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Modified forms of vasopressin and oxytocin in a bovine pineal preparation

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A bovine pineal acid extract displays a vasotocin-like bioactivity in several bioassays, and is recognized by antibodies against the Pro-Arg-Gly-amide ending common to vasopressin and vasotocin. By using molecular sieve filtration and reversed-phase HPLC, a vasopressin- and oxytocin-like peptide was isolated from this pineal preparation, while no evidence for a vasotocin-like peptide was obtained. The isolated neuropeptides contain a modified amino acid at position 2. This structural difference with authentic pituitary vasopressin and oxytocin may alter their biological and immunological properties, which have been interpreted as vasotocin-like, and thus underlies the controversy concerning the existence of vasotocin in the mammalian pineal gland.

Peptide isolation; Neuropeptide; Oxytocin; Vasopressin; Post-translational modification; (Bovine pineal)

1. INTRODUCTION

The report of Milcu et al. [1] which described a peptide in bovine pineal glands with the biological and chromatographic properties of [8-arginine]-vasotocin (AVT) created the controversy on the existence of AVT as a mammalian pineal peptide. Over the last 20 years data from biological, immunological and chemical studies have been interpreted as evidence for or against the presence of this alleged peptide in the mammalian pineal organ (review [2]). The repeatedly reported detection of AVT has mainly been based on a vasotocin-like activity and immunoreactivity [1,3–5], while a number of reports have consistently disclaimed the presence of AVT in the mammalian pineal on the grounds of immunological or chromatographic properties [6–8]. Neacsu [9] prepared a peptidic fraction (termed E5) with antigonadotropic activity from bovine pineal glands. The author concluded that E5 consisted of 14 amino acids including those of AVT [9]. This bovine pineal preparation displayed AVT-like bioactivity in several bioassays [8,10], and reacted with antisera against Pro-Arg-Gly-amide endings, the carboxyl-terminal tripeptide common to AVT and [8-arginine]vasopressin (AVP) [11]. In contrast, Pévet et al. [8] failed to detect any AVT, AVP or oxytocin (OXT) in the pineal E5 extract by radioimmunoassays (RIA). In the scope of our work on the pineal gland we purified neuropeptides from the bovine E5 preparation. Here we report the isolation and characterization of pineal AVP- and OXT-like peptides, while no evidence for AVT was obtained. It is shown that AVP- and OXT-like peptides from the bovine pineal contain a modified amino acid. This structural difference with the authentic hypophyseal neuropeptides AVP-(1–9) and OXT-(1–9) may alter their biological and immunological properties, which may have been interpreted as AVT-like and thus underlies the controversy con-

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cerning the existence of AVT as a mammalian pineal peptide.

2. MATERIALS AND METHODS

The preparation of the bovine pineal acid extract (E5) is described elsewhere [9], and was obtained from Dr P. Pévet (NIH, Amsterdam). Purification of neurohypophyseal hormone-like structures and their dissociation from accompanying carrier proteins were performed by gel filtration in formic acid as in [12]. Details of the procedures and antigenic properties of the radioimmunoassays have been described [13–16]. Reversed-phase HPLC was carried out with a Perkin-Elmer model series 4LC system; the HPLC columns were described in [11,17]. The gel isoelectric focusing was performed on polyacrylamide gel slabs (10 × 10 × 0.1 cm) containing 2% Servalyte AG 2-11 (Serva, Heidelberg) [18]. The amino acid composition of the isolated neuropeptides was determined by performic acid oxidation and acid hydrolysis (6 M HCl containing 0.1% thioglycolic acid, 16 h at 110°C), followed by *o*-phthaldialdehyde (OPT) derivatization and separation of the OPT-amino acids by HPLC [19,20].

3. RESULTS

Fig.1A shows representative dilution curves of AVP determination in the bovine pineal E5 preparation, using the AVP antiserum raised against the COOH-terminal tripeptide ending common to AVP-(1–9) and AVT-(1–9) [13]. Using radioimmunoassays for the 20-membered ring structure of AVP [14] and AVT [15], only amounts of immunoreactive AVP-like material could be measured in the extract (fig.1B). Antigenic similarities of pineal OXT-like constituents with standard OXT-(1–9) could also be measured in the RIA for OXT [14].

A portion corresponding to 20.0 mg protein of the bovine pineal E5 preparation was fractionated by gel filtration on a Sephadex G-100 column eluted in 0.1 M formic acid containing 0.1% (w/v) bovine serum albumin (fig.2A), followed by Sephadex G-50 superfine filtration in 1% formic acid of the peptide pool with an AVP and OXT immunoreactivity (fig.2B). This material was retained on Sep-pak and could be eluted with 80% methanol. By this procedure, the immunoreactive material was considerably purified and concentrated. Final purification was achieved by subse-

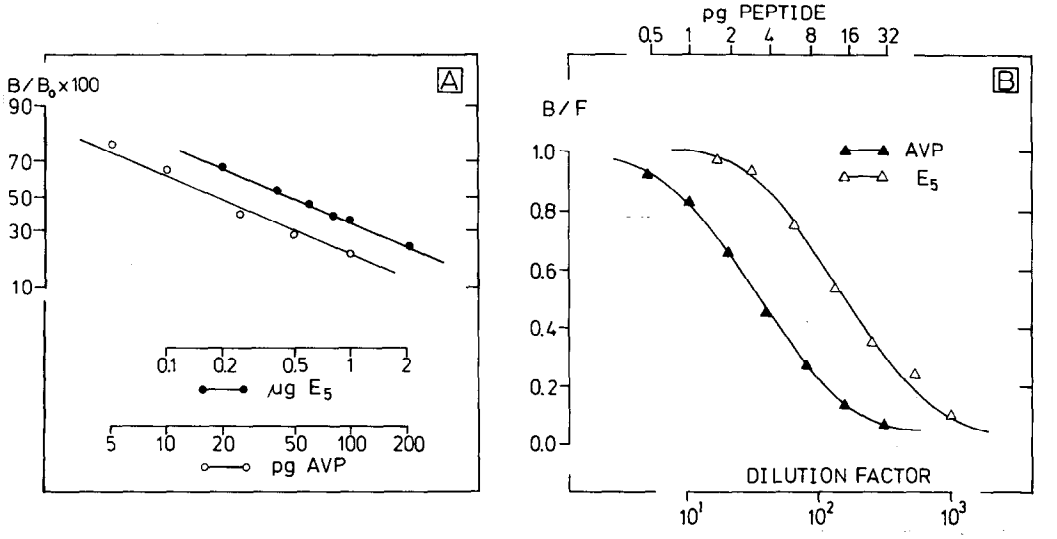

Fig.1. Standard curve and cross-reactivity curves of the bovine pineal E5 extract for the vasopressin RIAs (see text). (A) Log-logit plot of dilution and standard curves using the AVP antibody raised against the Pro-Arg-Gly-amide ending [13]. (B) Inhibition of binding to ^{125}I-AVP to the AVP antiserum for the ring structure of the neuropeptide [14] by pineal compounds. Each point of the curves represents the mean of three determinations. *B*, % binding observed; B_0, % initial binding; *F*, % non-binding.

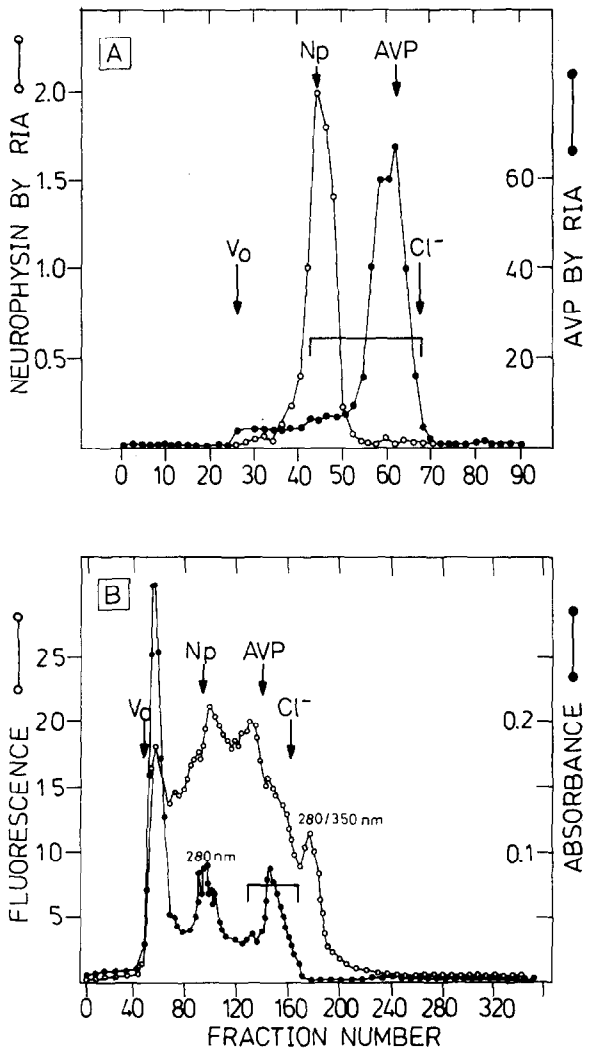

Fig.2. Fractionation of the bovine pineal E5 fraction by molecular sieve filtration. (A) 20.0 mg bovine pineal E5 dissolved in 2.0 ml of 0.1 M formic acid was applied on Sephadex G-100 (1.6 × 58 cm) run in 0.1 M formic acid containing 0.1% (w/v) BSA at a flow rate of 80 ml/h; fraction size, 2.0 ml. The presence of neurophysin and peptides with a COOH-terminal Pro-Arg-Gly(NH_2) sequence was assayed by radioimmunoassay [13,16]. Arrows indicate elution positions of standard AVP-(1–9) and bovine pituitary neurophysin (NpII). (B) Rechromatography of the peptide pool with AVP immunoreactivity (fractions 42–68; A) on Sephadex G-50 superfine (1.6 × 98 cm) eluted with 1% (v/v) formic acid at 1.0 ml/h; fraction size, 1.0 ml. The neurohormone-like activity assayed by RIA in the indicated area (fractions 129–166) was collected for subsequent purification on HPLC.

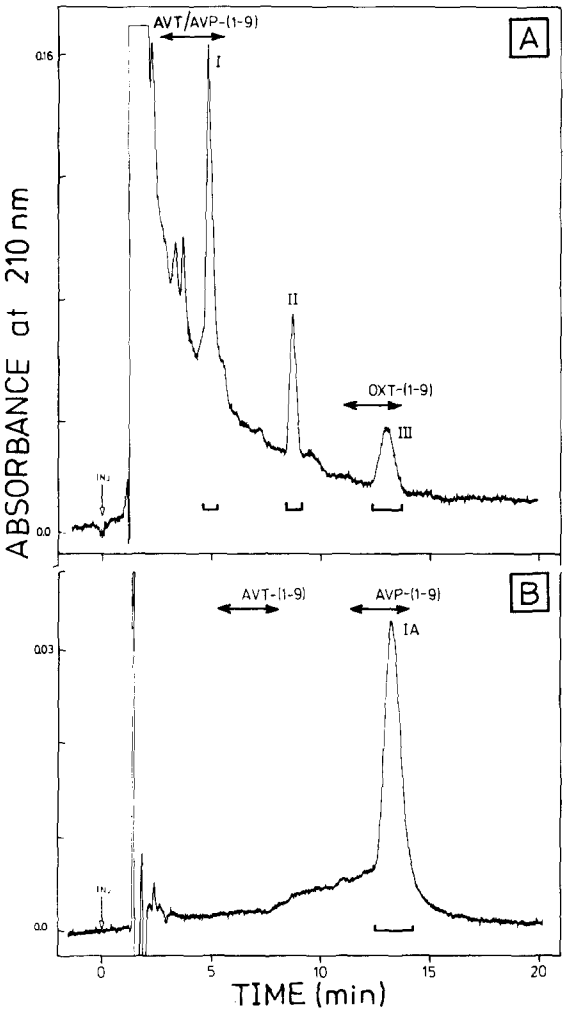

Fig.3. Final purification of bovine pineal neuropeptides by reversed-phase HPLC. The material obtained from the immunoreactive AVP- and OXT-like pool (fractions 129–166, fig.2B) was passed through a Sep-pak C18 cartridge eluted with methanol. The dried methanol effluent was dissolved in 10 mM ammonium acetate (pH 4.15) and applied through multiple injections to a μBondapak C18 column (0.39 × 30 cm). (A) Fractionation of 0.9 nmol AVP- and 1.1 nmol OXT-like material by isocratic elution with 0.15% (v/v) acetic acid in methanol/10 mM ammonium acetate (pH 4.15) (36:64) at a flow rate of 2.0 ml/min. Component III was used for structural analysis. (B) Rechromatography of component I (A) by isocratical elution with 0.15% (v/v) acetic acid in methanol/10 mM ammonium acetate (pH 4.15) (1:3) at a flow rate of 2.0 ml/min. Component IA was subsequently submitted to amino acid analysis. Arrows indicate elution positions of synthetic peptides under the same conditions as for (A) and (B) on a separate μBondapak column and served as a reference chromatogram.

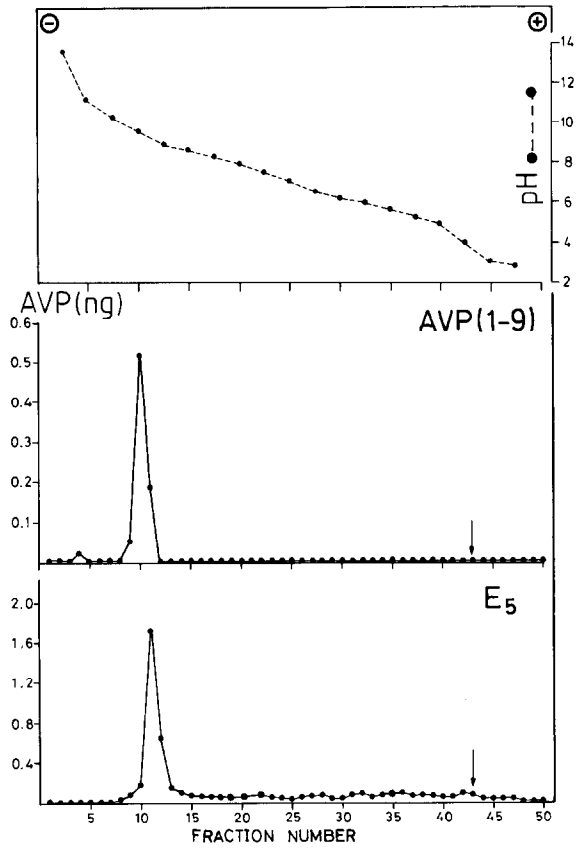

Fig.4. Gel isoelectric focusing in a 5% polyacrylamide gel slab containing 2% carrier ampholytes in the pH range 2–12 [18]. (Top) Synthetic AVP (1.0 ng immunoreactive AVP applied, recovery 86%). (Bottom) Bovine pineal component IA (fig.3B) (7.0 ng AVP-like material by RIA, recovery 86%), with subsequent RIA for AVP (ng/2 mm slice). Arrow indicates sample application spot.

quent reversed-phase HPLC steps on a μBondapak C18 column [17], and yielded approx. 4.5 nmol AVP-like peptide and 5.6 nmol OXT-like peptide by radioimmunoassay [13–15]. These natural components, detectable in the HPLC profile by UV absorbance at 210 nm (fig.3), had comparable retention times to synthetic AVP-(1–9) and OXT-(1–9) in three solvent systems, and showed that the material was pure. An AVT-like peptide remained undetectable above background levels by RIA [15] during all chromatographic purification steps. Using parallel polyacrylamide gel slab isoelectric focusing patterns of immunoreactive AVP-like material after HPLC (component IA, fig.3B) and synthetic AVP-(1–9) yielded nearly coincidental isoelectric points at pH 9.2–9.4 (fig.4).

The isolated peptides were further analyzed for amino acid composition after performic acid oxidation and acid hydrolysis by pre-column derivatization with OPT and reversed-phase HPLC [19,20]. The analysis showed that the isolated pineal AVP- and OXT-like peptides contained all the constituent amino acid residues in the molar ratios expected for authentic AVP-(1–9) and OXT-(1–9), respectively, except for tyrosine (fig.5). However, another OPT derivative was detected in the HPLC profile, which was not observed when synthetic AVP-(1–9) and OXT-(1–9) were oxidized and hydrolyzed in the same way, and did not correspond to any of the other common amino acids of peptide and protein hydrolysates in AVP-(1–9) and OXT-(1–9) (fig.5). The unknown OPT derivative, when calculated as a tyrosine residue, occurred in a molar ratio as expected for tyrosine in AVP-(1–9) and OXT-(1–9) (fig.5). In order to obtain information about the structure of the unknown residue in the pineal neuropeptides, hydrolysates of several synthetic peptides with derivatized tyrosine residues and amino acids with structures related to tyrosine were analyzed by the OPT derivatization-HPLC method [20]. After oxidation and hydrolysis of cholecystokinin octapeptide, which is sulfated at the hydroxyl group of the tyrosine residue, and of the AVP antagonist [d$(CH_2)_5$,2-(*O*-methyl)-Tyr^2]AVP [21], underivatized tyrosine was detected by amino acid analysis. Oxidation and hydrolysis of α-methyl-*p*-tyrosine and 3-methoxy-tyrosine failed to generate an OPT derivative with similar retention time to the unknown residue. DOPA (3-hydroxytyrosine), which is a natural intermediate of dopamine biosynthesis from tyrosine, eluted well before tyrosine on HPLC.

In order to evaluate a possible artifactual derivatization of the tyrosine residue in pineal AVP and OXT during tissue extraction [[^{3}H]Tyr^2]OXT (1 μCi; spec. act. 20 Ci/mmol) was added to 15 mg wet wt pineal tissue, and extracted with water or 1 M acetic acid and left at room temperature for 30 min. The extract was lyophilized and fractionated by reversed-phase HPLC using a methanol/ammonium acetate (10 mM, pH 4.15) system [19]. The [[^{3}H]Tyr^2]-

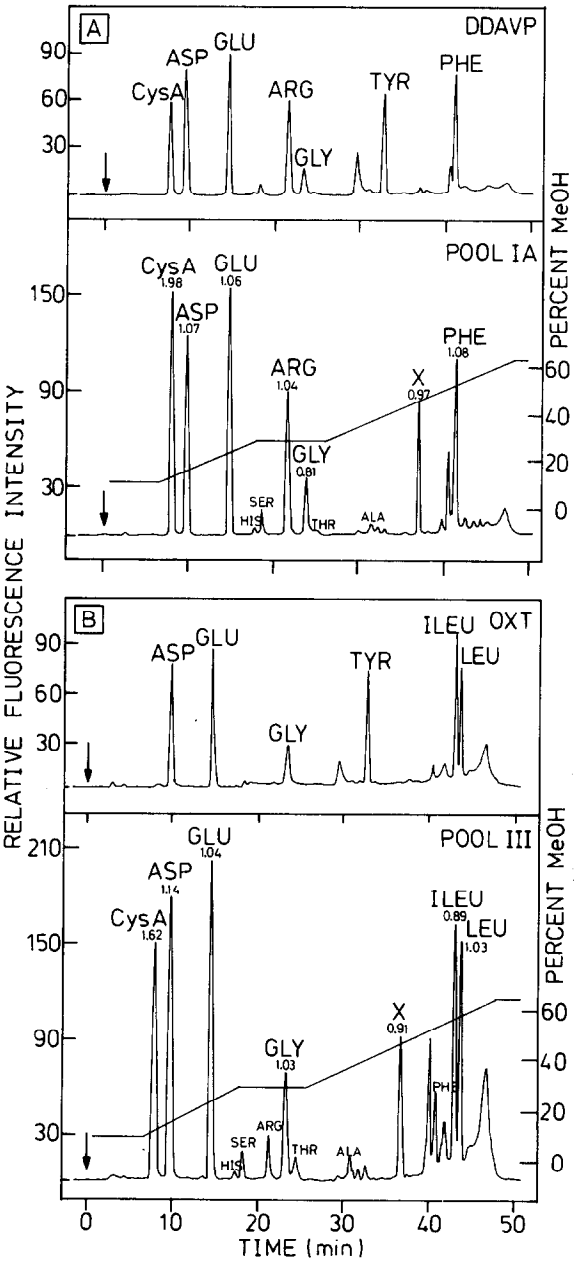

Fig.5. Elution profiles of OPT derivatives of amino acids on a CP-Spher C18 column (0.46 × 25.0 cm) by HPLC. (A) Upper: amino acid analysis of 500 pmol synthetic 1-desamino-D-arginine[8]-AVP (DDAVP); lower: separation of OPT derivatives of residues from pineal AVP-like material (peak IA, fig.3B). (B) Upper: analysis of 500 pmol synthetic OXT-(1–9); lower: fractionation of the pineal OXT-like peptide (peak III, fig.3A). Elution was carried out with linear gradients between 0.1 M sodium citrate/HCl (pH 5.9) containing 2% THF and methanol as described [19] at a flow rate of 1.0 ml/min. Amino acids are indicated by conventional abbreviations except cystic acid (Cys A). Component X represents the unknown OPT-amino acid derivative of bovine pineal AVP- and OXT-like peptides.

OXT-containing fractions of each extraction method were subjected to amino acid analysis and elution of ^{3}H-labeled residues was monitored. In the amino acid analysis system a single radioactive peak co-eluted with the tyrosine standard. Elution of labeled components at the position of the unknown residue in pineal neuropeptides did not occur. Thus, it is unlikely that the unknown amino acid residue in the isolated bovine pineal AVP- and OXT-like peptides has been generated by exposure to the pineal homogenate during extraction procedures.

4. DISCUSSION

From these experiments, it is concluded that the bovine pineal E5 preparation contains modified forms of AVP-(1–9) and OXT-(1–9), while AVT is an undetectable constituent. The modification possibly is a derivatized tyrosine residue located in residue 2 of these peptides. The data indicate that the unknown residue is different from a tyrosine residue alkylated or sulfated at the hydroxyl group of the Tyr side chain, methylated at the α-position, or hydroxylated at position 3 of the ring structure, but the identity of the residue remains unknown. Definite identification of the modified residue must await the isolation of larger quantities of the AVP- and OXT-like peptides from bovine pineal glands to allow mass-spectrometric analysis. Such quantities are at present not available. These modified forms of AVP and OXT may have altered biological and immunoreactive properties. For instance, changes in the tyrosine residue of AVP-(1–9) and OXT-(1–9) can alter the potency of the peptides in bioassays and generate antagonistic properties [22]. These altered properties may have led to misinterpretations in biological and immunological studies. It cannot be excluded as yet that these modified forms of AVP-(1–9) and OXT-(1–9) have been responsible for the AVT-like bioactivity of mammalian pineal gland extracts [1,8,10]. In addition, the structural modification may cause loss of immunoreactivity to specific antibodies against the N-terminal positions of these peptides, but does not affect immunoreactivities with C-terminal directed antibodies [11,12]. Thus, it can explain the contradictory observations of the presence of AVT in the pineal gland [1,3–5], and the findings that bioactive material in pineal extracts behaved differently from synthetic AVP and OXT in the isolation procedures [1,3]. The derivatization of AVP and OXT in the pineal gland may represent a post-translational modification occurring in the AVP- and OXT-containing fibers of the mammalian pineal organ [23], and suggests that an enzymatic system exists which can change the chemical nature of the tyrosine residue. This enzymatic system must be different from tyrosine hydroxylase which converts tyrosine into DOPA. Derivatized forms of AVP and OXT may also occur in other AVP- and OXT-containing areas of the brain. These peptides may have novel biological activities in the brain, different from the thus far recognized central activities of AVP-(1–9) and OXT-(1–9).

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