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Soluble and Membrane-Bound Leucyl- and Arginyl-Aminopeptidase Activities in Subcellular Fractions of Young and Adult Rat Brains

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The subcellular distribution of soluble and membrane-bound leucyl- and arginyl-aminopeptidase activities were analyzed in one and five month old rat brains using Leu- and Arg-2-naphthylamide as substrates. Both soluble leucyl- and arginyl-aminopeptidase activities showed the highest levels in the synaptosomal fraction in the two groups of rats. The highest levels of membrane-bound leucyl- and arginyl-aminopeptidase activities were found in the microsomal fraction in the two ages studied. There were no differences between the two ages in soluble leucyl- and arginyl-aminopeptidase activities. However, a significant decrease in both membrane-bound enzymatic activities was evidenced in the synaptosomal fraction of older rats. Developmental changes of these aminopeptidase activities in a determined subcellular localization, may reflect modifications in their effect on neuropeptides susceptible to be hydrolyzed in this particular location.

Key words: Leucyl aminopeptidase, Arginyl aminopeptidase, Subcellular distribution, Brain development.

Proteases and peptidases play a major role in the metabolism of biologically active neuropeptides (11). Leucyl aminopeptidase (LeuAP), through its exopeptidase activity, has been demonstrated to be active on dynorphins (2) and substance P (7); while arginyl aminopeptidase (ArgAP), by means of its endopeptidase activity, seems to act by hydrolysing neurotensin, bradykinin, angiotensin I, substance P, luliberin and somatostatin at internal bonds (14). However, the identification of a neuropeptide degrading activity is not a sufficient criterion by itself to conclude that a biological function of

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these enzymes exist. To better understand the mechanisms which regulate the intracellular and extracellular concentrations of these peptides, it is important to determine the subcellular localization of the neuropeptide degrading enzymes (1). On the other hand, according to the stages of brain development categorized by McIlwain (16), around the twenty-fifth day of age in the rat, the myelination is completed and the brain attains its full morphological and functional maturity. Since developmental changes in several proteolytic enzymes and neuropeptides have been evidenced (8, 9, 12, 13, 15, 17) prior 1 month of age, it is interesting to investigate putative age related changes in the subcellular localization of these enzymes at the time when maturation of the brain has been completed.

In the present study we have analyzed the subcellular distribution of soluble and membrane-bound LeuAP and ArgAP activities and also their eventual modifications between young (1 month old) and adult (5 month old) male rat brains, using Leu- and Arg-2-naphthylamide (LeuNNap and ArgNNap) as substrates.

Materials and Methods

The brains of young and adult male Sprague-Dawley rats were perfused with saline solution under equithensin anesthesia, quickly removed and homogenized in 10 volumes of 0.32 M sucrose (homogenate). Subcellular fractions were obtained according to the method of Krueger (10). Briefly, from the crude mitochondrial pellet (P_2) (12,500 × *g*), by subfractionation in density gradients, myelin, synaptosomal and mitochondrial fractions were obtained. Synaptosomal, mitochondrial, microsomal (P_3) (100,000 × *g*) fractions as well as samples from the homogenate and the crude nuclear pellet (P_1) (1000 × *g*), were homogenized in 10 mM Tris HCl buffer (pH 7.4 and ultracentrifuged (100,000 × *g*, 30 min, 4 °C); from the resultant supernatants and the one previously obtained at 100,000 × *g* (S_3) corresponding to cytosol, soluble enzymatic activity and proteins were assayed per triplicate. The pellets and myelin were homogenized in 10 mM Tris HCl (pH 7.4) plus 1 % of Triton-X-100 to obtain, after further ultracentrifugation (100,000 × *g*, 30 min, 4 °C), supernatants from which membrane-bound activity and proteins were determined also per triplicate.

LeuAP and ArgAP activities were measured fluorometrically using LeuNNap and ArgNNap as substrates, according to the modified method of Greenberg (6): 10 µl of each supernatant were incubated during 30 min at 25 °C with 1 ml of substrate solution (1 mg/100 ml of LeuNNap or ArgNNap, 10 mg/100 ml BSA and 10 mg/100 ml dithiothreitol in 10 mM phosphate buffer pH 7.4). After incubation, the enzymatic reaction was stopped by adding 1 ml 0.1 M acetate buffer (pH 4.2). The quantity of 2-naphthylamine released as a result of the enzymatic activity was determined fluorometrically at 412 nm of emision wavelength with an excitation wavelength of 345 nm. Proteins were quantified by the method of Bradford (3). Specific soluble and membrane-bound LeuAP and ArgAP activities were expressed as nmol of LeuNNap, and ArgNNap hydrolyzed per min per mg of protein. For the statistical analysis the Student *t* test was performed.

Results

The levels of soluble and membrane-bound LeuAP and ArgAP activities in the different subcellular fractions of young and adult rat brains are shown in figure 1. Soluble LeuAP and ArgAP activities exhibited the highest levels in the synaptosomal fraction of both young and adult rats, and the differences among fractions

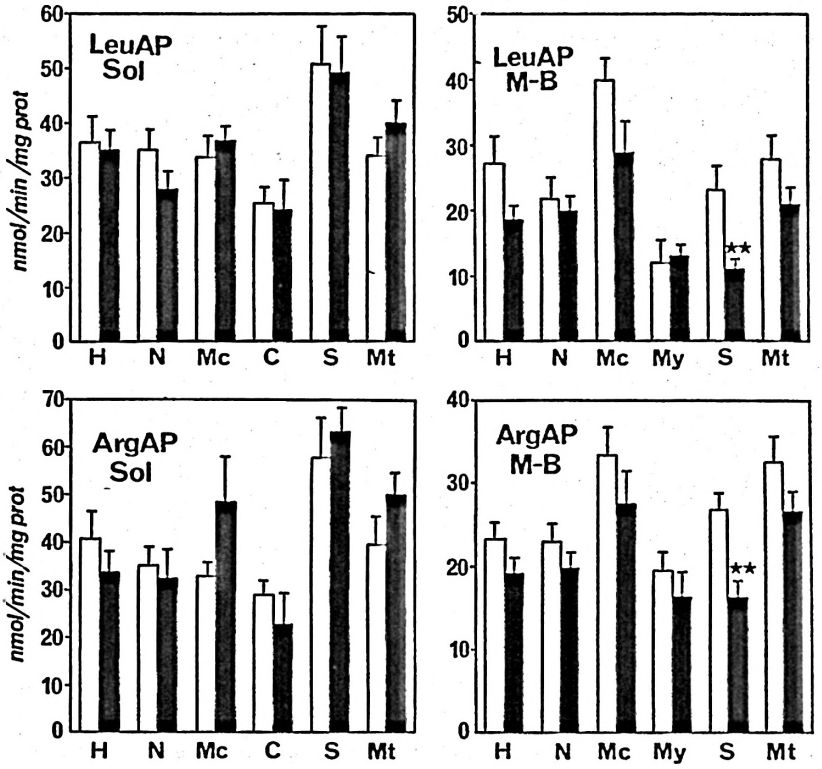

Fig. 1. *Specific soluble (Sol) and membrane-bound (M-B) LeuAP and ArgAP activities in homogenate (H), nuclear (N), microsomal (Mc), cytosol (C), myelin (My), synaptosomal (S) and mitochondrial (Mt) fractions from brain of one month old (n = 5-8, open bars), and five month old rats (n = 3-10, filled bars).*

Bars represent mean ± SEM levels expressed as nmol of LeuNNap or ArgNNap hydrolyzed per min per mg of protein. ** $p < 0.025$.

were more evident when ArgNNap was used as substrate.

No differences were disclosed between the two ages in soluble LeuAP or ArgAP activities.

Soluble LeuAP activity was significantly higher than a membrane-bound one in nuclear ($p < 0.05$) and synaptosomal ($p < 0.01$) fractions of young rats and in the homogenate, synaptosomal and mitochondrial fractions ($p < 0.01$), of adult ones.

In adult rats, soluble ArgAP activity was significantly higher than membrane-bound one ($p < 0.01$) in all fractions tested, except in the microsomal one. In young animals, however, this same result occurred only in the homogenate ($p < 0.01$), synaptosomal ($p < 0.01$) and nuclear ($p < 0.025$) fractions.

On the other hand, The highest levels of membrane-bound LeuAP activity were found at microsomal level in the two groups of age tested. Membrane-bound ArgAP also exhibited high activity in the microsomal fraction as well as at mitochondrial level.

Both membrane-bound LeuAP and ArgAP activities showed a tendency to diminish with age in the majority of frac-

tions assayed, and this decrease achieved statistical significance ($p < 0.025$) at synaptosomal level.

Discussion

The highest levels of soluble LeuAp and ArgAP activities exhibited in the synaptosomal fraction of both young and adult rats, support the hypothesis postulated by GREENFIELD and SHAW (7) that LeuAP is released from neurons to contribute to the degradation of Substance P.

On the other hand, the highest levels of membrane-bound LeuAP activity found at microsomal level in the two groups of age tested, may be strongly related to the biotransformation of precursor peptides.

It could be argued that the fall in the specific activity of membrane-bound enzymes detected in adult rats, when compared to that of young animals, may be due in part to the increase with age in the protein content. However, it is unlikely that this circumstance affects the present results since the most striking developmental changes in protein content take place before 30 days of age and no significant differences are found in older ages (12, 13). Moreover, a decrease in specific membrane-bound activity was observed, not evident in soluble one, which even showed an increasing trend in older animals.

Although it has been proposed that LeuAP activity, determined by the use of arylamide substrates, is involved in the degradation pathway of several neuropeptides, it should be considered that the use of these substrates to assay this enzyme in crude extracts, might also reflect the activity of other aminopeptidases which act preferentially on arylamide derivatives (15). ArgAP specifically hydrolyzes arginyl and lysyl NNap derivatives (15) and also shows a significant soluble endopeptidase activity in degrading physiologically active neuropeptides (14). On the other hand, the endopeptidase activity which hydrolyzes neurotensin at Arg^8-Arg^9 internal bond, is involved in the inactivation of the peptide in the rat brain synaptic membranes (4). The aminopeptidase activity of this enzyme appears to be restricted to dipeptides and tripeptides with basic aminoterminal residues.

Changes prior to 1 month of age in the enzymes cleaving LeuNNap and ArgNNap have been detected in the different subcellular fractions of rat brain (12), but no modifications between young and adult animals, with ArgNNap as substrate, have been reported (13). Other proteolytic enzymes have been demonstrated to change in the developing rat brain, as pyroglutamate aminopeptidase specific activity, which shows the highest levels in cytosol, diminishes from neonatal to adult ages (17), and prolidase activity also presents developmental changes in myelin and mitochondrial fractions as well (9).

In view of the putative role of LeuAP and ArgAP in the biotransformation and/or inactivation of neuropeptides, it is of particular interest the evidence of high soluble activity in the synaptosomal fraction at both ages, and the demonstration of age related changes in membrane-bound activities in this same fraction, which could reflect a functional modification in their action over susceptible substrates.

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Resumen

Se estudia la distribución subcelular de la actividad de leucina- y arginina-aminopeptidasa soluble y unida a membrana en cerebro de ratas jóvenes de un mes y adultas de cinco meses de edad, utilizando Leu- y Arg-2-naftilamida como sustratos. En ambas edades, los máximos niveles de actividad de leucina- y arginina-aminopeptidasa solubles se encuentran en la

fracción sinaptosomal y los de las unidas a membrana, en la fracción microsomal. No existen diferencias en la actividad de leucina y arginina aminopeptidasa solubles entre las ratas de uno y cinco meses. Sin embargo, la fracción sinaptosomal de las ratas adultas muestra una disminución significativa en ambas actividades enzimáticas unidas a membrana. Los cambios producidos en la actividad aminopeptidásica, en una determinada localización subcelular, podrían reflejar modificaciones en su efecto sobre los neuropéptidos susceptibles de ser hidrolizados, en esa particular localización.

Palabras clave: Leucina aminopeptidasa, Arginina aminopeptidasa, Distribución subcelular, Desarrollo cerebral.

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