tetraphosphate (Ap_4A) and adenosine 5',5'''-P^1,P^5-pentaphosphate (Ap_5A) are stored in and released from rat brain synaptic terminals. In the present study we investigated the hydrolysis of dinucleotides (Ap_4A and Ap_5A) in synaptosomes from the cerebral cortex of adult rats. Ap_4A and Ap_5A, but not Ap_3A, were hydrolyzed at pH 7.5 in the presence of 20 mM Tris/HCl, 2.0 mM $MgCl_2$, 10 mM glucose and 225 mM sucrose at 37°C. The disappearance of the substrates measured by FPLC on a mono-Q HR column was both time and protein dependent. Since synaptosome integrity was at least 90% at the end of the assay, hydrolysis probably occurred by the action of an ecto-enzyme. Extracellular actions of adenine dinucleotides at central nervous system terminate due to the existence of ecto-nucleotidases which specifically cleave these dinucleotides. These enzymes in association with an ATP diphosphohydrolase and a 5'-nucleotidase are able to promote the complete hydrolysis of dinucleotides to adenosine in the synaptic cleft.

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- Ap_nAase

Adenosine 5',5'''-P^1,P^4-tetraphosphate (Ap_4A) and adenosine 5',5'''-P^1,P^5-pentaphosphate (Ap_5A) are stored in secretory granules of chromaffin cells (1) and in the rat brain synaptic terminals (2), being released in a calcium-dependent process (2). Furthermore, the presence of high-affinity receptors for diadenosine polyphosphates (Ap_nA) in neural tissues has been reported (3,4). Diadenosine polyphosphates are considered to be a new class of neurotransmitter substances and extracellular neural modulators (5-7). Thus, an enzyme able to hydrolyze extracellular dinucleotides may be a mechanism to control their life span and thus the duration and extent of receptor activation (5).

A diadenosine polyphosphate hydrolase (Ap_nAase) has been described in chromaffin cells and the action of this enzyme together with an ecto-apyrase and an ecto-5'-nucleotidase can promote the complete extracellular hydrolysis of the dinucleotides Ap_4A and Ap_5A to adenosine (5,8,9). Recently, Mateo et al. (10) have shown a similar association of enzymes in the electric organ of *Torpedo* (peripheral nervous system). With respect to the central nervous system, previous studies have demonstrated that extracellular ATP is hydrolyzed to adenosine by the action of an ecto-ATP diphosphohydrolase (ecto-apyrase) and an ecto-5'-nucleotidase in intact synaptosomes from rat brain (11-13).

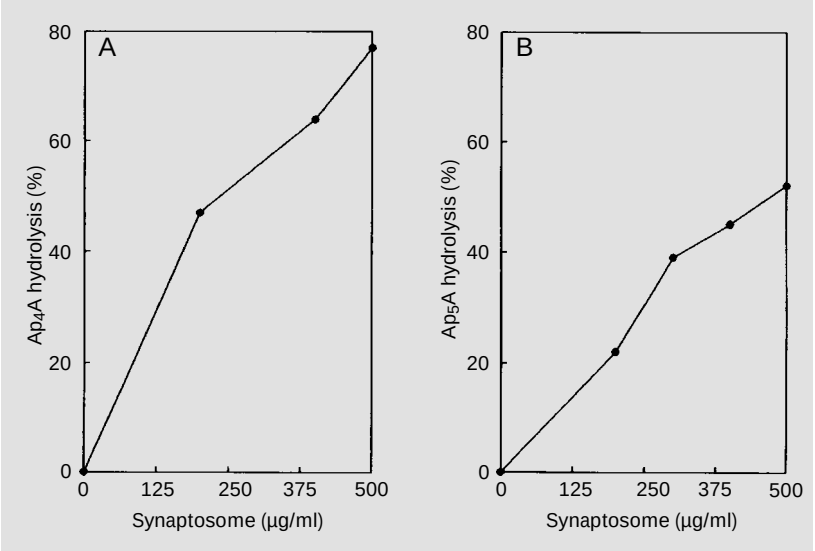

Figure 1 - Ap_4A (A) and Ap_5A (B) hydrolysis as a function of synaptosome protein concentration. The assay mixture contained 20 mM Tris/HCl, pH 7.5, 2.0 mM $MgCl_2$, 10 mM glucose and 225 mM sucrose in a final volume of 200 µl. The samples were incubated for 60 min at 37°C. The data are representative of three independent experiments with different synaptosome preparations.

The aim of the present study was to demonstrate ecto-diadenosine polyphosphate hydrolase activity (ecto-Ap_nAase) in synaptosomes from cerebral cortex of adult rats. The role of this enzyme activity in the purinergic system is discussed.

Adult male Wistar rats (230-250 g) from the local breeding colony were used. Rats were maintained on a 12-h light/12-h dark cycle in a constant temperature room. Synaptosomes were isolated as described by Nagy and Delgado-Escueta (14). Ecto-Ap_nA hydrolase activity was measured by the disappearance of Ap_nA (Ap_3A, Ap_4A and Ap_5A) using the fast protein liquid chromatography (FPLC) system. Briefly, synaptosome fractions were incubated in 0.2 ml of a reaction mixture containing 20 mM Tris/HCl, pH 7.5, 2.0 mM $MgCl_2$, 10 mM glucose and 225 mM sucrose. After preincubation for 10 min at 37°C the reaction was started by the addition of 20 µl diadenosine polyphosphates to provide a final concentration of 100 µM in a final volume of 200 µl. The reaction was stopped by the addition of trichloroacetic acid to give a final concentration of 5%. Samples were chilled on ice and centrifuged at 14,000 *g* for 7 min. Samples of 50 µl were diluted ten times with deionized water and injected into a Mono Q HR 5/5 (1 ml) column from Pharmacia (Uppsala, Sweden). Elution buffers were filtered through HAW04700 Millipore membranes and degassed prior to use. Before use, the column was equilibrated at 1 ml/min with 50 mM H_3PO_4 buffer, pH 3.5, neutralized with NaOH. Nucleotides were detected by absorbance at 254 nm. For analytical purposes the Mono Q column was eluted with a linear gradient of 0.5 M H_3PO_4 (10-100%), pH 3.5, neutralized with NaOH, at a flow rate of 1.0 ml/min. The retention times of all nucleotides were determined before sample analysis.

Lactate dehydrogenase (LDH) was assayed according to Keiding et al. (15). Synaptosome integrity was estimated by measuring the activity of the cytosolic enzyme LDH after incubation of the synaptosome fraction at 37°C at two different incubation times (0 and 80 min), and comparing it to the total enzyme activity in synaptosomes lyzed with 0.1% Triton X-100. The results are reported as percentage of LDH activity in the intact synaptosome fraction compared to the LDH activity in the lyzed synaptosomes (100%).

Protein content was determined by the method of Bradford (16), using bovine serum albumin as standard.

Synaptosomes from cerebral cortex of adult rats were able to hydrolyze the dinucleotides Ap_4A and Ap_5A. The reaction was monitored by determining the decrease of substrate in the reaction mixture by FPLC. The hydrolysis of Ap_4A and Ap_5A increased as a function of protein concentration and incubation time. The disappearance of both substrates was linear in the range 200 to 500 µg protein/ml in the incubation medium (Figure 1A,B). In another series of experiments we evaluated the rate of breakdown of both dinucleotides as a function of the time of

incubation after the addition of a fixed protein amount (85 µg) (Figure 2A,B). The velocity was constant up to 80 min of reaction. It is noteworthy that the substrate Ap_3A was not degraded by the synaptosomal fraction under our assay conditions.

The integrity of the synaptosome fraction was determined by measuring intrasynaptosomal LDH activity (17). The LDH values indicated 10% synaptosome disruption in our preparation after 80 min of incubation. We conclude that the majority of the synaptosomes maintain their integrity during the assay and, therefore, that the degradation of Ap_4A and Ap_5A by synaptosomes from cerebral cortex of adult rats is probably due to an ecto-activity, which does not hydrolyze Ap_3A.

Enzymes which hydrolyze ATP and ADP (ecto-apyrase) and AMP (ecto-5'-nucleotidase) have been fully characterized in the peripheral and central nervous systems (11-13,18). However, the enzyme ecto-diadenosine polyphosphate hydrolase, which is able to degrade Ap_nA compounds, has been only recently studied (8-10). Since initial evidence for the presence of a diadenosine polyphosphate hydrolase is the disappearance of Ap_4A and Ap_5A as a function of protein concentration and incubation time, the results presented here in Figures 1 and 2 indicate the existence of this enzyme activity in the central nervous system for the first time.

Another characteristic of this enzyme which was studied in the present investigation was its ecto-localization. Our results, together with other studies (8-11), suggest that the hydrolysis of Ap_nA to adenosine may occur by the sequential action of ectoenzymes involved in the extracellular degradation of nucleotides.

It will be of interest to extend our study to better characterize this enzyme activity and its possible participation in an enzyme chain together with an ecto-apyrase and an ecto-5'-nucleotidase to degrade the dinucleotides Ap_5A and Ap_4A to adenosine in the synaptic cleft.

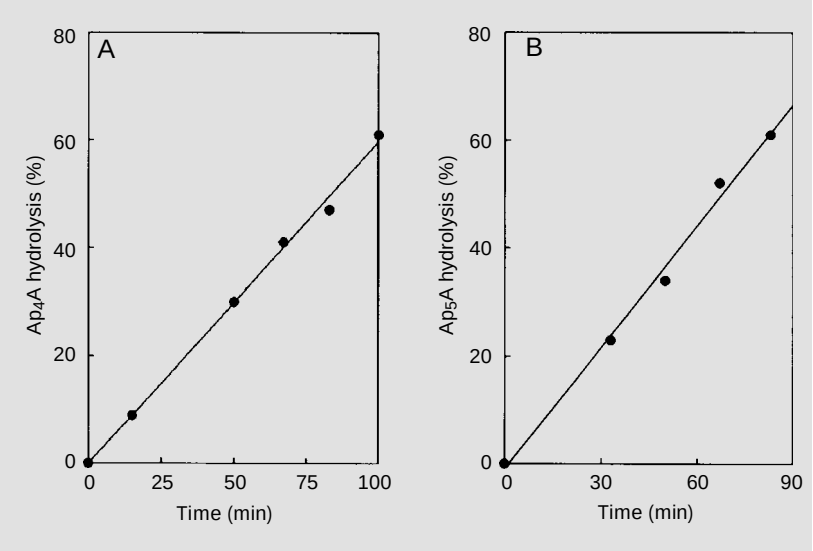

Figure 2 - Ap_4A (A) and Ap_5A (B) hydrolysis by synaptosomes as a function of time. The enzyme assay was carried out as described in the Legend to Figure 1, with 85 µg protein/200 µl at 37ºC. The data are representative of three independent experiments with different synaptosome preparations.

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