Quantitative detection and biochemical characterization of high-M_r forms of vasopressin in the rat hypothalamo–post-hypophysial system

Effect of water deprivation and rehydration

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1. INTRODUCTION

The peptide hormones oxytocin (OT) and vasopressin (AVP) and their carrier proteins the neurophysins (Np), are cysteine-rich components of peptidergic neurosecretory neurons in the hypothalamo-hypophysial system (HHS) [1]. The hormones and their associated neurophysins are synthesized as common precursor in the perikarya of the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus [2,3]. In [4], the sequence of a cDNA encoding the nonapeptide AVP and N_p II was published. The mRNA corresponded to a transcription product of 147 amino-acid residues; this length is in good agreement with results demonstrating a 20 000 M_r precursor for AVP in the hypothalamus [5,6] and with the size of N_p precursors synthesized in a cell-free translation system [7,8].

Up to now all the detection procedures of high-M_r precursors were based upon immunological cross-reactivity of anti-AVP and/or anti-N_p antibodies with these proteins. These experiments give only a qualitative estimation since cross-reactivity ratios between the peptides and their precursors are unknown. Furthermore, some precursor forms could escape detection if the antigenic determinants are buried in the tertiary structure. This may account for the discrepancies observed between the above data [5–8] and that in [9–12] indicating the existence of 80 000 M_r precursor forms and several intermediates with M_r between 80 000–20 000.

We have described a method for quantitating the AVP sequence included in higher molecular precursor that relies on revealing the antigenic determinant of AVP by trypsin cleavage and glycinamide linkage [13].

Combining this technique with [35S]cysteine labeling of the proteins synthesized in vivo, we investigated the maturation scheme of the AVP precursors in rat hypothalamic nuclei. The dynamics of the maturation was also approached by applying hydration–dehydration cycles to the animals.

Abbreviations: AVP, arginine vasopressin; OT, oxytocin; N_p, neurophysin; SON, supraoptic nucleus; PLH, posterior lobe of hypophysis; M_r, apparent relative molecular mass; SDS, sodium dodecyl sulfate; HHS, hypothalamo–hypophysial system; TPCK trypsin, Tos Phe CH_2Cl-treated trypsin

2. MATERIALS AND METHODS

2.1. *Animals and tissue collection*

Sprague-Dawley rats (250–300 g body wt) were used. Some were dehydrated for 4 days by water deprivation and anesthetized with Nembutal (0.4 ml/100 g body wt). They were then injected bilaterally adjacent to SON with 10 μCi [35S]cysteine (New England Nuclear) 500 Ci/mmol in NaCl 0.15 M with 10^{-2} M dithiothreitol as in [14].

Some of the animals were rehydrated for 3 h

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after the injection then decapitated. The SON were dissected out according to the Palkovitz technique [15], and the posterior lobe of the hypophysis removed in < 1 min. The tissues were immediately homogenized by sonication in HCl 0.1 N and stored at −80°C until used.

2.2. *Gel electrophoresis*

Gel electrophoresis was adapted from the method in [16]. Briefly polyacrylamide slab gels ($15 \times 15 \times 0.2$ cm) were made of 15% acrylamide, 0.4% bisacrylamide, 5 M Urea, and 0.9 M acetic acid. Electrode compartments contained 0.9 M acetic acid, 5×10^{-2} M KCl.

The homogenates were thawed and precipitated by trichloroacetic acid (5% final). The protein pellet was extensively washed with ether and redissolved in sample buffer (8 M urea, 0.9 M acetic acid, 1% Triton X-100) and left overnight at room temperature before being changed onto the gel. Electrophoresis was carried out for 24 h at 35 V. M_r-determinations were based on a series of gel in which extracts from tissues were run in slots adjacent to the following M_r marker proteins: transferrine (80 000); bovine serum albumin (68 000); soybean trypsin inhibitor (21 000) and cytochrome *c* (12 700).

2.3. *Analysis of radioactivity profiles*

After electrophoresis each track was cut into 2 mm slices which were individually solubilized in 200 μl 30% H_2O_2 at 60°C, and counted in the presence of 10 ml aquasol (New England Nuclear) in an Intertechnic liquid scintillation counter. The radioactivity of each slice was expressed as the percentage of the total radioactivity recovered into the gel track.

2.4. *Detection of the AVP precursors*

AVP sequences in precursor molecules were detected after reconstitution of the antigenic determinant by tryptic hydrolysis followed by derivatisation with glycinamide as in [13]. The gels were fixed in 10% acetic acid, then washed extensively with activation buffer (0.1 M Tris, 2×10^{-2} M NaCl, 0.1 M $CaCl_2$, pH 8). They were cut into 2 mm slices which were digested with 25 μg trypsin (TPCK treated trypsin 259 units/mg, Worthington) in 250 μl of activation buffer at 22°C for 5 h. The reaction was stopped by addition of 35 μg soybean trypsin inhibitor (Worthington). The supernatant was then subjected to the amidation procedure. To a tube containing glycinamide (1 M glycinamide in 0.1 M morpholine ethane sulfonic acid, pH 5.4) 150 μl of the sample and 15 μl carbodiimide (0.75 M) were added. The reaction was complete and almost immediate. The samples were then diluted in incubation buffer (10^{-2} M phosphate, 0.15 M NaCl, 1 g/l bovine serum albumin, 0.1 g/l NaN_3, pH 7.4). The precursors were quantified by an AVP radioimmunoassay [17].

3. RESULTS

3.1. *Radioactivity pattern in SON and PLH*

Since N_p and AVP are known to be rich in cysteine we first attempted to enumerate the protein species synthesized in the SON and transported to the PLH. After injection of this ^{35}S-labeled amino acid into the SON of rats, both SON and PLH were dissected out from the same animal and the proteins were fractionated by acid–urea electrophoresis.

The classical SDS–PAGE system was tried as well (not shown). This technique was not very convenient because the presence of SDS in the medium prevented subsequent immunological analysis.

Three hours after the injection of [^{35}S]cysteine 5 major peaks of radioactivity were detected in SON of M_r 80 000, 65 000, 50 000, 35 000, 17 000 (fig.1). At this time only 2 major peaks were seen in PLH with M_r 17 000 and 10 000 (fig.1). The last one corresponded to the position of neurophysin. These results agree with [14,18] where labeled N_p was present in the PLH 3 h after cysteine injection and unchanged profiles were shown for up to 12 h. The presence of high-M_r forms in the SON but not in the PLH, indicated a possible precursor product relationship between the forms synthetized in the SON and the neurophysin revealed in the PLH. After dehydration alone and dehydration–rehydration, the labeling patterns of the SON were very similar, only the relative amounts of radioactivity incorporated into the different proteins changed. The N_p which was predominant in PLH was almost absent in normal SON but represented a minor peak in dehydrated animals (see SON, fig. 1A,B); the data were consistent with an accelerated maturation process which in these situations would take place into the SON.

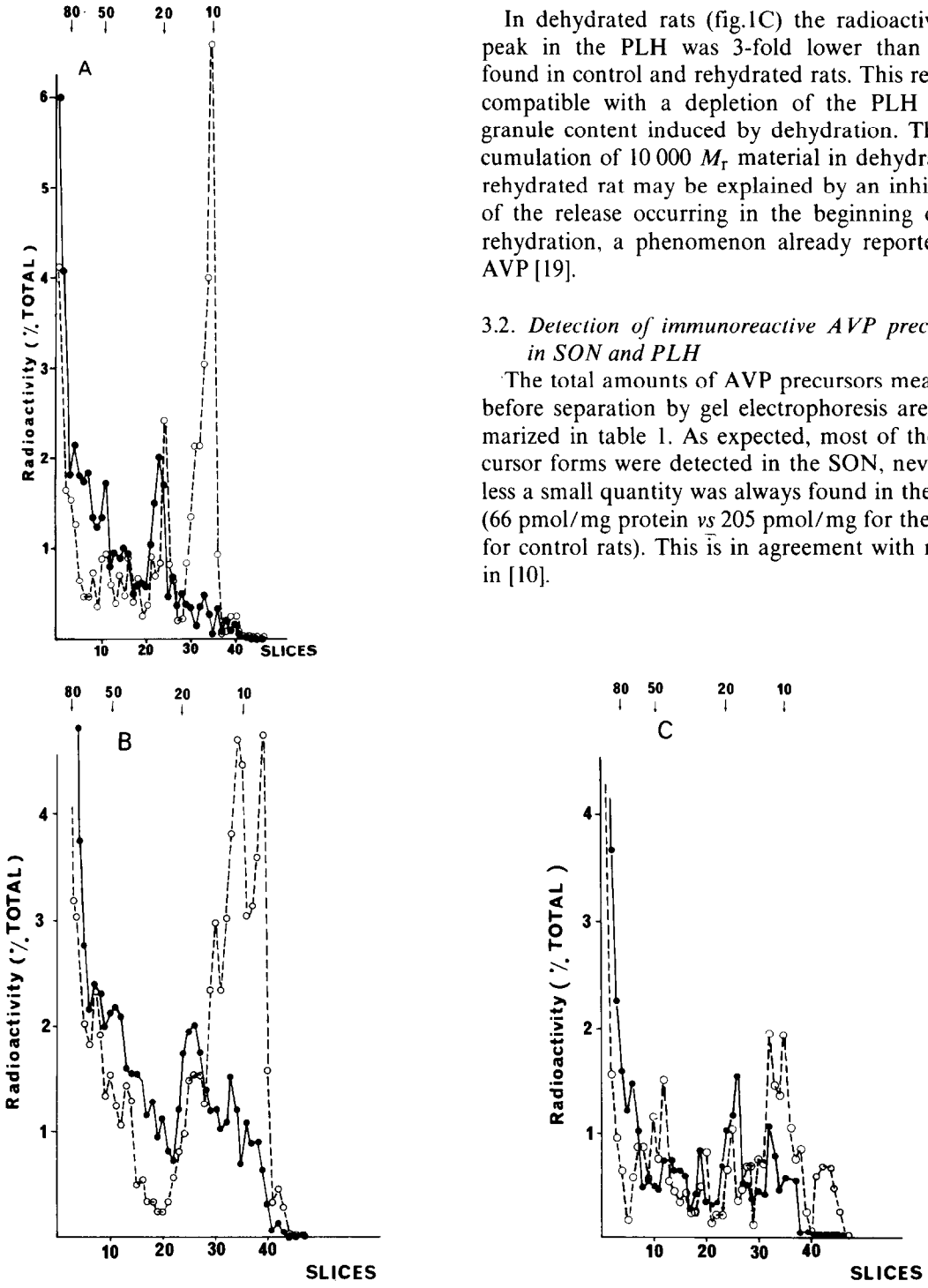

Fig.1. Acid–urea polyacrylamide gel electrophoresis of [^{35}S]cysteine-labeled proteins extracted from SON and PLH (3 h after injection of [^{35}S]cysteine into the SON: (A) control rat; (B) rat dehydrated for 4 days and 3 h rehydrated; (C) rat dehydrated for 4 days before injection. Radioactivity of each band was expressed as a percentage of the total; (•—•) SON; (○—○) PLH.

In dehydrated rats (fig.1C) the radioactive N_p peak in the PLH was 3-fold lower than those found in control and rehydrated rats. This result is compatible with a depletion of the PLH of its granule content induced by dehydration. The accumulation of 10 000 M_r material in dehydrated–rehydrated rat may be explained by an inhibition of the release occurring in the beginning of the rehydration, a phenomenon already reported for AVP [19].

3.2. *Detection of immunoreactive AVP precursors in SON and PLH*

The total amounts of AVP precursors measured before separation by gel electrophoresis are summarized in table 1. As expected, most of the precursor forms were detected in the SON, nevertheless a small quantity was always found in the PLH (66 pmol/mg protein *vs* 205 pmol/mg for the SON for control rats). This is in agreement with results in [10].

Table 1
Amount of immunoreactive AVP precursors

	Control rat	Dehydrated rat	Dehydrated–rehydrated rat
SON	205 ± 16	222 ± 28	313 ± 4.8
PLH	66.5 ± 4.3	46 ± 11.5	77.5 ± 13.8

The nervous structures were punched out according to the technique in section 2. They were homogeneized in 300 μl 0.1 M HCl. Each sample was precipitated by trichloroacetic acid (5% final). After centrifugation, the pellet was washed extensively by ether, then solubilized by 250 μl activation buffer and submitted to tryptic cleavage and amidation-linkage as in [13]. After dilution, the sample was assayed for its immunoreactivity. The results are given as AVP equivalents (pmol/mg protein; proteins were determined by the Lowry technique: SON 65 μg; PLH 200 μg). Data are expressed as means ± SD, $n = 6$.

The dehydration–rehydration treatment induced an increase of the precursor amount in the SON (8% and 33% for dehydrated and dehydrated–rehydrated rats, respectively). On the basis of these data, we chose dehydrated–rehydrated rats for the determination of the M_r for AVP precursors. Gel electrophoresis and subsequent treatments in view to detect AVP immunoreactivity were conducted as in section 2.

In SON (table 2) we observed 5 AVP immunoreactive forms with respective M_r-values of 80 000, 64 000, 34 000, 17 000, 8000, the 17 000 and 8000 being the predominant species. Hence, all the peaks observed after [^{35}S]cysteine labeling, except the one 50 000 M_r exhibited immunoreactivity.

In PLH (table 2) only 3 AVP immunoreactive forms were detected at 80 000, 34 000 and 8000 M_r, respectively, the last one bearing most of the immunoreactivity (45% of the total for PLH). In this case, the 17 000 M_r form detected by [^{35}S]cysteine labeling could not be detected by the AVP antibodies.

Table 2
Identification and quantification of AVP precursors in SON and PLH

M_r ($\times 10^{-3}$) ± SD		80 ± 0.8	64 ± 2	34 ± 1	17.6 ± 2	< 8 ± 0.5
Immuno-reactivity (pmol/band)	SON	0.50	1.10	0.75	2.30	4.70
	PLH	2.30	–	2.90	–	4.00

The immunoreactivity was expressed as AVP equivalents (pmol/band) and for half a structure (SON), and one structure (PLH). The molecular weight of precursors was determined by comparison with marker proteins (see section 2). Data are expressed as mean ± SD. Number of experiments was $n = 4$.

4. DISCUSSION

The quantitative determination of AVP sequence in protein is sensitive and precise enough to allow the quantification of AVP precursors in a single hypothalamic nucleus without any step of purification and concentration. In [13], we studied the spatial distribution of the precursor content and showed the regular decrease of the ratio precursor/hormone along the hypothalamo–hypophysial axis [13]. Another application is the follow-up of the precursor contents during the hydration–dehydration cycles (in preparation). Here, we have tried to clarify the distribution of the different molecular species of precursors, taking advantage of the fact that the quantification of vasopressin has been fairly adapted after acrylamide gel electrophoresis. For example, the precursor forms from SON (table 1, column 3) were around 20.3 pmol/structure while after gel separation 18.8 pmol/structure were recovered. A good correlation is found between [^{35}S]cysteine labeling, revealing at least neurophysin and vasopressin precursors, and the detection of AVP immunoreactivity after artificial maturation. The series of proteins bearing the AVP sequence (M_r 80 000, 65 000, 34 000, 17 000) are close parallels with that of neurophysin precursors in PLH [9,11,20]. A

comparable series has also been described in [12]. That these proteins represent authentic precursors is questionable since the messenger analysis [4–8] has only revealed a common precursor for AVP and neurophysin at 20 000 M_r. They are no longer fortuitous artefacts since they were found consistently by several groups, and in our hands with a high reproducibility and a fair dependence on the hydration state. Our experiments, performed in non-reducing conditions to preserve the full immunoreactivity of the AVP sequence, do not allow us to exclude aggregates stabilized by disulfide bridges. This explanation also holds for [12] but not for the observation [11] of a 80 000 M_r precursor in reducing conditions. Another possibility would be that high-M_r forms are not biosynthetic precursors of vasopressin but molecules containing an entire or partial AVP sequence in the same way as β-endorphin exhibits a Met-enkephalin sequence without being its precursor [21]. Only protein sequencing of these high-M_r forms will give the answer to this question.

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