Nature of the Immunoreactive Neurophysins in Ectopic Vasopressin-producing Oat Cell Carcinomas of the Lung

DEMONSTRATION OF A PUTATIVE COMMON PRECURSOR TO VASOPRESSIN AND NEUROPHYSIN

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ABSTRACT In an attempt to delineate the nature of the immunoreactive neurophysins in oat cell carcinomas of the lung with ectopic vasopressin production, tumor neurophysins were characterized by gel filtration and by electrophoresis. In all of the five tumor tissues, activities of both vasopressin and nicotine-stimulated neurophysin (NSN) determined by radioimmunoassay were demonstrated. A small amount of oxytocin as well as estrogen-stimulated neurophysin was detected in three of the tissues. When tissue extract was subjected to Sephadex G-50 gel filtration in 0.2 N acetic acid, the major portion of immunoreactive NSN emerged in the fractions corresponding to the molecular size of 10,000. The migration pattern of NSN in these fractions on electrophoresis was qualitatively the same as that of NSN extracted from human posterior pituitary glands. In addition to this major neurophysin, immunoreactive NSN with the molecular size of 20,000 was consistently demonstrated in three tumor extracts. This high molecular weight form of neurophysin represented 6.5–8.7% of total NSN immunoactivities in each tumor extract and its elution profile was not changed when analyzed under denaturing conditions in 6 M guanidine hydrochloride. On electrophoresis, it migrated near the gamma globulin region; however, the peak was broad suggesting that it consists of more than two different molecular populations. A substantial portion of the high molecular weight NSN appears to be a glycoprotein judging from its binding to concanavalin A. When the high molecular weight form of neurophysin

was incubated with trypsin, essentially all of the activities were converted into NSN with the molecular size of 10,000. Moreover, an equimolar amount of vasopressin was liberated after the treatment, the elution pattern of which closely resembled that of synthetic arginine vasopressin. When a lower concentration of trypsin was used, some of the 20,000-dalton neurophysin exhibited activities of both NSN and vasopressin. Since the antivasopressin serum used in this study appeared to be directed toward the ring-portion side of vasopressin, these results suggest that this 20,000-dalton neurophysin is, in all probability, a common precursor to vasopressin and neurophysin, and that vasopressin may be located in the middle of the precursor molecule.

INTRODUCTION

The association of a malignant tumor and the inappropriate secretion of antidiuretic hormone (ADH)¹ (1) is now recognized frequently. Although a number of other mechanisms have been proposed, tumor production of ADH may account for the inappropriate ADH syndrome in patients with neoplasms. Since the first demonstration of vasopressin-like material in a bronchogenic tumor by Amatruda et al. (2), several papers have documented that ADH in tumor tissue extracts is both biologically and immunologically similar to arginine vasopressin (AVP). The most definitive evidence for tumor production of vasopressin was provided by George et al. (3), who showed that the hormone could be synthesized *in vitro* by bronchogenic

This study was presented in part at the Sixth International Congress of Endocrinology, Melbourne, Australia, 10–16 February 1980.

Received for publication 30 December 1980 and in revised form 7 April 1981.

¹Abbreviations used in this paper: ADH, antidiuretic hormone; AVP, arginine vasopressin; Con A, concanavalin A; ESN, estrogen-stimulated neurophysin; LVP, lysine vasopressin; NSN, nicotine-stimulated neurophysin.

carcinoma; similar results have been reported for a uterine tumor (4).

Neurophysins, carrier proteins for neurohypophyseal hormones, have been shown to exist in the posterior pituitary gland and to be released in response to stimuli that may stimulate the secretion of vasopressin and oxytocin. The experiments conducted in animals so far suggest, but do not establish, that neurophysins may be synthesized in the hypothalamus concomitantly with vasopressin and oxytocin (5, 6). However, no consensus is obtained regarding the presence of neurophysins in ectopic vasopressin-producing tumors. Pettengill et al. (7) found that an oat cell tumor of the lung in culture produced vasopressin without concurrent synthesis of any neurophysin. In contrast, Hamilton et al. (8) reported that neurophysin was present in extracts of three tumors from patients with inappropriate ADH syndrome. Similarly, Legros (9) found both vasopressin and neurophysin in two tumor extracts. Although these latter observations may support the view that neurophysin is produced in ectopic vasopressin-producing tumors, the exact nature of neurophysins in tumor tissues is not known. Our study was undertaken to clarify the nature of neurophysins and octapeptide hormones in tumor tissues from patients with ectopic production of vasopressin.

METHODS

Tissues and plasma. Five patients, between 58 and 74 yr, with oat cell carcinoma of the lung were studied. All of the subjects exhibited a typical clinical picture of inappropriate ADH syndrome. Heparinized blood was collected for the analysis of vasopressin and neurophysins from four of the patients on ad lib. intake of water. Plasma was immediately separated by centrifugation and stored at -20°C until assayed. Tumor tissues were obtained at autopsy and kept at -70°C until analyzed. The time intervals between death and the collection of tissues were within several hours. Fresh human pituitary glands were kindly supplied by Dr. Inui at the Tokyo Metropolitan Examiners' Office. Posterior lobes were carefully separated before extraction.

Extraction of tissues. Tumor tissues and posterior pituitary glands were sectioned while still frozen, and minced and homogenized in ice-cold 0.1 N HCl (10) with a Teflon pestle. The final pH was adjusted to 1.5 with concentrated HCl and insoluble material was removed by centrifugation at 2,300 g for 15 min at 4°C . The supernate was subjected to gel filtration or assayed for neurophysins and octapeptide hormones after dilution with an appropriate volume of the assay buffer.

Gel filtration. Gel filtration of tissue extracts was performed at 4°C on a column (1×50 cm) of Sephadex G-50 superfine (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Uppsala, Sweden) equilibrated with 0.2 N acetic acid or 6 M guanidine hydrochloride by descending flow. Flow rate was 1.2 ml/h and each 0.4-ml fraction was collected. The column was calibrated with blue dextran, chymotrypsinogen A, ^{125}I -labeled human growth hormone, cytochrome c, ^{125}I -labeled human nicotine-stimulated neurophysin (NSN) (11), $\alpha(1-24)$ ACTH, oxytocin, lysine vasopressin (LVP), and AVP.

In each experiment, a tracer amount of ^{125}I -labeled NSN was included in the sample to compare the running rates of neurophysins and octapeptides. After gel filtration, immunoreactivities of neurophysins and hormones were localized by diluting the fractions and carrying out radioimmunoassays. When the purification of NSN from a larger volume of tumor tissue extract was attempted, a 2.6×100 -cm column packed with Sephadex G-50 superfine and equilibrated with 0.2 N acetic acid was also used before rechromatography on a small column as described above.

Electrophoresis. Electrophoresis of tissue extracts was done at 4°C using a column (1.7×35 cm) of Biogel P-2, 200–400 mesh (Bio-Rad Laboratories, Richmond, Calif.) equilibrated with 0.05 M Tris-HCl buffer (pH 8.6). Usually 0.7 ml of the sample was applied to the column and electrophoresis was performed at a current of 6.6 mA/cm² for 20 h. When lyophilized eluates from exclusion chromatography were subjected to electrophoresis, human serum albumin and gamma globulin were incorporated into the sample and served as internal markers. After electrophoresis, the column was eluted with the same buffer, and fractions of 0.5 ml were collected. Migration pattern was monitored by extinction at 280 nm by a spectrophotometer and the distribution of neurophysins was determined by radioimmunoassay of the resulting fractions.

Affinity chromatography with concanavalin A (Con A). A small column (total volume: 0.5 ml) of Con A-Sepharose (Pharmacia Fine Chemicals) was prepared in a disposable plastic syringe. The column was washed before use with 0.1 N acetic acid and then equilibrated with Con A buffer (0.05 M phosphate buffer, pH 7.0, containing 0.5 M NaCl, 1 mM MgCl_2 , and 1 mM CaCl_2). Samples were dissolved in the buffer and applied to the column at 4°C . The column was washed with 10 ml of the buffer and specifically bound material was eluted with 0.2 M α -methyl-D-mannopyranoside (grade III, Sigma Chemical Co., St. Louis, Mo.) in Con A buffer. Recovery of immunoreactive NSN was >90%.

Trypsin treatment. Sephadex eluates containing a putative neurophysin precursor were pooled, lyophilized, and trypsinized under different conditions. The reaction mixture contained the precursor dissolved in 200 μl of 0.05 M phosphate buffer (pH 7.4) to which 50 μl of freshly prepared solution of DPCC-treated trypsin (Sigma Chemical Co.) at a concentration of 0.5 or 0.05 mg/ml was added. The samples were incubated at 37°C for 0.5 or 2 min, and the reaction was terminated by acidification to pH 2.8 with acetic acid. As a control, a portion was treated in the same manner without the addition of trypsin. To determine the recovery of neurophysin after trypsin treatment, 8.30 ng of NSN was incubated with trypsin at a final concentration of 100 $\mu\text{g}/\text{ml}$ for 2 min. In the reaction mixture determined by radioimmunoassay NSN was 8.38 ± 0.29 ng (mean $\pm$ SEM, $n = 5$) in the control and 7.55 ± 0.35 ng (mean $\pm$ SEM, $n = 5$) in the trypsin-treated mixture, respectively. When 2.10 ng of synthetic AVP was similarly treated, the vasopressin found was 2.12 ± 0.05 ng (mean $\pm$ SEM, $n = 5$) in the control and 1.99 ± 0.04 ng (mean $\pm$ SEM, $n = 5$) in the trypsin-treated mixture. The mean recoveries after trypsinization were, therefore, 90.1% for NSN and 93.8% for AVP. In this experiment, aprotinin was included in the assay buffers at a concentration of 500 U/ml to avoid further tryptic digestions during radioimmunoassay.

Radioimmunoassays. NSN and estrogen-stimulated neurophysin (ESN) (11) were measured using immunological materials kindly donated by the National Institute of Arthritis, Metabolism, and Digestive Diseases and the National Pituitary Agency, U. S. Public Health Service. The general procedure

of neurophysin radioimmunoassay is described in detail elsewhere (12).

Vasopressin was radioimmunoassayed as reported (13) using ^{125}I -labeled synthetic AVP (Sigma Chemical Co.) and an antiserum developed in a rabbit by multiple injections of LVP coupled with bovine serum albumin. This antiserum reacted on an equimolar basis with AVP and LVP (Sigma Chemical Co.). Pressinamide was also active in the displacement of ^{125}I -labeled AVP from the antiserum with a relative potency of 152% at 50% binding on a molar basis. The slope of the dose-response curve constructed with pressinamide, however, was different from that of AVP, which was steeper than the former. AVP was ~ 40 times more potent at inhibiting binding of ^{125}I -AVP to the antiserum than deamino-dicarba-AVP and at least 10^5 times more potent than oxytocin (Sandoz Ltd., Basel, Switzerland), arginine vasotocin (Calbiochem-Behring Corp., San Diego, Calif.), proline-arginine-glycine- NH_2 (Pro-Arg-Gly- NH_2), ϵ -aminocaproyl-Pro-Arg-Gly- NH_2 , tocinaamide, deamino-dicarba-oxytocin, and deamino-dicarba-vasotocin. Thus, the antiserum appeared to be directed toward the ring-portion side of the vasopressin molecule. Pressinamide and tocinaamide used in this study were kindly provided by Dr. W. H. Sawyer, College of Physicians & Surgeons of Columbia University, New York. Pro-Arg-Gly- NH_2 and ϵ -aminocaproyl-Pro-Arg-Gly- NH_2 were generous gifts of Dr. Moore, University of Calgary, Canada. Deamino-dicarba derivatives were purchased from the Protein Research Foundation, Osaka, Japan.

Radioimmunoassay of oxytocin was performed using ^{125}I -labeled synthetic oxytocin (Sandoz Ltd.) and an antiserum generated in a rabbit against oxytocin covalently linked to bovine serum albumin by carbodiimide condensation. In this radioimmunoassay, the cross-reactivity of AVP, LVP, and neurophysins was negligible, whereas the binding of ^{125}I -oxytocin to the antiserum exhibited a dose-related inhibition in the range of 2 to 200 pg of oxytocin.

Statistical analysis. Statistical significance was determined by Student's unpaired *t* test.

RESULTS

Table I shows plasma levels of vasopressin and neurophysins in four patients with oat cell carcinoma of the lung who developed inappropriate ADH secretion. Plasma vasopressin concentrations were consistently elevated in spite of hyponatremia and decreased plasma osmolality, which is in good agreement with the diagnosis. Plasma NSN levels were high in three of them (patients 2–4), whereas the concentration was

within the normal control range (0.7 ± 0.1 ng/ml, mean $\pm$ SEM, $n = 40$) in the remainder (patient 1), although only a single determination of plasma NSN was possible in this patient. Molar ratios of NSN to vasopressin, calculated on the basis of the assumption of the molecular weight of neurophysin to be 10,000, ranged from 3.8 to 33.6 (19.2 ± 6.1 , mean $\pm$ SEM). Plasma levels of ESN were, on the other hand, enhanced in two patients (patients 2 and 3) compared with normal controls (1.2 ± 0.1 ng/ml, mean $\pm$ SEM, $n = 38$).

The concentrations of neurophysins as well as octapeptide hormones in tumor tissues obtained from these subjects and another male patient, aged 64 yr, with oat cell carcinoma of the lung (patient 5) are given in Table II. In all of the five tissues, the presence of a significant amount of both vasopressin and NSN was demonstrated. The mean molar ratio of NSN to vasopressin calculated as described above was 3.16 ± 1.06 (mean $\pm$ SEM), which was not significantly different from that of NSN to vasopressin in human posterior pituitaries (1.62 ± 0.12 , mean $\pm$ SEM, $n = 10$). Oxytocin and ESN were detected, though quantitatively far less than vasopressin and NSN, in three of the tissue extracts (patients 1–3). However, ESN thus determined may be overestimated because of slight cross-reaction of abundant NSN in the ESN radioimmunoassay.

Fig. 1a illustrates the elution pattern of an extract of pooled human posterior pituitary glands from a column of Sephadex G-50 superfine equilibrated and eluted with 0.2 N acetic acid. The major portion of immunoreactive NSN was coeluted with ^{125}I -labeled NSN in the fractions corresponding to the molecular size of 10,000. The peak was broad and asymmetrical, suggesting the heterogeneity of neurophysin molecules. In the ascending limb of the NSN peak, a peak of immunoreactive ESN was demonstrated. In addition, two small peaks of both NSN and ESN were observed.

TABLE I
Plasma Levels of Vasopressin and Neurophysins in Patients with Ectopic Production of Vasopressin

Case	Vasopressin	NSN	ESN	Molar ratio
	pg/ml	ng/ml	ng/ml	NSN/Vasopressin*
1. 58 yr, male	19.2	0.72	<0.5	3.8
2. 67 yr, male	625	121	13.3	19.3
3. 68 yr, male	19.3	3.9	2.8	20.2
4. 74 yr, male	54.1	18.2	0.72	33.6

* Calculations were based on the assumption of the molecular weight of NSN to be 10,000.

TABLE II
Neurophysins and Hormones in Ectopic Vasopressin-producing Tumor Tissues

Tumor extract*	NSN	ESN	Vasopressin	Oxytocin	Molar ratio† NSN/Vasopressin
	ng/g tissue		ng/g tissue		
1	2,265	18.0	153	0.11	1.48
2	1,490	68.7	38.5	2.46	3.87
3	1,280	124	18.2	0.87	7.03
4	154	<5	7.59	<0.1	2.03
5	137	<5	9.78	<0.1	1.40

* The number of tumor tissue extract coincides with that of the patient in Table I.

† Calculations were based on the assumption of the molecular weight of NSN to be 10,000.

FIGURE 1 Elution profiles from Sephadex G-50 in 0.2 N acetic acid of immunoreactive neurophysins, vasopressin, and oxytocin in extracts of human posterior pituitaries and tumor tissues from patients with inappropriate ADH secretion. (a) Extract of pooled human posterior pituitary glands. (b) Tumor extract 1. (c) Tumor extract 2. (d) Tumor extract 3; see Table II for further identification of tumor tissue extracts. V_0 , void volume; V_t , total volume; ^{125}I -NSN, the peak fraction of ^{125}I -labeled NSN added to the samples as an internal marker.

One was found in the exclusion volume and the other eluted before the major peak of neurophysins. However, nearly all of the immunoactivities were co-eluted with ^{125}I -labeled NSN when these fractions were separately pooled and rechromatographed. The distribution of vasopressin and oxytocin on gel filtration was identical to that of synthetic hormones.

When extracts of ectopic vasopressin-producing tumors were subjected to gel filtration on a Sephadex G-50 column and eluted with 0.2 N acetic acid, immunoreactive NSN emerged in one major peak and two small peaks (Fig. 1 b-d). The main NSN peak C was again broad, which suggests the presence of different molecular species and its elution pattern was similar to the major NSN peak of posterior pituitary extract. In addition to this peak, a small but significant amount of NSN immunoactivity was consistently demonstrated in the fractions that corresponded to the molecular size of 20,000 (peak B in Fig. 1b-d). This peak represented 8.7% (Fig. 1b), 6.9% (Fig. 1c), and 6.5% (Fig. 1d) of total NSN immunoactivities in each tumor tissue extract. Radioimmunoassay of ESN in eluted fractions, on the other hand, demonstrated a small peak of ESN in two tumor extracts, which was eluted slightly earlier than the major NSN peak C (Fig. 1c and d). In the remaining tumor extract, the presence of any ESN peak was difficult to establish because of a small quantity of ESN in the tissue and cross-reaction of NSN in the ESN radioimmunoassay (Fig. 1b). The elution profile of immunoreactive vasopressin was identical to that of synthetic AVP. In accordance with the existence of a small amount of ESN, a minute peak of oxytocin was observed in the fractions corresponding to synthetic oxytocin.

To determine whether peak B neurophysin consists of aggregated forms of neurophysin, the fractions were combined, lyophilized, and incubated in 6 M urea at 4°C overnight. Rechromatography revealed that this maneuver did not alter the elution profile of peak B neurophysin. Furthermore, the presence of the NSN peak B was also demonstrated when a tumor tissue extract (1 in Table II) was chromatographed on a column of Sephadex G-50 equilibrated with 6 M guanidine hydrochloride (Fig. 2a). NSN fractions in peak B separated by gel filtration in 0.2 N acetic acid were then pooled, lyophilized, and analyzed under denaturing conditions in 6 M guanidine hydrochloride. As shown in Fig. 2b, NSN activities emerged in the region corresponding to the molecular size of 20,000. In contrast, most of the activities coeluted with ^{125}I -labeled NSN when peak A neurophysin in the exclusion volume was rechromatographed. Thus, immunoreactive neurophysin with the apparent molecular size of 20,000 was not smaller

FIGURE 2 Elution profiles from Sephadex G-50 in 6 M guanidine hydrochloride of tumor tissue extract from a patient with inappropriate ADH secretion and peak B neurophysin separated by Sephadex G-50 gel filtration in 0.2 N acetic acid. (a) Tumor tissue extract 1; see Table II for further identification of tumor tissue extract. (b) Fractions containing peak B neurophysin obtained by Sephadex G-50 gel filtration of tumor extract were combined, lyophilized, and rechromatographed in 6 M guanidine hydrochloride. V₀, void volume; V_t, total volume; ^{125}I -NSN, the peak fraction of ^{125}I -labeled NSN added to the samples as an internal marker.

neurophysin aggregated or bound to other proteins, while it appeared that the largest immunoreactive neurophysin that emerged in the void volume of a Sephadex G-50 column was neurophysin bound to proteins. For the purpose of identification, NSN appearing in peak B will be referred to as a high molecular weight form of neurophysin.

Fig. 3 shows a series of dose-response curves elicited by neurophysin in the NSN radioimmunoassay. A high molecular weight form of neurophysin showed a curve of similar shape and slope as those of 10,000-dalton neurophysin from posterior pituitary and tumor tissue extracts.

Neurophysins separated by exclusion chromatography were then analyzed by column electrophoresis. Fig. 4a illustrates the migration pattern on electrophoresis of immunoreactive neurophysins when the fractions containing the major neurophysin peak in human posterior pituitary extract were pooled, lyophilized, and subjected to the column electrophoresis. ESN was eluted in a single peak, while two peaks

FIGURE 3 Dose-response curves of human posterior pituitary and tumor neurophysins in the NSN radioimmunoassay. Neurophysins were separated by Sephadex G-50 gel filtration in 0.2 N acetic acid before assay. The picograms of standard NSN (National Institutes of Health) and micro-liters of each neurophysin fraction are plotted on the abscissa on a logarithmic scale.

(I and II) were observed in immunoreactive NSN, which was consistent with the finding of a rather wide and asymmetrical peak on gel filtration (Fig. 1a). Column electrophoresis of peak C neurophysin in a tumor tissue extract obtained by gel fractionation (Fig. 1b), on the other hand, revealed that the migration pattern was qualitatively the same as that of NSN in posterior pituitary extract (Fig. 4b). ESN was present in only a minute quantity of this tissue extract.

Fig. 4c depicts the electrophoretic pattern of a high molecular weight form of neurophysin in a tumor extract (Fig. 1b). Although the major portion of NSN activity was located near the gamma globulin region, the peak was broad, suggesting that a high molecular weight form of neurophysin consists of more than two different molecular populations.

To characterize a high molecular weight form of neurophysin, 16.2 g of tumor tissue (1 in Table II) was extracted in 0.1 N HCl, followed by fractionation in 0.2 N acetic acid using a larger column of Sephadex G-50. NSN activity was located by radioimmunoassay and a high molecular weight form of neurophysin was further purified by rechromatography on a 1 × 50-cm Sephadex G-50 column equilibrated with 0.2 N acetic acid and lyophilized. When a portion was subjected to affinity chromatography with Con A, ~32% of the activities was retained to Con A-agarose and was eluted with α -methyl-D-mannopyranoside (Fig. 5a). When neurophysin with the molecular size of 10,000 separated by electrophoresis (Fig. 4b) was ap-

FIGURE 4 Migration patterns of immunoreactive neurophysins on column electrophoresis. (a) The major neurophysin peak in posterior pituitary extract separated by gel filtration. (b) Peak C neurophysin in tumor extract. (c) Peak B neurophysin in tumor extract. See Fig. 1 for further identification of each neurophysin fraction. Alb indicates the peak of albumin and γ -Glob, that of gamma globulin added to the samples as internal markers.

FIGURE 5 Affinity chromatography on Con A-agarose. (a) A high molecular weight form of neurophysin in tumor tissue extract separated by Sephadex G-50 gel filtration. (b) Tumor tissue neurophysin with greater mobility on column electrophoresis (NSN-I in Fig. 4b). (c) Tumor tissue neurophysin with less mobility on column electrophoresis (NSN-II in Fig. 4b). The elution buffer containing 0.2 M α -methyl-D-mannopyranoside was added at the time indicated by the arrow.

plied to the column on Con A-agarose under identical conditions, no appreciable amount of NSN bound to Con A (Fig. 5b and c). The result suggests that some of the high molecular weight forms of neurophysin in ectopic vasopressin-producing tumor tissue are glycosylated.

Tryptic digestion of a high molecular weight form of neurophysin under controlled conditions was then used to determine whether neurophysin and vasopressin-like peptide could be generated by this procedure. When a high molecular weight form of neurophysin was incubated with trypsin at a final con-

centration of 100 μ g/ml for 2 min and the reaction mixture was fractionated on a Sephadex G-50 column in 0.2 N acetic acid, virtually all of the activities were converted into NSN with the molecular size of 10,000 (Fig. 6b), whereas the control eluted as a single NSN peak in the region of molecular size of 20,000 (Fig. 6a). In the control, a small peak of vasopressin immunoactivity was observed which was coeluted with a high molecular weight form of neurophysin (Fig. 6a). On a molar basis, it corresponded to $5.35 \pm 0.07\%$ (mean $\pm$ SEM, $n = 5$) of neurophysin activity in each fraction. However, no vasopressin immunoactivity was found in the fractions corresponding to synthetic AVP. In sharp contrast, a definite peak of immunoreactive vasopressin was observed after trypsin treatment, the elution pattern of which closely resembled that of synthetic AVP. The molar ratio of neurophysin to vasopressin generated by trypsinization approximated one. Vasopressin activity that was observed in the region corresponding to a high molecular weight form of neurophysin in the control was lost after the treatment.

FIGURE 6 Elution profiles from Sephadex G-50 in 0.2 N acetic acid of a high molecular weight form of neurophysin after trypsinization. (a) Control. (b) A high molecular weight form of neurophysin was treated with trypsin at a final concentration of 100 μ g/ml for 2 min. Vo, void volume; Vt, total volume; 125 I-NSN, the peak fraction of 125 I-labeled NSN added to the reaction mixture as an internal marker before gel filtration.

A high molecular weight form of neurophysin was then subjected to limited proteolysis under milder conditions. When trypsin at a final concentration of 10 $\mu\text{g/ml}$ with the reaction time of 0.5 min was used, the elution profile of a high molecular weight form of neurophysin remained largely unchanged, although a small portion of the activity was converted to 10,000-dalton neurophysin. However, a distinct peak of immunoreactive vasopressin appeared in the descending limb of the neurophysin peak. In addition, another peak of vasopressin, albeit small, was observed that eluted before synthetic AVP (Fig. 7a). Increasing the exposure time to trypsin to 2 min (Fig. 7b) resulted in an increase in conversion of a high molecular weight form of neurophysin into a smaller product. Moreover, immunoreactive vasopressin increased in its height and emerged in two major peaks; one co-emerged with synthetic AVP, while the other eluted in the fractions identical to the small vasopressin peak that was observed after trypsinization for 0.5 min (Fig. 7a). After the incubation with trypsin at a concentration of 100 $\mu\text{g/ml}$ for 0.5 min (Fig. 7c), the 20,000-dalton neurophysin peak disappeared and was recovered in a 10,000-dalton component. Vasopressin activity, on the other hand, emerged in one major peak in the region of synthetic AVP.

DISCUSSION

The foregoing results have shown that neurophysins are present in all of the ectopic vasopressin-producing tumors examined in this study and suggest that neurophysin synthesis may occur in association with ectopic production of vasopressin. The results are consistent with the previous reports by Hamilton et al. (8) and by Legros (9), but are at variance with the finding of Pettengill et al. (7). The latter investigators have grown an oat cell tumor of the lung in tissue culture and found that the tumor produced vasopressin without concomitant synthesis of any NSN, using the same neurophysin radioimmunoassay as used in our study. The apparent discrepancy between their study and ours is difficult to explain. A mechanism might exist in some exceptional tumor cells whereby vasopressin can be biosynthesized without neurophysin.

Exclusion chromatography of pooled human posterior pituitary extracts revealed almost all of the neurophysin activities to be in the fractions corresponding to the molecular size of 10,000 daltons, which is in good agreement with the molecular weight estimate of human neurophysins in previous reports (14, 15). On electrophoresis, they were separated into two NSN peaks and one ESN peak. The elution profile of tumor tissue neurophysins from a column of Sephadex G-50, on the other hand, was essentially the same as that of

FIGURE 7 Elution profiles from Sephadex G-50 in 0.2 N acetic acid of a high molecular weight form of neurophysin after trypsinization. (a) Trypsin at a final concentration of 10 $\mu\text{g/ml}$ for 0.5 min. (b) Trypsin at a final concentration of 10 $\mu\text{g/ml}$ for 2 min. (c) Trypsin at a final concentration of 100 $\mu\text{g/ml}$ for 0.5 min. V_o , void volume; V_t , total volume; ^{125}I -NSN, the peak fraction of ^{125}I -labeled NSN added to the reaction mixtures as an internal marker before gel filtration.

neurophysins in the posterior pituitaries with the exception that a high molecular weight form of neurophysin was demonstrated in the former. No qualitative difference was again obtained in the migration pattern of 10,000-dalton neurophysin on electrophoresis between tumor tissue and posterior pituitary extracts. The similarities in physicochemical properties of neurophysins and octapeptide hormones in tumor tissues and posterior pituitaries suggest that ectopic vasopressin-producing tumor could be an excellent model for the study of vasopressin and neurophysin biosynthesis in human hypothalamus.

A high molecular weight form of neurophysin was

unequivocally demonstrated in tissues of ectopic vasopressin production. It has a molecular size of 20,000 daltons on gel filtration and migrates near the gamma globulin region on electrophoresis. The dose-response curve constructed with varying amounts of a high molecular weight form of neurophysin was parallel with that of 10,000-dalton neurophysin of both posterior pituitary and tumor sources in the NSN radioimmunoassay. When a high molecular weight form of neurophysin was treated with dilute trypsin, essentially all of the immunoreactive NSN was converted to 10,000-dalton neurophysin, which indicates that the former may be a precursor protein to the latter. Moreover, vasopressin immunoactivities appeared and its elution profile was not distinguishable from that of synthetic AVP. More than a decade ago, Sachs and his colleagues (5, 6) proposed that vasopressin and its neurophysin are derived from a common precursor protein synthesized in the hypothalamus. Processing of the putative precursor is believed to occur during intraaxonal transport to the posterior pituitary gland, where the hormone and its neurophysin are stored for subsequent release. Although a number of experiments conducted in animals thus far supports this hypothesis, no substantial evidence has been forthcoming. Recently, bovine hypothalamic messenger (m)RNA has been shown to direct the synthesis of possible neurophysin biosynthetic precursors in a cell-free system (16–18). Similarly, it has been reported that labeled cysteine injected adjacent to the supraoptic nucleus of the rat was rapidly incorporated into a 20,000-dalton protein that, in time, was converted to a 12,000-dalton protein (19, 20). Along with the findings that the 20,000-dalton protein reacts with antineurophysin serum (21) and that trypsin liberates neurophysin and vasopressin-like peptide from the protein (22), these authors suggested that this 20,000-dalton protein may be a precursor to neurophysin and vasopressin in the rat. Viewed in this light, the high molecular weight form of neurophysin in ectopic vasopressin-producing tumors demonstrated in this study may be a common precursor to vasopressin and neurophysin, although the possibility that neurophysin is part of one 20,000-dalton protein and vasopressin part of another could not be completely excluded. If indeed the high molecular weight form of neurophysin is a common precursor, then each precursor molecule should contain one molecule of vasopressin and one molecule of neurophysin, because the molar ratio of vasopressin and neurophysin generated by trypsin approximated one and the recovery of neurophysin was not different from that of vasopressin under the present experimental conditions. Whether a precursor exists for oxytocin and its neurophysin was not established in this study, because the small amount of oxytocin and ESN present

in ectopic vasopressin-producing tumors prevented us from doing such an experiment.

The high molecular weight form of neurophysin possesses some immunoactivity of vasopressin (Fig. 6a). On a molar basis, it corresponded to 5% of neurophysin activity. After the exposure to trypsin at a concentration of 10 μ g/ml for 0.5 min, the elution profile from exclusion chromatography of the 20,000-dalton neurophysin was largely unchanged. However, a definite peak of immunoreactive vasopressin appeared in its descending limb (Fig. 7a), which suggests that some of the precursor molecules acquired vasopressin activity by trypsinization. Because the antigenic determinant of the antivasopressin serum used in this study mainly resides in the ring-portion side of vasopressin molecules, the result suggests that vasopressin may be located in the middle of the precursor molecule and a peptide extended at the amino terminus of vasopressin was removed by limited proteolysis. However, final confirmation of the presence of vasopressin and neurophysin molecule in the same precursor protein must await determination of its amino acid sequence. Of additional interest in this regard is the elution profile of vasopressin generated by trypsin. It emerged in a minor peak before synthetic AVP (Fig. 7a). Increasing the exposure time to trypsin resulted in an increase in this peak in height and an appearance of the vasopressin peak in the region of synthetic AVP (Fig. 7b), which may indicate that vasopressin of larger molecular size is an intermediate when the precursor is converted to a vasopressin-like peptide by trypsin. The nature of the intermediate remains unknown. It should be noted, however, that the peptides obtained by trypsinization may not be identical to vasopressin and its neurophysin biosynthesized in tumor tissues because tryptic cleavage is likely only to be the first step in posttranslational processing. Moreover, AVP is amidated at its carboxyl terminus. Multiple enzymes including carboxypeptidase B-like enzyme (23, 24) and amidating enzyme may participate in the complete conversion of the precursor to vasopressin and its neurophysin in tumors with ectopic vasopressin production.

Electrophoresis revealed that a high molecular weight form of neurophysin consists of at least two different molecular populations (Fig. 4c). We have also found that a substantial portion of the high molecular weight form of neurophysin is a glycoprotein, judging from its adsorption to Con A, a finding consistent with the observation by Lauber et al. (25) that their tentatively identified neurophysin precursor in mouse hypothalami was bound by Con A-Sepharose. Very recently, Brownstein et al. (26) showed that a [35 S]cysteine-labeled putative precursor of vasopressin-related neurophysin in rat hypothalami bound to Con A. Polymorphism of the glycoprotein hor-

mones and glycoprotein in general is a common phenomenon and is usually recognized on the basis of charge differences (27). It appears to be caused by variations in the carbohydrate side chains that are built on to each completed polypeptide molecule, step by step, through the action of a number of specific enzymes. It seems reasonable to speculate, therefore, that carbohydrates may play an important part in the structural modifications responsible for the observed heterogeneity of a high molecular weight form of neurophysin. Whether this microheterogeneity could be a direct result of the biosynthesis of the glycoprotein or a result of alteration of the carbohydrate side chains during purification in acidic condition is not certain. If in fact a high molecular weight form of neurophysin is a glycoprotein, its molecular size of 20,000 daltons may be overestimated, because glycoproteins are known to be eluted on gel filtration earlier than expected from their molecular sizes.

An equimolar amount of vasopressin and neurophysin was liberated from the precursor after the treatment with trypsin. In concordance with this finding, vasopressin and neurophysin are thought to exist in the hypothalamus at a 1:1 M ratio (28). Hence, one would predict the molar ratio of vasopressin and its neurophysin in tumor tissue extracts to be one. However, this is not the case. Neurophysin was measured in excess of vasopressin in all of the tumor tissues studied. This could not be explained solely by the existence in extracts of a high molecular weight form of neurophysin that is reactive to neurophysin antiserum, but shows little vasopressin immunoactivity because this form of neurophysin accounted for <10% of the total neurophysin activity in each tumor extract. Similarly, the cross-reactivity of ESN in the NSN radioimmunoassay may not be a plausible explanation for this finding in view of the presence of a small quantity of ESN in tumor tissue extracts. Of relevance is the molar ratio of vasopressin to NSN in posterior pituitary extracts. Neurophysin was again measured in excess of vasopressin. Vasopressin has been shown to be labile in neutral condition and to be readily degraded through the action of proteolytic enzymes. This property of vasopressin may account for the apparent abundance of neurophysin in extracts of both tumor tissues and posterior pituitaries.

There is ample evidence that most, if not all, ectopic hormones, precursors, and degradation products differ from their normal glandular counterparts only in their proportions. A close resemblance in physicochemical properties of hormones and neurophysins in ectopic vasopressin-producing tumors and in human posterior pituitaries may support this thesis. In human hypothalamus, vasopressin and its neurophysin may be produced from the 20,000-dalton precursor(s) demonstrated in this study.

ACKNOWLEDGMENTS

We thank Dr. K. Kosaka for his support and Dr. A. Itabashi for his cooperation.

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