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Amidating processing enzyme complex for bioactive peptides (PAM) shows differences in specific activity and form in secretory granules isolated from the proximal and distal parts of the hypothalamo-neurohypophyseal tract in rats

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In rats the PAM specific activity in hypothalamic and neurohypophyseal extracts was 0.58 ± 0.8, respectively 1.78 ± 0.6 nmol · mg prot.$^{-1}$ · h^{-1} (n = 5). PHM specific activity in the soluble part of the granules was higher in the neurohypophyseal than in the hypothalamic granules, and the fraction of total PHM and PAL present in the soluble part increased with the distance from the hypothalamus from some 45% to approx. 85%. Western blots of membrane and soluble granule fractions showed prevalence of higher mol. wt. forms in hypothalamic granules. It would appear that higher mol. wt. forms of PAM are processed by proteolytic enzymes during transport in the neuron and that non-neural cells in the neurohypophysis have a considerable PAM activity.

Neuroendocrine protein, Amidating enzyme; Posttranslational modification, Secretory granule

1. INTRODUCTION

Bioactive neuropeptides are synthesized as higher molecular weight precursor proteins which undergo several posttranslational modifications, often including endoproteolysis, limited exopeptidase digestion and C terminal amidation. Processing of the prohormones in the hypothalamo-neurohypophyseal tract goes on after packaging or during transport in the neurons [1,2]. It probably involves prohormone convertases (e.g. PC1, PC2) in the secretory granules [3,4]. PC2 has been demonstrated to be present in the neurointermediate pituitary [5]. Also a carboxypeptidase H has been shown to be present in granules. Necessary cofactors for processing enzymes such as calcium, cobalt and copper are present in high concentrations in neurosecretory granules [6]. The amidation of the neurohypophyseal hormones vasopressin and oxytocin from their glycine-extended precursors is catalyzed by the sequential action of PHM and PAL. The two enzymes are *part of a bifunctional, integral membrane protein precursor*, PAM. It has been suggested that amidation is rate limiting in the series of processing steps and that the amount and activity of PAM may be regulated in different ways [7–9].

Generally, it appears that at least 7 different proteins are produced from spliced PAM mRNA and that tissue specific processing generates further diversity, comprising membrane and soluble forms of different sizes [10,11]. There are considerable differences in the occurrence of these forms between e.g. the neurointermediate and the anterior pituitary lobe [11]. High activities of PAM are found in secretory granules prepared from the neurointermediate lobe of the hypophysis from rats [12] and ox [13] and from the hypothalamus of rats [14]. In the neurointermediate lobe PAM activity is mainly soluble and present in forms between 75 and 43 kDa [11]. It has been a problem for the interpretation in such studies that neurointermediate pituitaries have not been separated in their 2 different tissue components. In the neurointermediate pituitary of the rat, approximately equal levels of PAM activity are found in the intermediate and neural parts [15,16]. There are no reports on characteristics of PAM in the hypothalamus or the pituitary stalk but Takahashi et al. [17] have purified soluble forms of PHM (36 kDa) and PAL (41 kDa) in a crude granule fraction prepared from rat brain.

There have been no previous direct reports on possible posttranslational processing of pro-PAM or PAM

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Abbreviations PAM, peptidylglycine α-amidating monooxygenase; PHM, peptidylglycine α-hydroxylating monooxygenase; PAL, peptidyl-α-hydroxyglycine α-amidating lyase; PMSF, phenylmethylsulfonyl fluoride; AVP, arginine vasopressin; SON, supraopticus nucleus; PVN, paraventricular nucleus

(PHM, PAL) during transport of the neurosecretory granules. To study PAM in the terminals of the neurosecretory neurons proper, separation of the PAM- and granule-containing pars intermedia from the neurohypophysis must be done. Also, it is necessary to take into consideration the fact that PAM immunoreactivity has been shown to be present in glial cells [18]. About 30% of the neurohypophysis consists of such cells (pituicytes) which, however, do not contain secretory granules or signs of secretory activity [19].

2. MATERIALS AND METHODS

Male PanWistar rats, weighing approx. 250 g, were given food and water ad lib

Due to the very small amount of protein in secretory granules prepared from 1 neurohypophysis, preparations had to be pooled from batches, for each of which 6 or 12 rats were generally used

2.1 *Dissection of tissues and preparation of extracts*

After decapitation, the pituitary and the brain were promptly removed Median eminence and pituitary stalk tissue (called stalk tissue) were isolated by cutting with 0 5 mm radius and 0 5 mm depth from the cut end of the pituitary stalk In some experiments, hypothalamic tissue was obtained as previously described by Roberts et al. [20], with a slight modification. In some experiments zones comprizing only the SON or PVN region were isolated. Neurohypophyseal tissue was carefully separated mechanically from pars intermedia by needles under a microscope.

Extracts from hypothalamus, stalk and neurohypophyseal tissue were prepared as by May et al. [12] with the following modifications tissues were homogenized in 20 mM TES, pH 7.4, containing 10 mM mannitol, 0.1% Triton X-100 and 30 μg/ml phenylmethylsulfonylfluoride. The homogenates were frozen, thawed and sonicated three times, then centrifuged for 1 h at 100,000 × g Supernatants were stored at −80°C until assayed and analysed by Western blotting. Individual hypothalami were homogenized in 1.0 ml medium, PVN, SON, stalk tissues and neurohypophyses in 0.3 ml respectively.

2 2. *Preparation of secretory granules*

Preparation of *hypothalamic secretory granules* was made employing a modification of the procedure of Emeson [14] using PMSF 0 03 mg/ml throughout 2 2 ml of the middle fraction was collected and further centrifuged at 100,000 × g for 1 h in a fixed angle rotor (Beckman, type Ti-50). The pellet, representing the granule fraction was resuspended in 250 μl of the homogenization buffer and recentrifuged at 100,000 × g for 1 h after freezing and thawing three times The supernatant represented the soluble fraction of hypothalamic granules. The pellet was washed with 0.1 M sodium carbonate once [21], then resuspended in 250 μl of homogenization buffer containing 0 1% Triton X-100 and sonicated for 5 s three times The supernatant obtained by centrifugation was the membrane associated fraction of hypothalamic granules. The preparations from approx. 30 rats were stored at −80°C and pooled for analyses. It was checked that the bands in the 0.8, 0.9 and 1.0 M sucrose region of the gradient showed comparable PHM activity

Secretory granules *from rat neurohypophyses* after removal of the intermediate lobe were isolated on 400 μl Percoll gradients centrifuged at 4°C in a standard bench-top microcentrifuge (O. Dich, Copenhagen, Denmark) by the method of Gratzl et al. [22], adapted to small scale and using PMSF as described above. The preparations from batches of up to a total of 100 rats were stored at −80°C and later pooled Before analysis, the pooled fractions were freeze-dried In some experiments, secretory granules were isolated by the sucrose method of Rouillé et al [4].

2.3. *Radioimmunoassay of arginine vasopressin (AVP)*

This was performed using a rabbit anti-Arg^8-vasopressin serum (RAS 8103) from Peninsula.

Table I

Distribution of PHM and PAL activity in normal rat neurohypophyseal (NG), stalk (SG) and hypothalamic (SONG and PVNG) secretory granules[a]

	Soluble fraction		Membrane fraction		Act. in soluble fraction/total activity[b] (%)
	Protein content in fraction (μg)	Spec. act. (nmol/mg prot h)	Protein content in fraction (μg)	Spec. act (nmol/mg prot. · h)	
NG:	71.6 ± 1 0		22.7 ± 0 2		
PHM		14.2 ± 0 1		3.4 ± 0.1	92.9
PAL		22 4 ± 1 2		20 8 ± 0 1	77.3
SG:	95.0 ± 2.9		77.1 ± 10.8		
PHM		7 2 ± 1 7		2.7 ± 0 3	76.7
PAL		23 9 ± 0.2		20 2 ± 0.9	59 3
SONG.	161.9 ± 15.0		200 7 ± 26 0		
PHM		3.3 ± 0.4		2 8 ± 0 8	48.7
PAL		24.3 ± 0.2		19.6 ± 1.0	50 0
PVNG:	124.7 ± 15.5		169.3 ± 23.4		
PHM		2.1 ± 0.1		2.0 ± 0.3	43.6
PAL		20 9 ± 0.9		16 4 ± 0.5	48.4

[a] The data represent the mean of three separate experiments ± S.E.M.

[b] = $\frac{\text{protein in S fraction } (\mu g) \times \text{spec act (nmol/mg protein)}}{\text{protein in S fraction} \times \text{spec act} + \text{protein in M fraction} \times \text{spec. act.}} \times 100\%$.

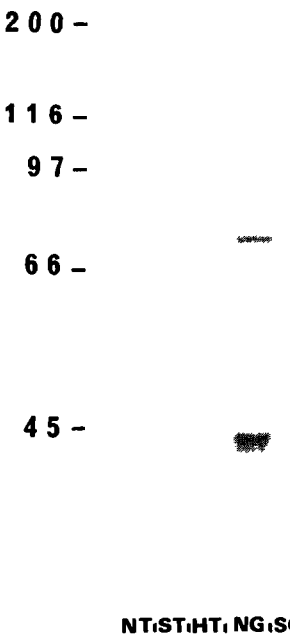

Fig. 1. Identification by Western blot analysis of forms of PAM protein in tissue extracts (NT, ST and HT) and granules (NG, SG and HG) from neurohypophysis (N), stalk (S) and hypothalamus (H). The samples were run on an 8% SDS-polyacrylamide gel with 0.25% *N,N'*-methylene-bis-acrylamide and 40 μg of protein was loaded on each lane, followed by transfer to nitrocellulose membranes. The immunostaining was performed by using a primary antibody to 75 kDa form of rat PAM (mouse monoclonal IgG) and rat adsorbed goat anti-mouse IgG (H + L) with alkaline phosphatase conjugate. The standard markers are indicated on the left.

2.4. *Activity assay for PAM, PHM and PAL*

These analyses were carried out using the methods of Eipper et al. [11,23].

Determination of protein concentration was done as in [24].

2.5 *Western blot analysis*

Samples of membrane, soluble fraction and tissue extract were run on 10% polyacrylamide-SDS gel with 0.25% *N,N'*-methylene-bis-acrylamide as described by Laemmli [25]. 15–50 μg of protein was loaded on each lane followed by electrophoretical transfer to nitrocellulose (BioRad) using buffer containing 12.5 mM Tris-base, 96 mM glycine (Merck), pH 8.4, and 20% methanol (Merck). Mol. wt was estimated by comparison with protein standards (BioRad). Immunostaining was performed by using a primary antibody to rat PAM (mouse monoclonal IgG_1; Unigene, 110 Little Falls Rd, Fairfield, NJ 07006, USA) raised against the 75 kDa form of rat PAM recognizing an epitope on the PHM part and detecting both PHM and PAL activities in high and low molecular forms of PAM [26]. Rat adsorbed goat anti-mouse IgG (H + L) and alkaline phosphatase (Caltag Lab., cat. no. M30208) were used.

It was checked that neurohypophyseal granules isolated by the modified Percoll method showed the same specific activity for AVP and bands on Western blotting as the granules isolated by the sucrose method of Rouillé et al [4].

3. RESULTS

3.1. *Specific activity of PHM, PAL and PAM in secretory granules and tissue from the supraoptic nucleus region, the paraventricular nucleus region, the stalk and the neurohypophysis*

As seen in Table I, the specific activity of PHM in the soluble fraction in neurohypophyseal granules was higher than in granules from the other regions ($P < 0.05$ for all). The specific activity of PHM in the membrane fraction did not differ in this respect. For PAL, the specific activity in the soluble and membrane fraction was comparable for granules from all the different regions, except for the membrane fraction from the PVN region, where it was lower ($P < 0.05$). For both PHM and PAL, the fraction of the 'total activity' which was present in the soluble part increased along the neuron, from approx. 45% to 85%.

In 5 experiments, the PAM spec. activity (nmol · mg prot.$^{-1}$ · h^{-1}) in tissue extract from the neurohypophysis was 1.78 ± 0.6, whereas it was 0.58 ± 0.8 in tissue extract from the hypothalamus. This difference was statistically significant ($P < 0.01$). When the sucrose gradient used for isolation of secretory granules from the neurohypophysis was divided into a lower and an upper half, more than 13% of the total specific activity was localized to the upper half, whereas all the rest of the activity was localized to the pellet.

3.2. *Prevalence of different PAM bands in Western blots of granules, their fractions and tissue extracts*

Figs. 1 and 2 show PAM reactive proteins in tissue extracts and granules from the hypothalamus, the stalk and the neurohypophysis.

In *hypothalamic granules* several bands above 75 kDa could be demonstrated. Marked ones at 135, 120, 105 and 84 were seen. Two bands at 64 and 58 were prominent both in the membrane and soluble fractions. In contrast to neurohypophyseal granules, no bands at 75 or 44 kDa were seen. In the membrane fraction 2 marked bands were seen at 40 and 38 kDa. In the soluble fraction there was a marked band at 40 kDa. In *hypothalamic tissue extracts* bands were very similar to the granular bands.

Granules isolated from stalk tissue showed bands resembling those in hypothalamic granules. The stalk tissue extract showed bands very similar to hypothalamic tissue extract. Two marked bands at 43 and 38 kDa could also be seen. In *neurohypophyseal granules*, bands at 84, 75, 46 and 44 kDa were prevalent. Thinner bands at 58 and 38 were also present in the soluble part. In *neurohypophyseal tissue extracts*, bands very similar to those seen in neurohypophyseal granules were seen.

In *pars intermedia granules* (Fig. 3) the strong bands at 75 and 44 kDa, seen in neurohypophyseal granules were also strong. *Pars intermedia tissue extract* showed predominance of bands at 75 and below 31 kDa.

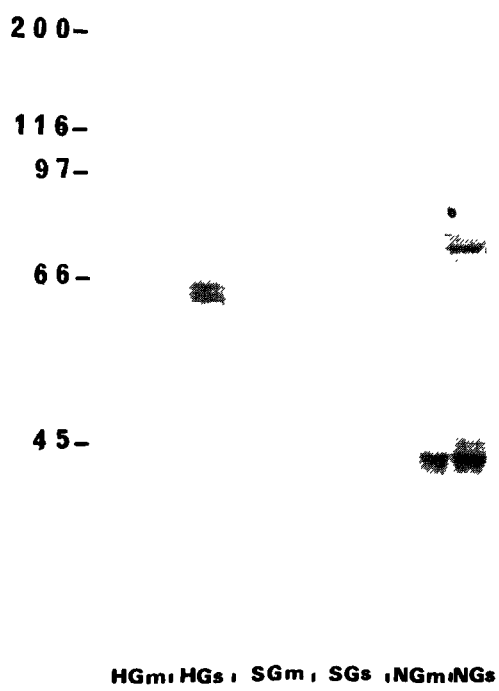

Fig. 2. Distribution of PAM proteins in membrane-associated fraction (Gm) and soluble fraction (Gs) of secretory granules from hypothalamus (H), stalk (S) and neurohypophysis (N) by Western blot analysis. The membrane-associated and soluble fractions of hypothalamic, stalk and neurohypophyseal secretory granules were obtained from 40 adult rats by the methods described in section 2. The samples were run on a 8% SDS polyacrylamide gel with 0.25% *N,N'*-methylene-bisacrylamide and 40 μg of sample protein loaded on each lane was run for electrophoresis. The molecular weight markers are indicated on the left.

4. DISCUSSION

To our knowledge the present experiments are the first ones in which granular contents in the proximal and distal part of the hypothalamo-hypophyseal nerve tract have been compared directly under normal (and stimulated) conditions.

4.1. *Quality of the granule preparation*

The removal of pars intermedia before preparation of neurohypophyseal granules is important since PAM immunoreactivity is abundant in the rat intermediate lobe [15]. This tissue contains granules of a size not very different from those in the neural lobe.

Immunoreactivity of PAM in the hypothalamus is concentrated in the area which was taken out for preparation by us [27]. The fact that granules from different parts of the granule band showed very similar PAM activity indicates that the band is homogenous and it would thus appear that such granules to a large extent represent granules that end up in the neural lobe.

4.2. *Comments on the PHM specific activity in hypothalamic tissue and hypothalamic granules versus neural lobe tissue and neural lobe granules*

The finding of higher PHM specific activity in neural lobe tissue than in hypothalamic is different from the findings in sheep [28] but in agreement with previous findings in rats [29,30], supporting the hypothesis that different processing may occur in different species. The high specific activity found in *secretory granules* in the neurohypophysis would be in agreement with a hypothesis that a considerable part of the final processing goes on in the distal part of the neuron.

The finding of a much higher specific activity of PAM in neural lobe tissue than in hypothalamic tissue is most likely explained by the presence of a highly active PAM in pituicytes (modified glial cells) in the neural lobe. This would be in accordance with the observation of Rhodes et al. [18] that PAM is found in normal and neoplastic glial cells and also with our recent findings (Hansen, Larsson and Thorn, unpublished) of the presence of large amounts of mRNA for PAM in the neurohypophysis as well as with the finding of May et al. [12] on neurointermediate lobe subcellular fractions. In a Percoll gradient they found up to 18% of the PAM activity present in a fraction reacting with Golgi markers (see also [28]). In the sucrose gradient used in some experiments by us we found more than 13% of the PAM activity in the part of the gradient containing RER.

4.3. *Comments on membrane to soluble forms in granules*

The finding that in neurohypophyseal granules only approx. 15% of the activity was membrane associated is in agreement with the findings by May et al. [12] on neurointermediate granules.

4.4. *Comments on the prevalence of different PAM reactive bands on Western blotting in granules and tissue from the different parts of the hypothalamus–neurohypophyseal tract*

From the Western blots it appears that a high prevalence of the 75 and 44 kDa bands is characteristic for granules from the neurohypophysis and pars intermedia as previously found for granules from unseparated neurointermediate lobes [11]. It is interesting that the patterns found in secretory granules from the hypothalamus in the present study showed similarities to the patterns found by Eipper et al. [11] in granules from the anterior pituitary by the common presence of several marked bands above 84 kDa. Also, Eipper et al. in the anterior pituitary found 2 marked bands at 45 and 42 kDa whereas we found in the hypothalamus granules 2 bands at 40 and 38 kDa. The function of PAM in the anterior pituitary is obscure since the major anterior pituitary hormones are not amidated. The 40 and 38 kDa bands found in hypothalamic granules in our experiments may be related to the 41 and 36 kDa bands found in a crude granule fraction from rat brain [17].

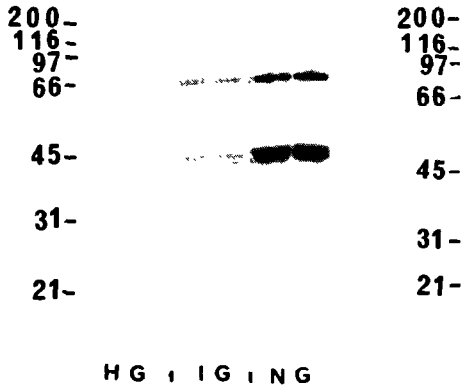

Fig. 3. Comparison of PAM proteins in hypothalamic (HG), pars intermedia (IG), neurohypophyseal (NG) secretory granules and pars intermedia tissue extract (IT) by Western blotting. The samples were run on a 10% SDS-polyacrylamide gel with 0.25% of *N,N'*-methylene-bis-acrylamide. 40 μg of proteins were loaded on a lane The molecular weight markers are indicated on the left

It would appear that in the anterior pituitary a processing of PAM similar to the one in the neurohypophyseal system might not occur.

The Western blot pattern in stalk extract is in agreement with the hypothesis that this tissue is highly concentrated in granules with a composition mainly as hypothalamic granules.

The fact that the extracts of pars intermedia tissue showed bands at 84 and below 32 kDa that were not seen in granule preparations also stress the importance of using secretory granules for analyses of characteristics of PAM in the cells. It is interesting that the PAM patterns of secretory granules from pars intermedia and the neurohypophysis showed considerable similarities. However, a band at 60 kDa in granules from pars intermedia was not present in granules from the neurohypophysis.

Many secretory proteins are reported to be degraded at some stage during their transit through the cellular secretory apparatus or during their stay in granules [3]. In a rat thyroid carcinoma cell line Beaudry and Berthelsen [26] found membrane forms of PAM which are processed to smaller new forms by splitting at the dibasic residues present. A 104 kDa protein transformed to 43 or 41 kDa. A 94 kDa form to a 75 kDa form. In AtT-20 cells endoproteolytic processing of membrane-associated PAM-precursors to generate soluble forms of PAM has been demonstrated by Milgram et al. [31].

The hypothalamo-neurohypophyseal system is unique by the movement of secretory granules by axonal flow over a long distance. The finding in our experiments of several PAM reactive bands with molecular weight higher than 75 kDa in hypothalamic granules but not in neurohypophyseal granules is in accordance with a hypothesis about high prevalence in hypothalamic granules of PHM/PAL with transmembranal and possibly cytoplasmic regions attached and processing of them to forms with lower molecular weight especially during transport through the distal part of the axons.

It is in accordance with such a hypothesis that in a separate set of experiments Western blots of the contents of isolated hypothalamic secretory granules which had been incubated at 37°C for 4 h showed a higher prevalence of lower mol. wt. bands than controls (unpublished) and that PAM immunoreactive proteins, resembling those present in the soluble fraction of neurohypophyseal granules were released to the medium together with PAM enzyme activity and AVP when isolated hemilobes from the neurohypophysis were stimulated by high potassium (unpublished).

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