EVIDENCE FOR HIGHER MOLECULAR WEIGHT IMMUNOREACTIVE FORMS OF VASOPRESSIN IN THE MOUSE HYPOTHALAMUS

Relationships with putative proneurophysins

Maryse CAMIER, Marc LAUBER, Jan MÖHRING+ and Paul COHEN,
with the technical assistance of Christine FAHY

Groupe de Neurobiochimie Cellulaire et Moléculaire, Equipe de Recherches Associée 693 du CNRS, Université Pierre et Marie Curie, 96 boulevard Raspail, 75006 Paris and *+Centre de Recherches International Merell Toraude, 67084 Strasbourg, France*

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1. Introduction

The neurohypophyseal nonapeptide hormones, oxytocin and vasopressin are synthesized in specialized nuclei of the hypothalamus. They are translocated to the neurohypophysis, the storage and excretion organ, within membrane-limited secretory granules where they are found as non-covalent complexes with the 10 000 mol. wt proteins, the neurophysins (reviewed [1–4]). The biogenesis of secretory protein components has been found to occur in the producing cells via larger biosynthetic precursors which generate the active molecules by post-translational processing. This appears to be the case for some peptide hormones [5–11]. As early as 1964, classical experiments of in vivo pulse followed by in vitro chase allowed Sachs and coworkers to obtain evidence that the appearance of radioactively labeled, and active, Arg^8-vasopressin (AVP) occurred after a lag period. Since this process was not inhibited by puromycin added during the chase, it was proposed that AVP was produced, first, as a larger inactive precursor molecule [12,13]. So far this hypothesis has not been demonstrated. Analogous methodology allowed similar conclusions with respect to neurophysin biogenesis, and a bifunctional prohormone, biosynthetic precursor common with AVP, was postulated [14]. In connection with the search in the hypothalamus for higher molecular weight putative proneurophysins [15,16] we have analyzed the radioimmunoassayable forms of AVP in mouse hypothalamic extracts fractionated according to the apparent molecular size of their protein components. We show that higher molecular weight forms of AVP of $\geqslant 30\,000$ and $\simeq 17\,000$ mol. wt can be detected together with the HMW neurophysin-like molecules of the corresponding apparent size. In addition, we show that a fraction of the immunoreactive species with $\sim 17\,000$ mol. wt crossreact both with anti-neurophysin and anti-vasopressin antibodies.

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2. Materials and methods

Phenylmethyl-sulfonylfluoride (PMSF), aprotinin, pepstatin, α-methyl-D-mannoside, bovine serum albumin were purchased from Sigma (St Louis, MO). Triton X-100 was from Calbiochem (San Diego, CA) and Bio-gel P-10 from Bio-Rad Laboratories (Richmond, CA). Insoluble concanavalin (con) A–Sepharose, Sephadex G-50 and activated CH–Sepharose 4B were supplied by Pharmacia (Uppsala). Human serum albumin (HSA) was from Behring (Marburg) and urea from Merck (Darmstadt).

Extraction and fractionation of mouse hypothalamic fragments were performed at 4°C. Hypothalamic fragments corresponding to the supraoptic and paraventricular nuclei of 50 mice were removed as in [15] and kept frozen in liquid nitrogen. Then the fragments were thawed and homogenized with a Potter Elvejhem in 5 ml 0.3 M sucrose containing 480 kallikrein inhibitor units (KIU)/ml aprotinin. The homogenate was centrifuged 15 min at 11 000 × *g* then the supernatant was collected and made 8 M in urea. The clear solution was centrifuged at 100 000 × *g* in a SW 50 rotor (Beckman L5 50) for 60 min and the supernatant was filtered on a Bio-gel P-10 column. The eluted fractions were tested for neurophysin and vasopressin immunoreactivities by a radioimmunoassay procedure (RIA).

The neurophysin RIA was carried out as in [15] using an antiserum (A_5IV) prepared by Dr Legros [17] (Liège, Belgium) and in the presence of 480 KIU/ml aprotonin. Bovine neurophysin II and ^{125}I-labeled bovine neurophysin II were used as standard and tracer respectively. The Arg^8-vasopressin RIA was conducted as in [18], using antiserum Bert (1/45 000 final dilution) and [^{125}I]AVP as tracer, allowing a minimal detection of 1–2 pg AVP. Under the conditions used 15% or 0.3% crossreactivity with Lys^8-vasopressin and oxytocin was observed. Less than 0.05% crossreactivity with neurophysin was detected.

Affinity chromatography on con A–Sepharose was performed as in [15] using con A buffer (50 mM Tris–HCl containing 1 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mg/ml bovine serum albumin, 1‰ Triton X-100, 400 KIU/ml aprotinin (pH 7.5).

Chromatography by immunoadsorption was with ammonium sulfate-precipitated anti-bovine neurophysin II antibodies (SII 03) prepared in this laboratory [19] and immobilized on CH–Sepharose 4B using the procedure recommended by the manufacturer.

3. Results and discussion

The pattern of the AVP-like material distributed in the eluate of the Bio-gel P-10 fractionation of the hypothalamic extracts made in 8 M urea allowed the detection of HMW forms of vasopressin (fig.1A). They were found to co-elute with the neurophysin-like material both in the 17 000 mol. wt and exclusion (⩾ 20 000 mol. wt) volumes of the molecular sieve column (fig.1A). The elution of urea rendered impossible the detection of free AVP by RIA. The

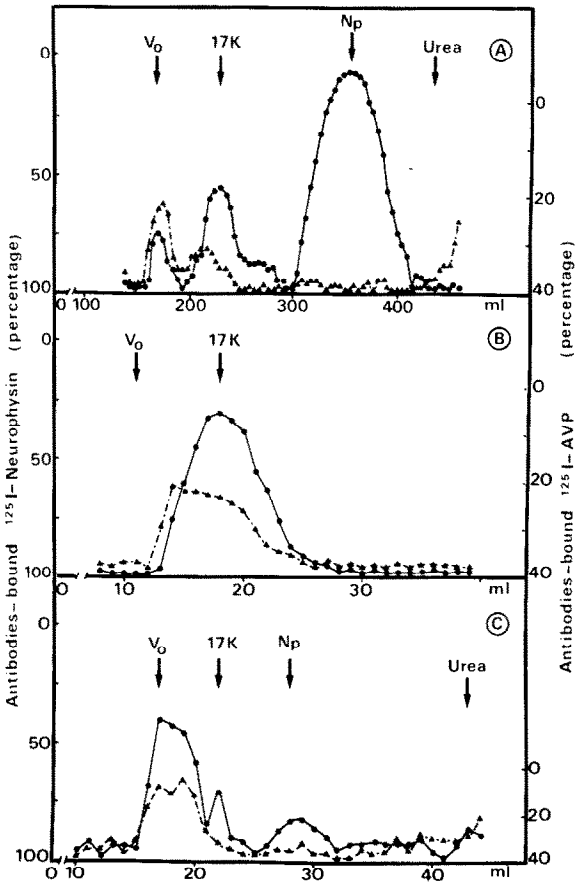

Fig.1. Immunological evidence for higher molecular weight forms of Arg^8-vasopressin. (A) Gel filtration fractionation (Bio-gel P-10, 40 × 4 cm column in 0.1 N HCOOH containing 0.1% Triton X-100, 0.5 mM PMSF and 1 mg/l pepstatin) of the hypothalamic extracts. (B) Gel filtration (Bio-gel P-10, 19 × 1.5 cm column equilibrated in the same eluent as above) of the 17 000 mol. wt species from (A) after exposure to 8 M urea for 16 h. (C) Sephadex G-50 filtration (30 × 1.5 cm column equilibrated in the same eluent as above) of the material recovered in the V_0 of the fractionation from (A) after exposure to 8 M urea for 14 h. The AVP-like (▲–▲) and neurophysin-like (●–●) immunoreactivities were determined every two fractions by RIA. The materials eluted in the V_0 of the Bio-gel P-10 and of the Sephadex G-50 correspond, respectively, to species with app. mol. wt ⩾ 20 000 and ⩾ 30 000.

elution profile of the AVP-like material recovered in the 17 000 mol. wt volume was not affected by exposure to 8 M urea (fig.1B). This demonstrates that the 17 000 mol. wt material is neither AVP aggregates nor AVP non-covalently attached to larger components. The material recovered in the exclusion volume of the same molecular sieve filtration (i.e., $\geqslant$ 20 000 mol. wt) represents a relatively small fraction of the neurophysin immunoreactivity detected in the extracts (fig.1A). After exposure to 8 M urea for 14 h, it was filtered on a Sephadex G-50 column. The elution pattern of fig.1C indicates that both neurophysin-like and AVP-like immunoreactive materials co-eluted together in volumes suggesting species with mol. wt $\geqslant$ 30 000. Two discrete peaks of neurophysin-like material with sizes $\sim$ 17 000 and 10 000 mol. wt, respectively, were observed (fig.1C) suggesting that these lower sized species were generated during the operations. These observations indicative of post-translational processing, will be detailed in [19].

Control experiments were performed as in [20] in order to assess that both neurophysin-like and AVP-like immunoreactivities detected in the exclusion volume of the molecular sieve filtration are real. It was found that they neither reflect protease activities affecting the integrity of the ^{125}I-labeled tracers nor are due to interference of other components in the RIA. This material was also tested for its ability to compete with the labeled neurophysin, or AVP, for binding to the corresponding antibodies. Serial dilutions of the 17 000 and $\geqslant$ 30 000 mol. wt materials gave characteristic plots (fig.2) suggesting unequivocally antigenic similarities with the reference components used as standards.

The 17 000 mol. wt neurophysin-like material (as in fig.1A) has been shown to exhibit strong interactions with a plant lectin, con A and can be selectively adsorbed on a covalent conjugate of the lectin (con A–Sepharose) [15]. This property, characteristic of many other secretory protein components, is believed to reflect some degree of protein glycosylation. It was therefore interesting to test for the possibility that the 17 000 mol. wt vasopressin-like material may carry carbohydrate moieties. The pattern of the experiment in fig.3 indicates that 90% of the 17 000 mol. wt neurophysin-like material was retained by the affinity adsorbant and was desorbed

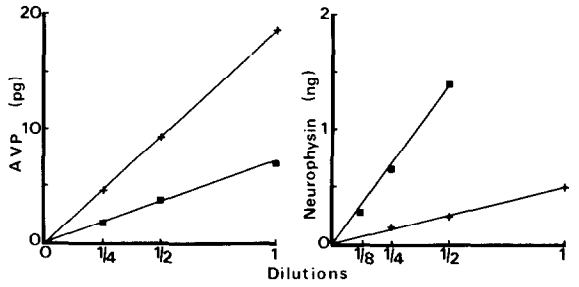

Fig.2. Immunological analysis of the AVP and neurophysin-like materials recovered in the 17 000 mol. wt (–■–) and exclusion (–+–) volumes of the Bio-gel P-10 fractionation of fig.1A. Serial dilutions of the material to be analyzed were evaluated for their capacity to compete with ^{125}I-labeled neurophysin or [^{125}I]AVP for binding to the corresponding antibodies. Immunoreactivities of the dilutions are expressed as AVP, or neurophysin, equivalents respectively calculated from corresponding standard curves (pg and ng per assay).

by irrigation of the con A–Sepharose conjugate successively by 0.2 M and 1 M α-methyl-mannoside. In contrast, the totality of the AVP-like material was not retained and was recovered, together with 10% of the neurophysin-like immunoreactivity, in the void volume of the affinity column. This observation might be taken as suggestive that while most of the 17 000 mol. wt neurophysin-like material is

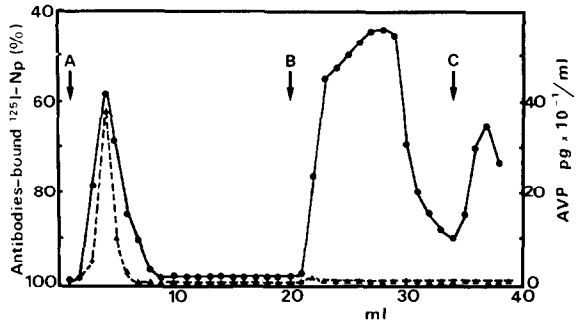

Fig.3. Affinity chromatography on a con A–Sepharose adsorbant of the 17 000 mol. wt immunoreactive material from fig.1A. The lyophilized sample was dissolved in the con A buffer and adsorbed on the column equilibrated in the same buffer. The column was then washed with the same buffer (A) then with 0.2 M (B) and 1.0 M (C) α-methyl-mannoside in con A buffer. AVP-like (▲–▲) and neurophysin (N_p)-like (●–●) immunoreactivities were measured by RIA on each fraction.

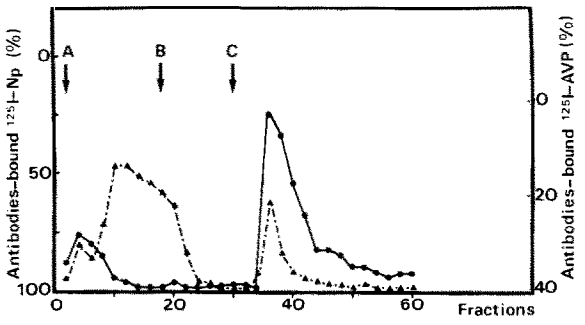

Fig.4. Affinity chromatography on anti-bovine neurophysin II antibodies immobilized on CH–Sepharose 4B. The 17 000 mol. wt material from fig.1A was lyophilized and applied on the column in 100 mM phosphate buffer, 100 mM NaCl, 0.1 HSA mg/ml (pH 7.5). The complex was washed (A) with the same buffer then with a 10 mM phosphate buffer (pH 7.5) (B) and desorption was conducted (C) by 1 M acetic acid. Both AVP (▲–▲) and neurophysin (N_p) (●–●) immunoreactivities were measured every 2 fractions (0.5 ml).

glycosylated to various extents, the 17 000 mol. wt material AVP-like is not. The latter may be substituted with other carbohydrate moieties which do not exhibit interactions with the con A immobilized on Sepharose.

It was interesting, however, to check if the AVP-like molecules carry also the neurophysin determinants in their sequence. The 17 000 mol. wt material (from fig.1A) was submitted to an affinity chromatography by immunoadsorption on a Sepharose–antineurophysin immune serum covalent conjugate. The pattern in fig.4 indicates clearly that a fraction of the original AVP-like material applied to the column was coadsorbed with 90% of the original neurophysin-like species and was desorbed only by a drastic pH change*. Control experiments showed that the immunoadsorbent retained $\leqslant 1.5\%$ of [^{125}I]AVP or $\leqslant 5\%$ of standard AVP as detected by RIA.

* Significant amounts of AVP-like immunoreactivity were recovered in the washing volume of the column (fig.4). Observations (not shown) indicate that the material responsible for this immunoreactivity is smaller than the species applied to the column and may correspond to fragments bearing the AVP sequence and derived by proteolytic cleavage of the larger molecules during the affinity chromatography procedure [19]

These observations, together with the analysis of the experiment on con A–Sepharose (fig.3), strongly suggest that a small proportion of the 17 000 mol. wt immunoreactive species may carry both the AVP and neurophysin antigenic determinants in their sequence. Furthermore the elution behaviour of the immunoreactive material recovered in the exclusion volume of the Bio-gel P-10 fractionation, after filtration on Sephadex G-50 clearly indicates that both AVP-like and neurophysin-like immunoreactivities co-elute in the exclusion volume (i.e., $\geqslant$ 30 000 mol. wt) (fig.1C). This is compatible with the existence of larger high molecular weight immunoreactive forms of neurophysin and AVP ($\geqslant$ 30 000 mol. wt) in the mouse hypothalamus. Consequently it is attractive to postulate that the intermediary size material (the 17 000 mol. wt neurophysin-like glycosylated species and the 17 000 mol. wt [neurophysin–AVP]-like hybrid molecules) may derive from the larger species by a post-translational process involving heterolytic cleavage. However a pulse-labeled 20 000 mol. wt, neurophysin-like material has been synthesized in the rat [21]. Neurophysin-containing molecules of 25 000 mol. wt [22] and 17 000 mol. wt [23] have been reported in the immunoprecipitable components of the cell-free translation products of hypothalamic mRNA from bovine [22] and rat or mouse [23], respectively. Although relationships with AVP-like higher molecular weight forms were not analyzed in [21–23] these forms may be comparable to the corresponding species described above and in [15,16]. The immunoreactive forms of $\geqslant$ 30 000 mol. wt could represent putative biosynthetic precursors common both to neurophysin and AVP. Because of the difficulties in obtaining reasonable amounts of the larger immunoreactive forms from the mouse hypothalamus this hypothesis will be investigated on sources from other species [19].

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