Short Contribution

Soluble and membrane-bound pyroglutamyl-peptidase I activity in the developing cerebellum and brain cortex

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ABSTRACT Developmental changes of soluble and particulate pyroglutamyl (pGlu)-peptidase I activities in the rat brain cortex and in the cerebellum are described in this work. The enzyme activity has been measured spectrofluorimetrically using pGlu-b-naphthylamide as substrate (in the presence and absence of EDTA and DTT, necessary activators of the enzyme) in both soluble and particulate fractions. In the soluble fraction of the cerebellum and brain cortex, pGlu-peptidase I activity is high in the perinatal period and decreases two or three folds subsequently, at a later stage in the cerebellum than in the brain cortex, reaching adult levels at the end of the first postnatal month. The decrease in the activity of pGlu-peptidase observed in this work coincides with increasing levels of brain thyroliberin concentration after the second postnatal week. The particulate pGlu-peptidase I activity, obtained after osmotic shock and high-salt treatment, shows less significant changes during brain development in the areas under study. It is suggested that cytosolic pGlu-peptidase I could play a part in the normal development of the rat central nervous system.

KEY WORDS: *pyroglutamyl-peptidase, cerebellum, development, aminopeptidase*

Several peptides, all of whose N-terminal endings are a pyroglutamic acid, have been characterized. Among these peptides we can find thyroliberin, luliberin, neurotensin, etc. The cyclization of the N-terminal glutamic acid allows them to have a longer half-life than other peptides of similar size, since the majority of the aminopeptidases, the most active proteolytic enzymes of the brain, are not capable of hydrolyzing the pGlu-X-bond.

However, there are some peptidases capable of hydrolyzing cyclic amino acid-x bond. Among this proteolytic group, two aminopeptidases have been described: one of them membrane-bound with high specificity on thyrotropin releasing hormone, called pGlu-peptidase II (Friedman and Wilk, 1986; O¨Connor and O´Cuinn, 1985) and the second one, pGlu peptidase I, which can hydrolyze, at least *in vitro*, the majority of peptides with pGlu N-terminal, including TRH, LHRH, neurotensin, bombesin etc. (Browne and O´Cuinn, 1983, O´Cuinn *et al.*, 1990). The latter enzyme has been described mainly in the cytosol. However, a recent work has also found pGlu-peptidase I activity in the particulate fraction (Alba *et al.*, 1995). In adult rats, pGlu-peptidase II seems to be the most important enzyme in the degradation of TRH (Charli *et al.*, 1989) and some authors have reported that pGlu-peptidase I plays no role in TRH degradation in the adult animal (Mendez *et al.*, 1990).

The function of the neuropeptides and their degrading enzymes during the development and in the adult animal does not seem the same. Peptides can act as autocrine factors in brain development (Zagon *et al.*, 1989) and intracellular peptidases (Gandarias *et al.*, 1997), including pGlu-peptidase I (Fuse *et al.*, 1990; Gandarias *et al.*, 1994b), undergo drastic changes during brain ontogeny. Other authors have also suggested that some cytosolic peptidases could be essential for cell growth and viability (Constam *et al.*, 1995).

TRH containing neurons may be involved in the development of rat cerebellum (Suzuki *et al.*, 1988) and TRH enhances the trophic action of endogenous glutamate in the cerebellum by amplifying several intracellular events (Casabona *et al.*, 1992)

Information concerning developmental alterations in the pGlu-peptidases in several brain areas (Aratan-Spire *et al.*, 1983, Fuse *et al.*, 1990; Vargas *et al.*, 1992; Gandarias *et al.*, 1994a,b) has been presented by several groups. However, little is known about the role of these enzymes in the cerebellum. Moreover, since particulate pGlu-peptidase I activity has only been described quite recently (Alba *et al.*, 1995), the information about the ontogeny of this form of the enzyme is lacking.

In the present work, we address possible changes in the ontogeny of both pGlu-peptidase I activities, soluble and particulate, in the cerebellum. We also report the ontogeny of these enzyme

Abbreviations used in this paper: DTT, dithiothreitol; E22, embryonic day 22; EDTA, ethylendiaminietetraacetic acid; LDH, lactate dehydrogenase; LHRH, luteinizing hormone releasing hormone; P0, P2, etc., postnatal days 0, 2, etc; pGlu-, pyroglutamyl-; TRH, thyrotropin releasing hormone.

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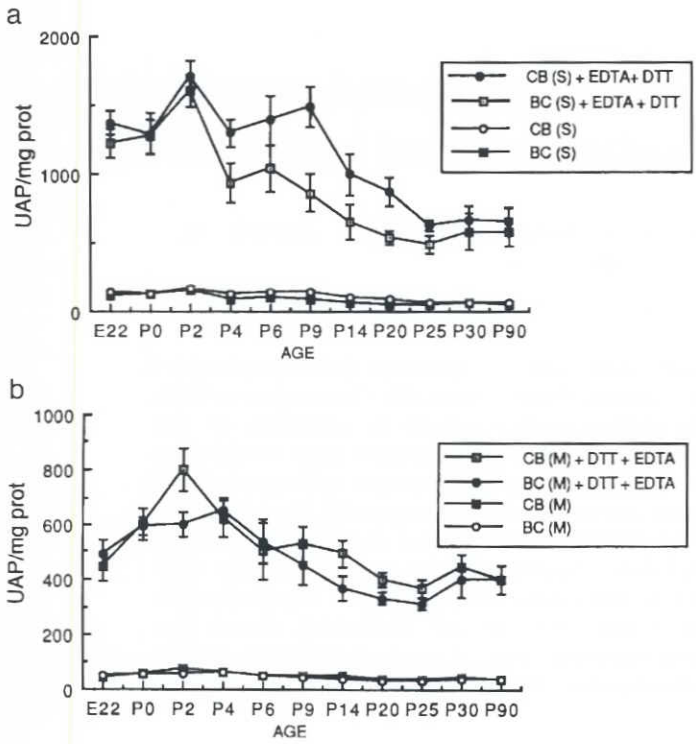

Fig. 1. Soluble (1a) and membrane-bound (1b) pGlu-peptidase I activity levels in the cerebellum (CB) and brain cortex (BC) of rats during different stages of development. *Values, recorded as units of peptidase activity per mg of protein, represent mean ± SEM.*

activities in the brain cortex, in order to compare the results obtained in the cerebellum with a brain area where developmental changes of soluble pGlu-peptidase I are quite well established.

Soluble and particulate pGlu-peptidase I specific activity levels in developing brain cortex and cerebellum are expressed in Figure 1. In both areas, soluble activity (1a) is high in the perinatal period and decreases subsequently 2-3 folds, reaching adult levels at P25-P30 (ANOVA test $p<0.001$). The decrease in the activity occurs later in the cerebellum than in the brain cortex (anova test $p<0.05$). Developmental changes observed in the particulate fraction (1b) are not so significant as in the soluble form (ANOVA test: cortex $p=0.04$; cerebellum $p=0.03$). However, it can be noted that we have found a peak of specific activity at P2-P4 depending on the brain area. After this, the activity decreases until P20, when adult levels are obtained. The values measured without using EDTA and DTT are also shown in Figure 1 (both a and b). In this case, the enzyme activities are more than 10-fold less, both in membrane and soluble forms.

Table 1 shows cortical and cerebellar lactate dehydrogenase (LDH) and pGlu-peptidase I activities in the pellets and supernatants after osmotic shock, salt treatments and centrifugations. We have found that, following osmotic shock and ultracentrifugation of cortical and cerebellar homogenates, while only 10% of all LDH activity is associated with the particulate fraction, 20-40% (depending on the age) of pGlu-peptidase I activity is particulate. Approximately 70% of the activity in the particulate fraction remains after salt treatment and posterior ultracentrifugation. However, LDH has no significant activity (2-3%) in these pellets.

Figure 2 shows soluble and membrane-bound pGlu-peptidase I activity levels per g of fresh tissue. The development pattern of soluble enzyme is very similar to that described for specific activities. In the membrane-bound-form, there are no significant changes of the activity/g tissue.

Results obtained in this research show that the brain cortex and the cerebellum have soluble and particulate pGlu-peptidase I activities during all the developmental stages assayed.

While pGlu-peptidase I activity in the cytosol has been well documented, there have been few references to the presence of particulate pGlu-peptidase I activity. However, in this work we have measured significant activity levels of the mentioned enzyme in the particulate fraction of the rat brain. We can discard the idea that the resultant activity is due to the action of pGlu-peptidase II because in the absence of EDTA and dithiothreitol (activators of pGlu-peptidase I) there is no significant quantity of pGlu-β-naphthylamide hydrolysis.

In view of the fact that a particulate pGlu-peptidase I activity had not been previously purified, it was important to ensure that the

TABLE 1

CEREBRAL CORTEX	HOMOGENATE	SOLUBLE OSMOTIC SHOCK	PARTICULATE OSMOTIC SHOCK	PARTICULATE SALT WASHING1 1	PARTICULATE SALT WASHING2
LDH	100	83	10	6	3
pGlu*	100	62-80	16-31	15-25	13-22
total protein	100	44	50	27	14
CEREBELLUM	**HOMOGENATE**	**SOLUBLE OSMOTIC SHOCK**	**PARTICULATE OSMOTIC SHOCK**	**PARTICULATE SALT WASHING1 1**	**PARTICULATE SALT WASHING2**
LDH	100	80	8	4.5	2
pGlu*	100	62-75	17-31	14-23	13-22
total protein	100	49	45	22	13

Total protein and LDH and pGlu-peptidase I activity levels in the osmotic-shocked homogenate, in the high speed supernatant and pellet and in the consecutive pellets obtained after treating the first pellet with high salt and high speed ultracentrifugation. Results are expressed as percentage of the amount of activity or protein detected in the homogenate.

*Depending on age analyzed.

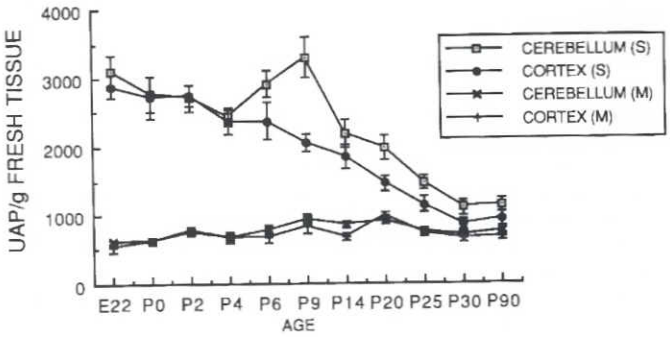

Fig. 2. Soluble (S) and membrane-bound (M) pGlu-peptidase I activity levels in the cerebellum (CB) and brain cortex (BC) of rats during different stages of development. *Values, recorded as units of peptidase activity per g of fresh tissue, represent mean ± SEM.*

activity under investigation was indeed associated with the membranes, and was not a loosely bound, non specifically associated soluble activity. The criteria set out by many researchers for a membrane enzyme were examined with reference to the pGlu-peptidase I in the rat brain. We found that, following salt-treatment and osmotic shock, approximately 70% of the original crude particulate pGlu-peptidase I remained associated with the particulate fraction (Table 1). This result suggests that the enzyme is not just the soluble pGlu-peptidase loosely associated with the membrane. Moreover the loss of lactate dehydrogenase activity in the particulate fraction after consecutive high-salt treatments agree with this hypothesis.

The present study also documents the developmental changes of pGlu-peptidase I activity in the brain cortex, and the cerebellum. In general, our results are consistent with previous studies (Fuse *et al.*, 1990; Gandarias *et al.*, 1994b) describing high brain levels of soluble pGlu-peptidase I activity in the perinatal period which decrease two or three fold to adult levels over the following two weeks. The present results expand previous brain findings to include differences in the developing cerebellum. In this area, the specific activity is also high in the perinatal days and low in the adult animal. However, the decrease in the activity occurs in later ages than in other brain areas, since soluble activity is relatively high until P9. This result agrees with the fact that cerebellum maturation is a relatively late event.

Intracellular peptide regulatory mechanisms are still poorly understood and several studies have demonstrated that in the adult animal the levels of several pGlu-containing peptides are not controlled by cytosolic peptidases (Mendez *et al.*, 1990). However, it has been proposed that neuropeptides (Cimino *et al.*, 1991) and their peptidases (Dauch *et al.*, 1993) during the earlier developmental stages may not be related to neurotransmission, but rather to phasic phenomena related to the development of the brain, probably by an autocrine mechanism (Zagon *et al.*, 1989). Moreover, intracellular peptidases can participate in the growth and viability of the central nervous system (Constam *et al.*, 1995).

Taking into account both these previous reports and our own findings we cannot discard the hypothesis that soluble pGlu-peptidase I has a role in brain development. These results support the idea that neuropeptidases, besides their well documented role in the peptide-degradation, could have a putative role in maturation or differentiation processes (Dauch *et al.*, 1993). The high levels of pGlu-peptidase I found in most developing tissues (Fuse *et al.*, 1990, Gandarias *et al.*, 1994b) also agree with this idea.

Since this enzyme is of broad specificity, the possible peptide/s susceptible to be degraded during brain development is/are unknown. However, it can be noted that decreasing levels of soluble pGlu-peptidase I activity in the cerebellum and brain cortex from the first postnatal week are coincident with the development of TRHergic system (Lamberton *et al.*, 1984; Gayo *et al.*, 1986, Bayliss *et al.*, 1994) during the same period. Therefore a possible role of this enzyme in the control of TRH action during brain development cannot be ruled out.

With respect to particulate activity, we have found more moderate changes of specific activity during the development. Since the changes practically disappear, if we analyzed the activity per g of fresh tissue, these may be due to changes in protein content. In general the activity levels, quite constant during the postnatal life, are quite similar to those found in the soluble form in adulthood.

The different developmental patterns of soluble and particulate pGlu-peptidase I activity seem to indicate that, although a previous work has reported that both forms of pGlu-peptidase I are the same enzyme (Alba *et al.*, 1995), their developmental regulation is not the same. Therefore there must be developmentally regulated posttranslational processes which can establish at each age the different activity levels of pGlu-peptidase I in the membrane or in the cytosol.

In summary, this study demonstrated developmental changes of soluble pGlu-peptidase I activity in the brain cortex and cerebellum. The results seem to agree with the idea that cytosolic peptidases could have a role in the development of different regions of the brain.

Experimental Procedures

Animals

Male Sprague-Dawley rats (n=10 per age), bred in our colony and maintained under conditions of controlled light (12 h) and temperature (24°C), with food and water *ad libitum* were used in this investigation. The ages of the animals were embryonic day 22 (E22), and postnatal days 0 (P0), 2 (P2), 4 (P4), 6 (P6), 9 (P9), 14 (P14), 20 (P20), 25 (P25), 30 (P30) and 90 (P90).

Sample preparation

In order to avoid proteolytic contamination from blood, animals from five groups were perfused with saline plus 50 mM phosphate buffer, pH 7.4, through the left cardiac ventricle under Equithensin anesthesia. The brains were quickly removed and cooled in dry ice and cerebella and brain cortices were taken by dissection. The samples were homogenized (in Tris HCl 10 mmol/l, pH 7.4) and ultracentrifuged (100,000 g, 35 min) to obtain the soluble fraction. The homogenization in a hipoosmotic solution allows us to disrupt all cellular and subcellular membranes. The supernatant solution was used for the analysis of soluble enzyme activity and protein quantification. The resultant pellets, washed with Na Cl and ultracentrifuged two twice to remove any loosely bound proteins, were homogenized in Tris-HCl 10 mM (pH 7.4), plus 1% of Triton X-100, to obtain, after centrifugation (100,000 g, 30 min, 4°C), supernatants. These were employed to detect particulate activity and proteins also in triplicate. All preparatory steps were carried out at 4°C.

Determination of pGlu-peptidase activity

pGlu-peptidase I activity was fluorometrically measured in triplicate using pGlu-β-naphtylamide as substrate, by the method of Greenberg

(1962), but with the recent modifications described by Alba *et al.* (1989): 10 µl (1 mg/ml) aliquots of soluble fractions were incubated with 1 ml of pGlu-β-naphtylamide (2 mg/100 ml), serum albumin (10 mg/100 ml) in 50 mmol/l phosphate buffer, pH 7.4. The enzyme activities were measured both in the absence and presence of DTT (5 mM) and EDTA (2 mM), necessary activators of pGlu-peptidase I and inhibitors of pGlu-peptidase II. The reaction was stopped by the addition of 1 ml 0.1 mol/l acetate buffer solution, pH 4.2. The β-naphthylamide released was determined by measuring the fluorescent intensity at 412 nm with excitation at 345 nm. Relative fluorescence was converted to picomoles of β-naphthylamine by comparison with a standard curve.

Assay for lactate dehydrogenase activity

Lactate dehydrogenase activity was determined spectrophotometrically by following NADH2 oxidation after pyruvate addition as modified by Bergmeyer and Brent (1972).

Data analysis

Results (mean ± standard error of the mean) were recorded as units of pGlu-peptidase I activity per mg of protein, measured by the method described by Bradford (1976), or per g of fresh tissue. One unit of peptidase activity is the amount of enzyme that hydrolyzes 1 picomol of pGlu-β-naphtylamide per minute. Statistical analysis was carried out by two way (brain area and developmental stage) ANOVA test.

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