Characterisation of an intermediate in neurophysin biosynthesis in the guinea pig

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In addition to oxytocin (OT), vasopressin (AVP) and their respective neurophysins (NPs), another [^{35}S]cysteine incorporating component is present in the guinea pig neurohypophysis. Gel filtration and Con A affinity chromatography revealed that this component was larger than NP and was glycosylated. NP-immunoreactivity was assessed using antisera which distinguish the OT- and AVP-related NPs. Whilst the anti-NP antiserum detected only one component (guinea pig NP), the anti-NP antiserum detected both NP and the glycosylated ^{35}S-labelled component. These results suggest that a significant amount of NP in guinea pig neural lobes bears a glycopeptide extension and represents a partially processed form of the AVP precursor in this species.

AVP *Precursor* *Neurophysin* *Glycopeptide* *Biosynthesis* *Intermediate*

1. INTRODUCTION

The neurohypophysial peptide hormones, oxytocin (OT) and vasopressin (AVP) are biosynthesised as part of larger precursors [1–3] together with their carrier proteins, the neurophysins (NPs). After biosynthesis in the magnocellular perikarya of the hypothalamic supra-optic and paraventricular nuclei, the precursors are packaged into neurosecretory granules and transported to the nerve terminals in the posterior pituitary glands. During transport, the precursors are processed by proteolytic cleavage and amidation [4,5] producing the mature neurosecretory products, stored in the neural lobe.

Hypothalamic injection of [^{35}S]cysteine has been used to establish several steps in the biosynthesis of these peptides in the dog and rat [6,7]. Radiolabel is incorporated into the cysteine-rich neurosecretory products which can be analysed after isolation from different parts of the hypothalamoneurohypophysial system [8]. Using these techniques in the guinea pig, we have recently demonstrated, in addition to the expected neurosecretory products, significant amounts of an additional biosynthetic component in the neural lobe [9], which we proposed was a partially processed form of the AVP-precursor. In this paper, we have now further characterised this component, and suggest that it contains the NP sequence with an additional glycopeptide attached at its carboxyl terminus [10,11].

2. MATERIALS AND METHODS

Under halothane/N_2O anaesthesia, guinea pigs (Hartley, 500–700 g) were given bilateral injections of [^{35}S]cysteine (1000 Ci/mmol, Amersham International) into the paraventricular nuclei (40 μCi, 2×2 μl) as in [9]. After 24 h the neural lobes were removed, homogenised in 100 mM HCl (200 μl/lobe) and centrifuged ($8000 \times g$, 2 min). Gel filtration was performed on a column (0.9×90 cm) of Sephadex G-75 (Pharmacia GB) equilibrated

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and eluted with 100 mM HCl containing 0.2% bovine serum albumin (BSA, fraction V, Sigma London), by upward flow at 6 ml/h. The ^{35}S radioactivity of the fractions was estimated by liquid scintillation counting. Reverse-phase high pressure liquid chromatography (HPLC) was performed on a Spectraphysics 8700 system using a column (70×4.6 mm) of Nucleosil 5 μC8, eluted with a gradient of acetonitrile (CH_3CN) in 0.1% trifluoroacetic acid (TFA) as in [9]. Buffer A, 0.1% TFA; Buffer B, 60% CH_3CN in buffer A.

Affinity chromatography on concanavalin A columns (2 ml, Con A-Sepharose 4B, Pharmacia) was carried out using a column buffer of the following composition (mM): $CaCl_2$, 1; $MnCl_2$, 1; $MgCl_2$, 1; Tris, 50; NaCl, 1000; Triton X-100, 0.1% (pH 7.4). Con A-bound material was eluted by the addition of 0.5 M α-D-methyl mannoside (Sigma, UK) to the column buffer.

NP-like immunoreactivity was tested using antisera against porcine NPs (unpublished) which are able to distinguish OT-related NP (NP_{OT}), from AVP-related NP (NP_{AVP}) and which cross-react with the equivalent NPs in the guinea pig. The assay procedure was as in [12]. Anti-NP_{AVP} serum was used at a dilution of 1:10000, anti-NP_{OT} at 1:60000. Porcine NP_{OT} and NP_{AVP} were used as tracers after iodination [13] and immunoreactivity in the samples was expressed in terms of their corresponding porcine NP standards.

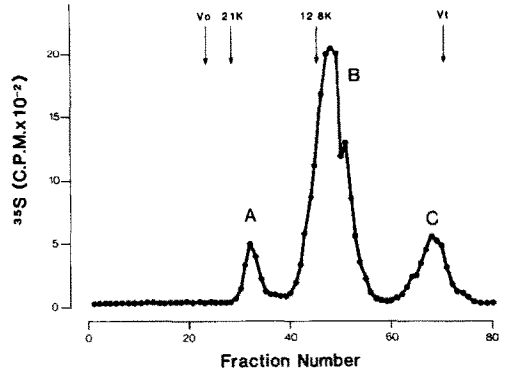

Fig.1. Gel filtration of [^{35}S]cysteine-labelled neural lobe extract. An acid extract of one neural lobe, removed 24 h after hypothalamic injection of [^{35}S]cysteine was chromatographed on a Sephadex G-75 column (90×0.9 cm). The column was eluted by upward flow at a rate of 6 ml/h. Fractions (1 ml) were collected and aliquots (250 μl) were measured for [^{35}S]cysteine radioactivity. The remaining portions of the fractions corresponding to the major radiolabelled components (peaks A–C) were pooled and lyophilized. The column was calibrated using thyroglobulin (V_o), soya bean trypsin inhibitor (21 kDa), cytochrome *c* (12.8 kDa) and [^{35}S]cysteine (V_t) as M_r markers.

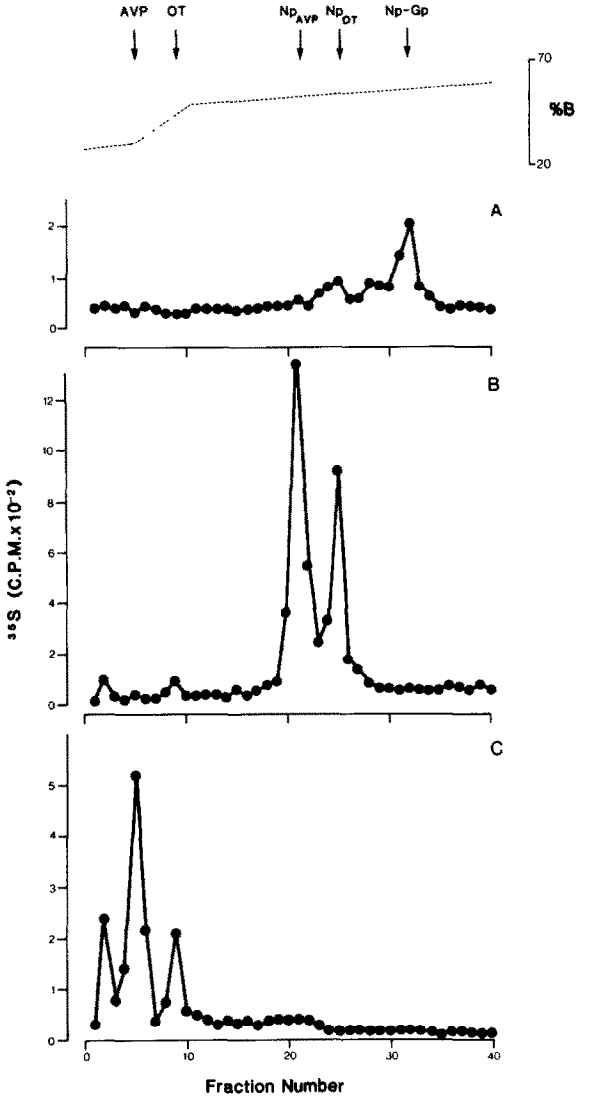

Fig.2. HPLC identification of radiolabelled components separated by gel filtration. Pooled, radiolabelled peaks A–C from fig. 1 were redissolved in water and subjected to HPLC. Fractions (1 ml) were collected and counted for ^{35}S radioactivity. Upper panel, HPLC of peak A, fig.1; middle panel, HPLC of peak B, fig.1; lower panel HPLC of peak C, fig.1. Arrows show the retention times of OT, AVP, NP_{AVP}, NP_{OT} and NP-GP. Dotted lines show elution gradient.

3. RESULTS

Sephadex G-75 chromatography was performed on acid extracts of neural lobes from animals injected 24 h previously with [^{35}S]cysteine (fig.1). Three major radioactive peaks emerged (A–C) with approximate M_r values of 18, 10 and <2 kDa. Fractions corresponding to these peaks were pooled, lyophilized, redissolved in water and subjected to reverse phase HPLC to identify the radiolabelled components (fig.2). The 18 kDa material eluted late in the gradient (fig.2A) in the position of the proposed neurophysin-glycopeptide intermediate NP-GP [9]. The 10 kDa material was further resolved (fig.2B) into NP_{AVP} and NP_{OT} whilst the low-M_r [^{35}S]cysteine-incorporating material contained AVP and OT (fig.2C). HPLC-purified NPs (fig.2B) and the NP-GP intermediate (fig.2A) were further chromatographed on Con A-Sepharose, and the results are shown in fig.3. As expected, ^{35}S-labelled NPs were not retained on the column, whereas the material from peak A did bind to Con A and was eluted with α-methyl mannoside. A neural lobe extract was then subjected to HPLC and fractions tested for NP-immunoreactivity (fig.4). The anti-NP_{OT} antisera detected only one component (NP_{OT}) whereas anti-NP_{AVP} immunoreactivity was found in the positions of both NP_{AVP} and the NP-GP component.

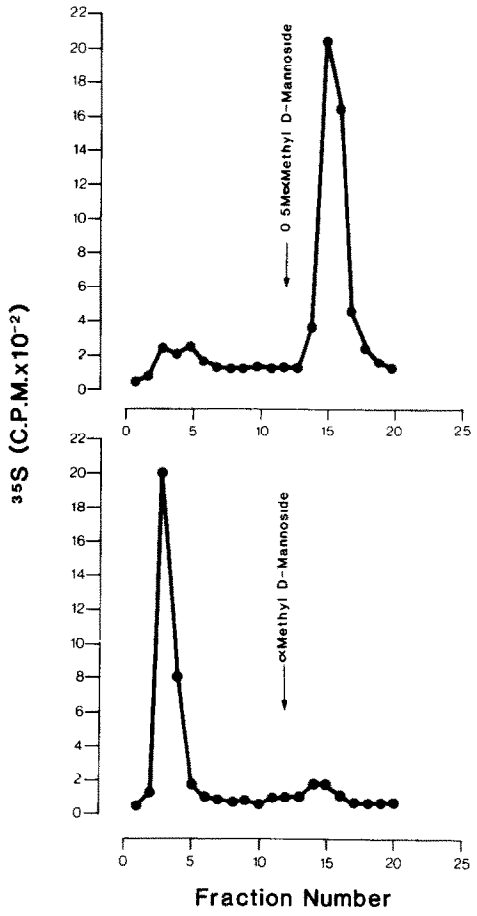

Fig.3. Con A-Sepharose affinity chromatography of NPs and NP-GP. ^{35}S-labelled material from HPLC (fig.2) was subjected to Con A-Sepharose affinity chromatography. Fractions (1 ml) were collected and counted for ^{35}S-radioactivity. Upper panel, Con A chromatography of ^{36}S-NPs. NP was not retained on the column. Lower panel, Con A chromatography of ^{35}S-NP-GP. Most of the ^{35}S counts in this fraction were retained on the column, and were eluted by α-D-mannoside (arrow).

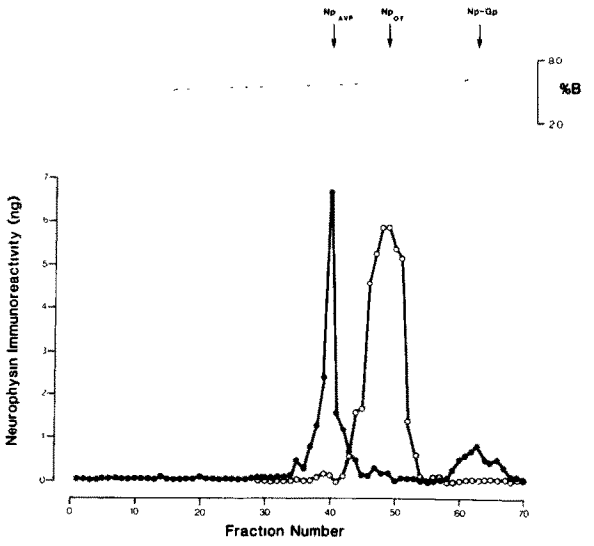

Fig.4. Neurophysin-related immunoreactivity in guinea pig neural lobes. An acid extract of a guinea pig neural lobe (1/3) was subjected to HPLC. Fractions (0.5 ml) were collected and aliquots (10 μl) from the fractions were assayed for NP_{OT} (open circles) and NP_{AVP} (closed circles). Immunoreactivity is expressed relative to the appropriate porcine NP standard. Arrows show the retention times of guinea pig NP_{AVP}, NP_{OT} and NP-GP. Dotted line shows elution gradient.

4. DISCUSSION

The primary structures of the bovine and rat AVP precursors, and the bovine OT precursor have recently been deduced from cDNA sequencing [14–16]. Both precursors contain the nonapeptide hormone sequence connected to the corresponding NP by a Gly–Lys–Arg sequence. The NP sequence in the AVP precursor is extended at its C-terminus by a 39 residue glycopeptide, whereas the NP sequence in the OT precursor terminates with a single additional histidine residue, and is therefore not glycosylated [3,17]. We have recently identified a component in the neural lobe of the guinea pig which appeared to be a biosynthetic intermediate in the AVP system [9]. This component could be separated from the classical neurohypophysial products (OT, AVP, NPs) by HPLC, in a peak which incorporated both [^{35}S]cysteine and [^{3}H]fucose, and which contained immunoreactive NP, but not OT or AVP. Isotope incorporation ratios suggested that this material was a partially processed form of the AVP precursor comprising NP with its C-terminal glycopeptide still attached.

In the present experiments, gel filtration and HPLC of ^{35}S-labelled pituitary extracts established that this material had a higher M_r than NP, consistent with it being a processing intermediate. Affinity chromatography on Con A-Sepharose confirmed that the ^{35}S-incorporating material was glycosylated, suggesting that it related to the AVP precursor, rather than the OT precursor. This assignment is further supported by measurements of the NP immunoreactivity of the component. Previous work showing the proposed intermediate to be NP-immunoreactive [9] used an antiserum which was specific for guinea pig NP but could not distinguish between NP_{AVP} and NP_{OT}. By using heterologous antisera which can differentiate between the guinea pig NPs we have now confirmed that the intermediate relates to the NP_{AVP} system. In view of the known structure of the AVP precursor in other species [14], the simplest explanation for our results is that significant amounts of a partially processed AVP precursor are present in the guinea pig neural lobe as an intact NP-GP sequence.

Rat neural lobes apparently contain little if any similar intermediate [18], but higher M_r [^{35}S]-cysteine-incorporating material has been observed in gel filtration studies of rat neural lobe extracts at early times after labelling [19], and glycosylated NP material has been reported in bovine pituitary extracts [20]. Perhaps the unusually high AVP : OT ratio in the guinea pig pituitary [21] favours the detection of AVP-related biosynthetic intermediates in this species. If the guinea pig AVP precursor has the same structure and cleavage sites as the bovine and rat precursors, then the link between AVP and NP contains a dibasic residue, a common site for precursor processing [4]. NP is linked to the glycopeptide via a single basic residue [14], a site which is not usually involved in processing [22,23]. In this context, it is interesting that processing would appear to begin with the removal of AVP, followed by cleavage of the C-terminal glycopeptide at a later stage [9].

Although the structure of this NP-GP component suggests it is a biosynthetic intermediate between the AVP precursor and the final neurohypophysial secretory products, it remains to be determined whether the pituitary content of NP-GP is further processed, or whether this molecule represents a mature neurosecretory product in the guinea pig neural lobe.

REFERENCES

[1] Sachs, H., Fawcett, P., Takabatake, Y. and Portanova, R. (1969) Recent Prog. Horm. Res. 25, 447–491.
[2] Pickering, B.T., Jones, G.W., Burford, G.D., McPherson, M., Swann, R.W., Heap, P.F. and Morris, J.F. (1975) Ann NY Acad. Sci. 248, 15–35.
[3] Russell, J.T., Brownstein, M.J. and Gainer, H. (1980) Endocrinology 107, 1880–1891.
[4] Geisow, M.J. and Smyth, D.G. (1980) in: The Enzymology of Post-Translational Processing of Proteins (Freeman, R.B. and Hawkins, H.C. eds) pp. 259–287, Academic Press, London.
[5] Bradbury, A.F., Finnie, M.D.A. and Smyth, D.G. (1982) Nature 298, 686–688.
[6] Sachs, H. and Takabatake, Y. (1964) Endocrinology 75, 943–948.
[7] Burford, G.D. and Pickering, B.T., (1973) Biochem. J. 136, 1047–1052.
[8] Gainer, H., Sarne, Y. and Brownstein, M.J. (1977) Science 195, 1354–1356.
[9] Robinson, I.C.A.F. and Jones, P.M. (1983) Neurosci. Lett. in press.

[10] Smyth, D.G. and Massey, D.G. (1979) Biochem. Biophys. Res. Commun. 87, 1006–1010.
[11] Seidah, N.G., Bennjannet, S. and Chretien, M. (1981) Biochem. Biophys. Res. Commun. 100, 901–907.
[12] Robinson, I.C.A.F., Woolf, C.N. and Parsons, J.A. (1981) J. Endocrinol. 90, 227–236.
[13] Salacinski, P., Hope, J., Clement-Jones, V., Sykes, J., Price, J. and Lowry, P.J. (1979) J. Endocrinol. 81, 131P.
[14] Land, H., Schütz, G., Schmale, H. and Richter, D. (1982) Nature 295, 299–303.
[15] Land, H., Grez, M., Ruppert, D., Schmale, H., Rehbein, M., Richter, D. and Schütz, G. (1983) Nature 302, 342–344.
[16] Schmale, H., Heinsohn, S. and Richter, D. (1983) EMBO J. 2, 763–767.
[17] Lauber, M., Camier, M. and Cohen, P. (1979) FEBS Lett. 97, 343–347.
[18] Swann, R.W., Gonzales, C.B., Birkett, S.D. and Pickering, B.T. (1982) Biochem. J., 208, 339–349.
[19] Russell, J.T., Brownstein, M.J. and Gainer, H. (1981) Brain Res. 205, 299–311.
[20] North, W.G., Mitchell, T.I. and North, G.M. (1983) FEBS Lett. 152, 29–34.
[21] Bisset, G.W., Errington, M.L. and Richards, G.D. (1973) Br. J. Pharmacol. 48, 263–272.
[22] Rosen, A.M. and Gether, D.M. (1977) Biochem. Biophys. Res. Commun. 78, 1060–1066.
[23] Itoh, N., Obata, K., Yanaihara, N. and Okamoto, H. (1983) Nature 304, 547–549.