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Identification of Oxytocin and Vasopressin from Neurohypophyseal Cell Culture†

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Our observation that dispersed cultures of neurohypophysis obtained from adult rats are capable of synthesizing and releasing oxytocin and vasopressin is unexpected, because in whole animals these hormones are known only to be stored, not to be produced in the posterior lobe of the pituitary. The hormone content of cell culture medium was elevated from 0 to 129 ± 14 pg/mg protein for oxytocin and from 0 to 42 ± 4 pg/mg protein for vasopressin during two weeks as determined by specific radioimmunoassay. By molecular mass and structure determination (tandem mass spectrometry) we have proved that the supernatant of the cell cultures contains not only immunologically but mass spectrometrically identified neurohypophyseal hormones. © **1998 John Wiley & Sons, Ltd.**

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Oxytocin (OT) (H-CYIQNCPLG-NH_2) and vasopressin (VP) (H-CYFQNCPRG-NH_2) are the only peptide hormones known to be secreted in significant amounts by mammalian neurohypophysis (NH). The OT is involved in regulating milk ejection and uterine contraction during birth, while VP controls water resorption of the distal tubules of the kidney, and potentiates ACTH release from the anterior pituitary stimulated by corticotropin releasing factor.[1] It serves as a neuropeptide in the central nervous system.[2] It is well documented that OT and VP are produced in the ribosomes of the cell body of supraoptic and paraventricular nuclei of the hypothalamus, packaged into neurosecretory granules, transported down the axons by axoplasmic flow to the ending in the posterior pituitary (NH), and stored in terminal dilations until their release.[3–5] Like many other hypothalamic releasing and inhibiting peptide hormones, OT and VP are synthesized as parts of large precursor molecules which, besides the hormones, contain the functionally linked carrier proteins, called neurophysin. Cleavage of the precursors occurs as they are being transported, and the storage granules in the ending contain free OT, VP and the corresponding neurophysin.

There is, however, accumulating evidence that cells outside of the hypothalamus, both in the brain and in peripheral organs, may also be physiologically important sources of the neurohypophyseal peptides.[6] There has been a number of reports that OT mRNA and VP mRNA can be detected in both the anterior and posterior pituitary glands of several species,[6–13] and it has been suggested that OT and VP could be synthesized locally.[14–16] However, data from different authors are rather controversial, and the presence of an authentic RNA is not indicative of peptide synthesis and secretion. The neural lobe of the pituitary is composed of termination of axons, of capillary vessels, and a neuroglia-like cell type called pituicytes.[17] Axons appear devoid of organelles and molecules involved in protein synthesis, and therefore they cannot be the site of neuropeptide production.

Mass spectrometry (MS) coupled with high performance separation methods offers unique opportunities for analysis of peptide mixtures.[18–20] Moreover, tandem MS can provide amino acid sequence information for predicted as well as novel peptides.

In the present study, we try to establish whether or not pituicytes, these totipotent cells mentioned above are capable of synthesizing OT and VP. We have prepared a monolayer cell culture from rat neurohypophysis, and used radioimmunoassay, LC/MS and MS/MS to characterize the immunological and chemical nature of the released peptides.

EXPERIMENTAL

Cell culture

The hypophysis of male Wistar rats weighing 180–230 g was removed under sterile conditions immediately after decapitation. The posterior lobe was carefully separated from the intermediate lobe under a preparative microscope. The tissue was digested enzymatically by 0.2% trypsin (Sigma, Germany) in phosphate buffered saline (PBS) for 60 min and 0.05% collagenase (Sigma) for an additional 60 min at 37 °C. The enzymatic hydrolysis was stopped by addition of 100 μg/mL trypsin inhibitor (Sigma). Mechanical disintegration of the tissue was performed on nylon blutex sieves, pore size of 100 μm, 80 μm and 48 μm in series. The dispersed cells were placed into 24 well plastic

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plates (Costar, US) coated with 5% rat tail collagene (Sigma). The starting cell density was 10^5 cells/mL of medium (Dulbecco's Modified Eagle Medium (DMEM) (Sigma) supplemented by 20% Foetal Calf Serum (Gibco, US), 100 μg/mL penicillin and 100 μg/mL streptomycin). The cell cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air. The culture medium was changed daily.

Radioimmunoassay

Hormone content of the cell culture supernatant was determined after 2 hours of changing medium. OT and VP were extracted from supernatant with LiChrosorb silica-gel powder (Merck, Germany) and were measured by specific radioimmunoassays, using methods previously reported from our laboratory.[21,22] The sensitivity of the assay for OT was 2 pg/tube, with less than 0.1% cross-reactivity with VP and 0.1% with vasotocin. The VP assay was also highly specific (cross-reactivity: 0.01% with OT and 0.1% with vasotocin) and sensitive (1 pg/tube).

Capillary chromatography

The capillary HPLC column was prepared employing 320 μm i.d. fused-silica capillary tubing obtained from Polymicro Technologues (Phoenix, AZ, USA). The column was packed with Nucleosil 5C18 100 Å reversed phase packing material, to a length of 15 cm. 10 μL samples were injected and washed for 15 min with solvent A. Micro-HPLC was performed using an Applied Biosystems (Foster City, CA, USA) 140C dual syringe pump. The flow rate from the pump was 150 μL/min. The solvent stream was split to produce final a flow rate of 1–2 μL/min. The mobile phase used for gradient elution consisted of (A) 0.2% acetic acid and (B) acetonitrile/water (80:20 v/v) containing 0.2% acetic acid. The gradient was linear from 5–95% B in 20 min.

ESI mass spectrometry

Mass spectra were recorded using a TSQ 7000 (Finnigan MAT, San Jose, CA, USA) equipped with a home-made micro-ESI ionization source.[23] Electrospray was performed by setting the high voltage to 2 kV. No sheath gas or sheath liquid was applied. The capillary temperature was held at 250 °C.

Automated tandem mass spectrometry

Tandem mass spectra were acquired using a modified Finnigan ICL script which monitors signal peaks and switches to product ion scan mode if the *m/z* value in the spectrum is found in the predefined list. The collision potential (V) was set to 1/35 of the *m/z* value of the parent ion. Argon was used as collision gas (2.5 mTorr).

RESULTS

Cell culture

The growth of cell cultures could be detected a few days after beginning culturing of the neurohypophyseal cells. The appearance of clumps was observed on days 2–4. A mass of encapsulated forms was seen on days 6–8. At about day 12–14, the culture became homogeneous, and polygonal, peripherally very dense cells with large nuclei were apparent. The electromicroscopic picture revealed the various sizes and intensities of secretory granules in the protoplasm around the large nuclei.

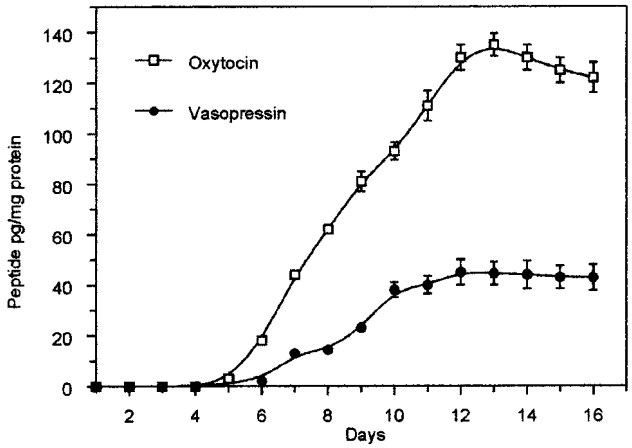

Figure 1. Two-hour hormone release in cell culture medium, as a function of the age of the culture. Oxytocin (□), vasopressin (●). (n = 10, mean ± S.E.M.)

Immunological characterization of released peptides

The time course of hormone production of the cell cultures was followed by determination of OT and VP content of the supernatant by specific radioimmunoassay (Fig. 1). During the preparation of the cell culture the residual hormone content of the posterior lobe was washed out with a high volume of medium, so that there was no detectable amount of any neurohypophyseal peptide in the first few days. On the fifth day immunoreactive OT and VP appeared in the medium, indicating their synthesis and release from pituicytes. The hormone content of the supernatant then increased and became constant for 12–14 days. Immuno-reactive (ir) OT has been detected at least 3 times higher in concentration (129 ± 14 pg/mg protein) than that of ir-VP (42 ± 4 pg/mg protein).

Identification of OT and VP by LC/MS

The chromatographic behaviour of neuropeptides extracted from the medium of neurohypophyseal cell culture with an age of 14 days, was investigated by reversed phase capillary chromatography connected to ESI-MS as detector (Fig. 2). A single major peak due to an ion at *m/z* 542.6 was obtained, which corresponds to the doubly charged molecular ion of 8-Arg-vasopressin (M_r = 1084.5), and it eluted with the same retention time as standard arginine vasopressin (12.44 min). The peak at 15.06 min in the mass chromatogram for the singly charged molecular ion of OT (*m/z* 1007.6) coincides with that of the peak of synthetic OT in a separate chromatogram. The hydrophobicity of OT is higher than that of VP, resulting in longer retention of OT on a reversed phase column.

Determination of molecular structure of OT and VP

Acquisition of tandem mass spectra during chromatographic analysis of cell culture extract resulted in identifying OT and VP (Fig. 3). Daughter ion spectra of the $[M + H]^+$ ion of OT show a series of N-terminal B ions, and the Y_3 ion;

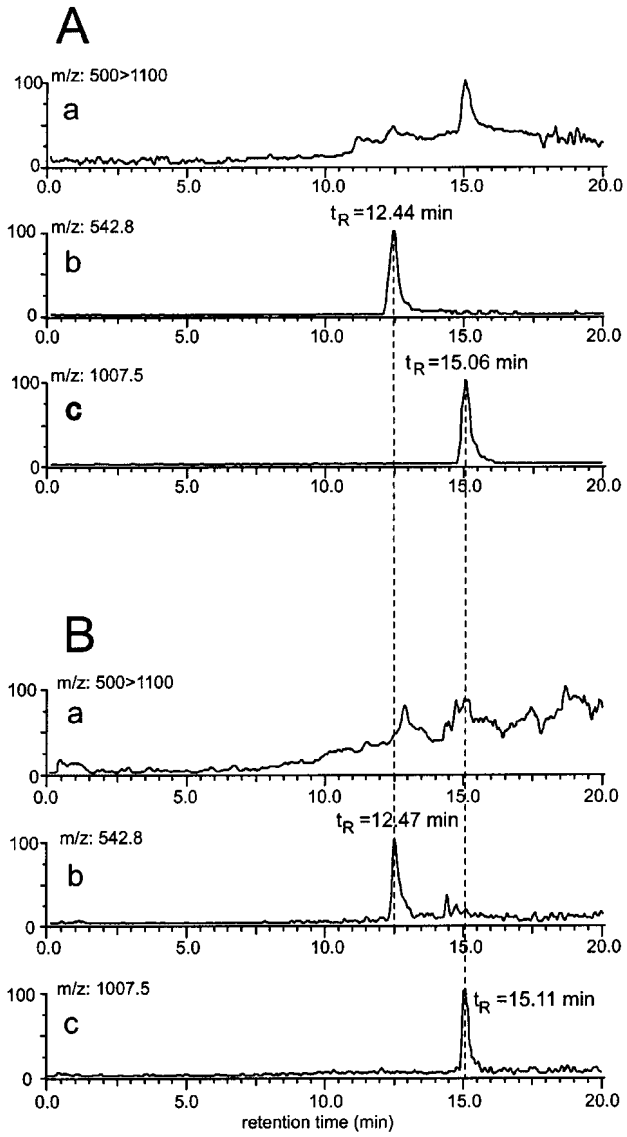

Figure 2. LC/MS analysis of a mixture of (a) synthetic oxytocin and vasopressin, and (b) of supernatant extract of neurohypophyseal cell culture. a, mass chromatogram of ions *m/z:* 500–1100, b, mass chromatogram of $[M + 2H]^{2+}$ ion (*m/z:* 542.8) of VP, c, mass chromatogram of $[M + H]^{+}$ ion (*(m/z:* 1007.5) of OT.

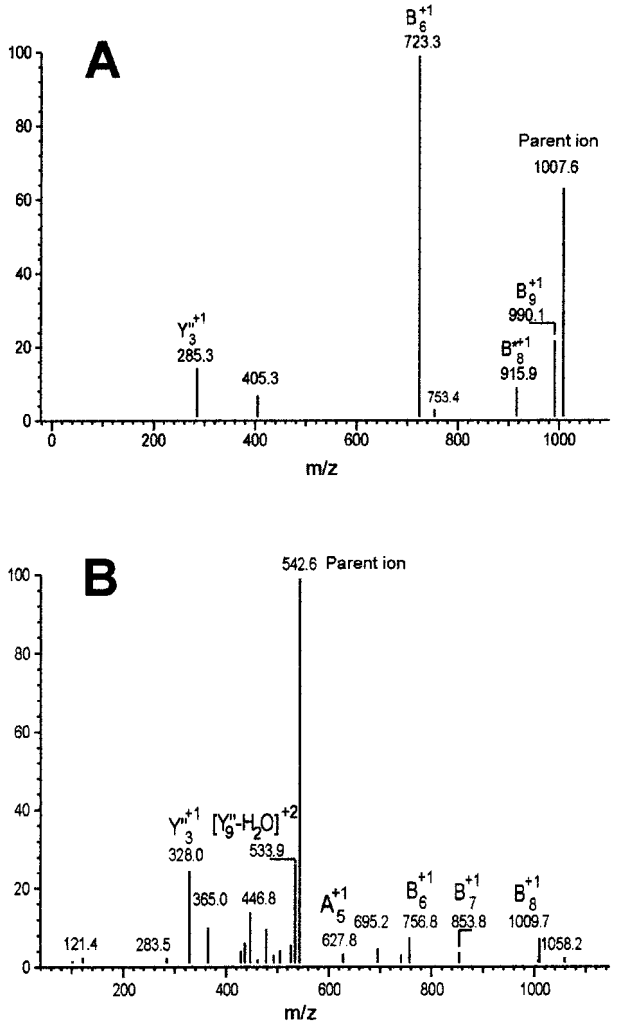

Figure 3. MS/MS analysis of OT and VP extracted from cell culture. (a) CID spectrum from 5 scans of $[M + H]^{+}$ ion of OT at *m/z:* 1007.5, (b) CID spectrum from 4 scans of $[M + 2H]2^{+}$ ion of VP at *m/z:* 542.8.

the abundant B_6 ion probably corresponds to the hexapeptide ring fragment. The tandem mass spectrum of the doubly charged molecular ion of VP shows again a series of N-terminal B ions, the A_5 and A_6 ions, and the C-terminal Y_3 ion.

DISCUSSION

Our observation that dispersed cell cultures of neurohypophysis obtained from adult rats are capable of synthesizing and releasing OT and VP is unexpected because in whole animals these hormones are known to be only stored and not to be produced in the posterior lobe of hypophysis. Until the 1970s there was considerable dispute as to whether or not these substances could be formed *de novo* in the posterior lobe. This was accepted to be unlikely since disconnection of the hypothalamo–hypophyseal tract resulted in loss of neurosecretory material, irrespective of whether the posterior lobe was left *in situ*, transplanted to an alternative site, or placed in tissue culture.[24,25]

VP and OT genes are abundantly expressed in magnocellular neurones of the supraoptic and paraventricular nuclei of hypothalamus.[26] The neurohypophyseal hormones are the products of independent genes.[27] Each gene comprises three exons separated by two intervening sequences. Each exon encodes one of the principal functional domains of the prohormone: hormone, carrier neurophysin and glycopeptide. In the case of the OT gene, the third exon comprises only the C-terminal part of the neurophysin plus a His. Rehbein *et al.*[27] reported that OT and VP genes transcribed and spliced in hypothalamic nuclei, and confirm that these cells are the real site of gene activity and peptide synthesis.

Using techniques dependent on the detection of messenger RNAs (mRNA), the expression of the OT and VP genes in extrahypothalamic cells has been unequivocally described.[6] Recent studies have shown the presence of OT and VP mRNAs in the pituitary gland of different species, but findings are divergent.[8–10,13–16,28,29] Terrier *et al.*[9] present *in situ* hybridization pictures that demonstrate VP mRNA in the anterior lobe, but no hybridization to the posterior lobe. This contrasts with the data of Murphy *et al.*,[16] who were unable to demonstrate OT and VP RNAs in the anterior lobe, but rather could identify them in the posterior lobe. Several confirming reports on the presence of these mRNAs

in the latter site[8,10,13,15,16] raise the question of the origin of OT and VP mRNAs, whether are they axonally transported from the magnocellular neuron cell bodies to the terminals or synthesized locally in the pituicytes of the posterior lobe. There is evidence supporting the axonal transport hypothesis, that is, under normal physiological conditions in intact animals most of these mRNAs are axonally transported down to the NL.[8,30] VP mRNA appears to be aggregated in a subset of distal axonal swellings and/or terminals of the posterior pituitary, and its axonal distribution does not precisely overlap that of vasopressin peptide. Furthermore, mRNA from rat hypothalamus and posterior pituitary are identical in their nucleotide sequence, the only difference being that the OT and VP mRNAs from the posterior lobe contain a shorter poly(A) tail than those found in the hypothalamus.[8,31,32]

However, there are also data supporting the alternative hypothesis, i.e. that the pituicytes of the posterior pituitary could be the site of OT and VP gene expression.[8,15,16,33] Pu *et al.*[15] demonstrated 10 fold increases of poly(A) tail truncated VP mRNA in rat posterior pituitary, 7 days after salt loading, and localized the mRNA in a subset of pituicytes. They have found not only the proliferation of pituicytes in the neural lobe after salt loading of stalk-transected animals, but an increased amount of VP mRNA in this site. There is other evidence that the pituicytes are sensitive to osmotic changes.[16,34] It has been supposed that the activation of VP gene expression in pituicytes may be due to osmosensitivity of these cells,[35] or the stimulus may come from blood-born factors secreted by other nerve cells in response to salt loading.[15] Our result, that in cell culture where the pituicytes have no connection to the hypothalamus, they can synthesize both OT and VP, may support the role of a factor in the activation of gene expression. The foetal calf serum in the cell culture medium contains many growth factors, and probably one of them is responsible for the liberation of the repressed function of these genes.

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