Proc. Natl. Acad. Sci. USA
Vol. 77, No. 8, pp. 5021–5024, August 1980
Physiological Sciences

Urotensin II: A somatostatin-like peptide in the caudal neurosecretory system of fishes

(urophysis/peptide hormone/neurohormone/osmoregulation/somatostatin)

DAVID PEARSON*, JOHN E. SHIVELY†, BRIAN R. CLARK†, IRVING I. GESCHWIND‡, MARYLYNN BARKLEY§, RICHARD S. NISHIOKA¶, AND HOWARD A. BERN¶

*Division of Neurosciences and †Division of Immunology, City of Hope Research Institute, Duarte, California 91010; ‡Department of Animal Science and §Department of Animal Physiology, University of California, Davis, California 95616; and ¶Department of Zoology and Cancer Research Laboratory, University of California, Berkeley, California 94720

Contributed by Howard A. Bern, May 15, 1980

ABSTRACT **Urotensin II, a peptide hormone from the caudal neurosecretory system of the teleost, *Gillichthys mirabilis*, was isolated by using classical chromatographic techniques and high-performance liquid chromatography (HPLC). Direct microtechniques for sequence determination were used to establish its structure. Urotensin II from *Gillichthys* is a 1363-dalton dodecapeptide with the amino acid sequence Ala-Gly-Thr-Ala-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val. This sequence is homologous with somatostatin in positions 1 and 2 and 7–9. The sequence has been verified by the production of a bioactive synthetic urotensin II. The possible chemical and physiological significance of its homology to somatostatin is discussed.**

The concept of a neurosecretory system in the caudal portion of the teleost spinal cord was first fully developed by Enami (1). In the goby *Gillichthys mirabilis*, it consists of two linearly arranged columns of neurosecretory cells, ventrolateral to the central canal and caudal to the fifth preterminal vertebra, which project axons into a well-defined neurohemal organ, the urophysis. An array of biological activities, including smooth muscle contraction, cardiovascular effects in fish, birds, and mammals, and osmoregulatory effects, have been described for factors derived from this system (2–6). At least three distinct, bioactive peptides are known to exist in the urophysis. An arginine vasotocin-like compound is present in some fish (7). Urotensin I (UI), a compound which has not yet been given full structural definition, is responsible for avian and mammalian hypotensive effects (6). Urotensin II (UII) has hypertensive (7), smooth muscle-contracting and osmoregulatory effects (6). Although the morphology and physiology of the caudal system have been investigated extensively during recent years, functional studies have been hampered in part by the fact that the chemistry of the active principles has not been fully defined; in addition, it has not been possible to develop immunological probes. Amino acid compositions of both UI and UII from the sucker *Catostomus commersoni* have been published by the Lederis group (6). The present work describes an isolation procedure for *Gillichthys* urotensin II and defines its structure. A preliminary report of portions of this work has been presented (8).

MATERIALS AND METHODS

Source of Urophyses. *Gillichthys mirabilis*, 10–15 cm long, were obtained from San Francisco Bay. The urophyses dissected from freshly killed animals were dried in acetone before storage in a desiccator at −4°C until used.

Isolation of UII. UII purification was carried out as described (8) with the following modifications. Gel-filtration chromatography on a 1 × 28 cm column packed with Bio-Gel P-6 (Bio-Rad), which was swollen in 0.2 M ammonium acetate buffer (pH 5.5), was performed at a flow rate of 100 μl/min. The column was pretreated with 1.0 ml of 10% (wt/vol) bovine serum albumin to minimize adsorptive losses of UII.

Bioassay. Biological activity of the hormone was determined by the trout hindgut contraction assay as described by Zelnik and Lederis (9) and Chan *et al.* (10).

Amino Acid Analysis. Peptide samples (2–10 μg) were hydrolyzed *in vacuo* for 24 or 48 hr in 3 M *p*-toluenesulfonic acid containing 0.2% 3-(2-aminoethyl)-indole (11). Amino acids were separated on a Beckman 121MB analyzer with a two-column system (12). Cysteine in the peptide was converted to either the *S*-methyl derivative by reaction with trimethyl phosphate after reduction with 2-mercaptoethanol (13) or to cysteic acid by performic acid oxidation (14). Tryptophan was quantitatively recovered either unreacted or as the *S*-methylated peptide but was destroyed by performic acid oxidation. The *S*-methylated peptide (10 nmol) was digested with carboxypeptidase A for 40 min and 3 hr, and the released amino acids were quantified on the amino acid analyzer.

NH_2-Terminal Sequencing. The *S*-methylated or performic acid-oxidized peptide (2–6 nmol) was subjected to automatic Edman degradations on a modified Beckman 890C sequenator. The modifications are similar to those described by Wittman-Liebold (15), including an improved reagent/solvent delivery system, improved vacuum system, reagent/solvent purification, and automatic conversion of anilinothiozolinone to phenythiohydantoin (>PhNCS) derivatives of amino acids (16). Peptides were retained in the spinning cup by the addition of 6 mg of polybrene, and >PhNCS derivatives of the amino acids were analyzed by high-performance liquid chromatography (HPLC) on a DuPont Zorbax ODS column. HPLC was performed on a Waters Associates chromatograph equipped with 254- and 313-nm detectors. Peaks were integrated and gradient elution was controlled with a Spectra Physics 4000 integrator system.

RESULTS

Isolation of Urotensin II. Initial fractionation of the acid-soluble compounds was carried out on Bio-Gel P-6 eluted with 0.2 M ammonium acetate, pH 5.5. Fig. 1 shows a typical elution profile, indicating that the UII was eluted with a V_e/V_0 of 3.0. If adsorptive interactions are ignored, this elution position would indicate a molecular size of less than 300 daltons. How-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: UI, urotensin I; UII, urotensin II; >PhNCS phenylthiohydantoin; HPLC, high-performance liquid chromatography.

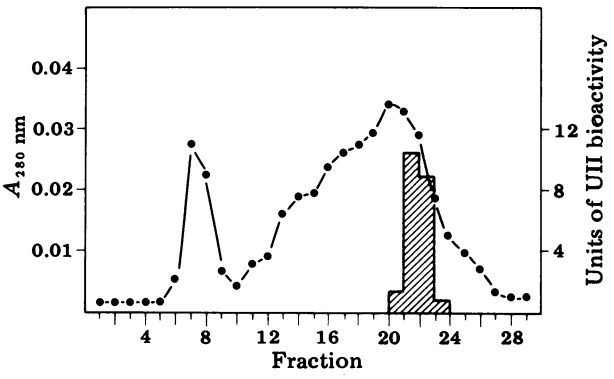

FIG. 1. Gel-filtration chromatography of urotensin II. The acid-soluble material from a urophysial extract was chromatographed on a 1.0 × 28 cm column of Bio-Gel P-6. Elution was carried out with 0.2 M ammonium acetate buffer, pH 5.5. ●—●, Absorbance of fractions of 280 nm; hatched area indicates UII bioactivity.

ever, we have observed that adsorptive interactions with Bio-Gel greatly retard the elution position of UII (and many other peptides), particularly at low pH. We have, in fact, observed a V_e/V_0 of greater than 8 when elution was carried out with dilute acetic acid. However, under these conditions severe decreases in the recovery of bioactivity occur.

Reverse-phase HPLC was carried out on the bioactive fractions from gel-filtration chromatography. Fig. 2 shows a typical elution profile. The reproducibility of this system was such that the hormone consistently had an elution time of 780 ± 15 sec. The bioactivity was associated with an UV absorbance peak at 254 nm.

Active fractions from the initial reverse-phase chromatography were pooled, lyophilized, and further purified on the same column by using a more gradual acetonitrile gradient. Bioactive fractions from this chromatographic step were pooled, lyophilized, and used for compositional and structural studies.

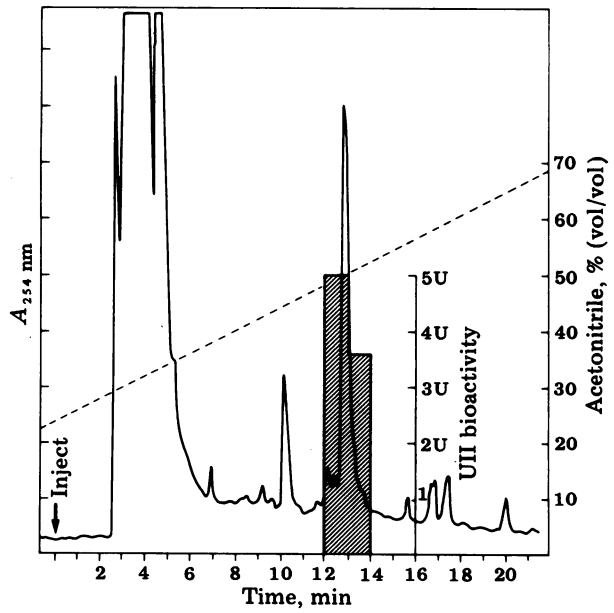

FIG. 2. Reverse-phase chromatography of urotensin II. Partially purified UII was reconstituted in 400 μl of 0.25% acetic acid and was chromatographed on a 0.26 × 25 cm micro-Zorbax ODS column at a flow rate of 1.0 ml/min with a gradient of increasing acetonitrile concentration (- - -) in an aqueous solution of 0.01 M sodium acetate containing 0.01% thiodiglycol. The hatched area shows UII bioactivity.

Table 1. Amino acid composition of urotensin II

Amino acid	Residues per molecule
Ala	1.9 (2.3)
Asx	1.3 (2.0)
Cys	0.9 (2.2)
Gly	1.3 (1.4)
Lys	1.2 (0.9)
Phe	0.8 (0.9)
Thr	1.3 (1.0)
Trp	1.3 (0.0)
Tyr	0.9 (0.4)
Val	0.8 (0.7)

Values for cysteine were determined for the *S*-methylated or performic acid-oxidized (shown in parentheses) peptide. Amino acids not shown were not detected above background levels.

Amino Acid Composition and Sequence. The amino acid composition of UII (Table 1) reveals that the peptide has a minimum of 12 amino acids and has a high likelihood of being a somatostatin analog. Compositional analysis of the *S*-methylated derivative showed a high recovery of tryptophan and tyrosine but only one residue of *S*-methyl cysteine per molecule. Subsequent analysis of the performic acid-oxidized peptide gave high yields of cysteic acid (but no tryptophan) and reduced yields of tyrosine. This analysis showed two residues of cysteine per molecule.

NH_2-terminal sequence analysis of *S*-methylated UII (2 nmol) (Fig. 3) gave an unambiguous sequence through cycle 10, but left some doubt as to the order of the last two residues, cysteine and valine. Subsequent sequence analysis of 4–6 nmol of *S*-methylated or performic acid-oxidized UII failed to resolve this problem. Digestion of *S*-methylated UII with carboxypeptidase A resulted in a rapid release of valine followed by a slower release of *S*-methyl cysteine and tyrosine (lysine was not released with this enzyme). These data strongly suggested that valine was COOH-terminal and cysteine was the penultimate residue.

To confirm the sequence of UII, two peptides were synthesized on a solid-phase Beckman peptide synthesizer. One peptide was identical to the proposed sequence in Fig. 3; the other was identical through position 10 but ended in Val-Cys. The former synthetic peptide had substantial biological activity and coeluted with authentic UII on reverse-phase HPLC, but the latter peptide had no activity and eluted on HPLC analysis with a retention time of 16.2 min, as opposed to 13.0 min for native UII. These data further confirm the proposed amino acid sequence for UII. Details of the synthesis, characterization, and biological-activity studies of the synthetic peptides will be presented elsewhere.

DISCUSSION

The present work reports two important advances in the study of caudal neurosecretion. First, the development of a rapid isolation method, which is applicable to the isolation of small amounts of hormone from fresh tissue, makes possible studies of UII biosynthesis and axonal transport. Because of the clear chemical relationship between somatostatin and UII, information on the biogenesis of urotensin II derived from studies of the isolated caudal neurosecretory system *in vitro* may also be relevant to study of the biogenesis of somatostatin.

Second, the structural definition of UII has made it possible both to prepare synthetic hormone and to expand our concepts of possible UII functions as a result of its homology with somatostatin. The availability of synthetic UII will provide large

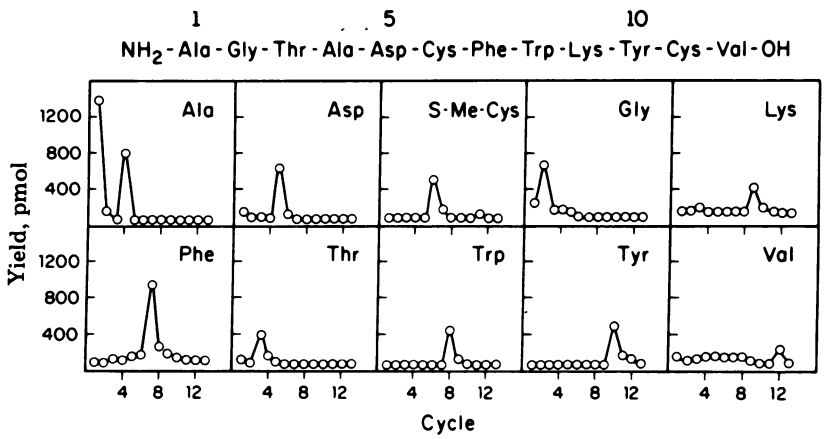

FIG. 3. Plot of yields from UII of >PhNCS-amino acids versus Edman degradation cycle. Cycles were analyzed by HPLC on a DuPont Zorbax ODS column. All amino acids were detected as >PhNCS derivatives at 254 nm except threonine, which was detected as the dehydro derivative at 313 nm. Yields were calculated by comparison with authentic standards after normalization of recoveries based on the inclusion of the internal standard >PhNCS-aminoisobutyric acid in the samples; >PhNCS-amino acids not shown were not present at significant levels above background. Cysteine was identified as either the *S*-methyl derivative (shown here) or as cysteic acid after performic acid oxidation (in this case tryptophan is destroyed); in either case, cysteine was not recovered in high enough yield to make an identification at cycle 11.

quantities of material for extensive physiological studies and for the production of antibodies.

The NH_2-terminal sequence analysis of UII demonstrates the sensitivity and utility of direct microsequencing with a modified spinning cup sequencer. Although Hunkapiller and Hood (17, 18) have reported successful sequence determinations of small peptides through the COOH terminus, it was difficult for us to obtain the last two residues of UII in high enough yield to permit an unambiguous assignment. We reasoned that performic acid-oxidized UII would be more strongly retained in the spinning cup by virtue of the ionic interaction of cysteic acid with the polycation carrier polybrene, but this was not the case. It may be that the contribution to peptide washout (organic solubility) of the aromatic residues at the COOH terminus of this peptide outweighed the ability of polybrene to retain even the performic acid-oxidized peptide in the spinning cup.

The amino acid composition and sequence analysis are in excellent agreement and indicate a molecular size of 1363 daltons for UII. The high yield of the NH_2-terminus argues that it possesses a single unblocked peptide chain. The homology between UII and somatostatin is striking (Fig. 4). Holladay *et al.* (19, 20) have studied the structure–function relationships of somatostatin and have deduced that the Phe^7-Trp^8-Lys^9 sequence is essential for biological activity. This sequence is also found in UII. In somatostatin, the presentation of this active site is maintained by the hydrophobic base-stacking interaction of phenylalanines at positions 6 and 10 (21, 22) as indicated by the broken line in Fig. 4. In urotensin II, this arrangement is assured by the disulfide bond between the cysteines at 6 and 10. Veber *et al.* (21) have synthesized a somatostatin analog with a cysteine disulfide ring in exactly this same arrangement, which resulted in increased potency over natural somatostatin in inhibiting the release of glucagon, insulin, and growth hormone. Thus, the structure of UII represents a surprising confirmation of what has already been demonstrated by synthetic improvements in the laboratory on a naturally occurring peptide hormone.

The preservation of the NH_2-terminal Ala-Gly sequence in UII suggests a possible contribution of these two "tail" residues to receptor binding. Previous studies have demonstrated that replacement with proline of Thr at position 10 but not of Asn at position 5 results in loss of biological activity for somatostatin (21). It is noteworthy that Asn^5 and Thr^{10} in somatostatin are replaced in UII with Asp^5 and Tyr^{10}, respectively. These replacements suggest that these are equivalent replacements in terms of the structural interactions between these and other residues. In fact, it has been suggested that Asn^5 interacts with Thr^{12} and Thr^{10} with the carbonyl of Phe^7 in somatostatin (21). In the light of these data, it is likely that Asn^5 or Asp^5 can hydrogen bond with Thr^{12} or Val^{12}, and Thr^{10} or Tyr^{10} can hydrogen bond with Phe^7 in the native peptides. The remaining amino acids appear to be nonessential by comparison of somatostatin with both synthetic analogs (23, 24) and with UII.

The fact that UII is a naturally occurring somatostatin analog raises interesting questions about the relative functions of these two hormones in fishes. An important similarity between these peptides involving control of Cl^- flux is emerging: somatostatin inhibits HCl secretion by the stomach (25) and also inhibits Cl^- excretion stimulated by vasoactive intestinal peptide in the elasmobranch rectal gland (26); UII inhibits Cl^- extrusion by the skin of the teleost, *Gillichthys mirabilis* (27). UII substitutes for somatostatin as an inhibitor of *in vitro* prolactin release in the fish *Tilapia* (unpublished results). Furthermore, as indicated in our discussion of chemical similarities between the hormones,

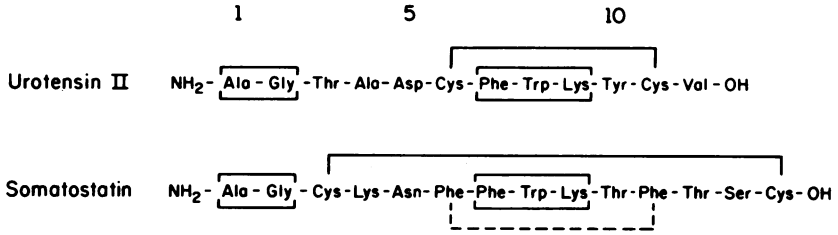

FIG. 4. Comparison of structures of UII and somatostatin. Homologous sequences are boxed. —, Disulfide bonds between cysteine residues; - - -, phenylalanine interactions.

a synthetic somatostatin analog, Cys-Phe-Trp-Lys-Thr-Cys, which is structurally more similar to UII than to somatostatin, shows very high somatostatin-like bioactivity in a variety of mammalian systems (21). Thus, it appears likely that UII will be shown to possess somatostatin-like activities in mammals.

It is important to note, however, that somatostatin and UII, despite their similarity, differ in important respects. Somatostatin does not appear to be active in the trout hindgut bioassay (unpublished observations), nor does it mimic the ability of UII to stimulate active Na^+ uptake by the *Gillichthys* urinary bladder (28). We anticipate that the relative roles of these two hormones will become apparent as physiological and pharmacological studies continue.

Note Added in Proof. Since preparation of this manuscript, we have become aware of a recent report (29) describing the presence of two somatostatin-like peptides in catfish pancreas. The smaller of the two, pancreatic somatostatin I, is 22 amino acids in length and has considerable homology with somatostatin, including the positions of cysteines and the critical 7–9 positions. Pancreatic somatostatin I is homologous with urotensin II only in the 7–9 positions.

We send our best wishes to Professor Lewis Kleinholz, a pioneer in the isolation of neurohormones, on the occasion of his seventieth birthday. We would like to acknowledge the expert technical assistance of Marsha L. Davis, Robert L. Gunther, Ursino Del Valle, Mark Levy, and James Dattilo. Portions of this research were supported by National Institutes of Health Grant NS13644 to D.P. and Grant HD-00394 to I.I.G. and M.B., by National Science Foundation Grants BMS-75-16345 and PCM-78-10348 to H.A.B. and by National Cancer Institute Grant 16434 to J.E.S.

1. Enami, M. (1959) in *Comparative Endocrinology*, ed. Gorbman, A. (Wiley, New York), pp. 697–724.
2. Lederis, K., Bern, H. A., Medakovic, M., Chan, D. K. O., Nishioka, R. S., Letter, A., Swanson, D., Gunther, R., Tesanovic, M. & Horne, B. (1974) in *Neurosecretion: The Final Neuroendocrine Pathway*, eds. Knowles, F. & Vollrath, L. (Springer, New York), pp. 94–103.
3. Chan, D. K. O. & Bern, H. A. (1976) *Cell Tissue Res.* **174,** 339–354.
4. Lederis, K. (1977) *Am. Zool.* **17,** 823–832.
5. Bern, H. A. & Lederis, K. (1978) in *Neurosecretion and Neuroendocrine Activity—Evolution, Structure, and Function*, eds. Bargmann, W., Oksche, A., Polenov, A. & Scharrer, B. (Springer, New York), pp. 341–349.
6. Yamamoto, K., ed. (1979) *The Caudal Neurosecretory System of Fishes*, 16th Gunma Symposium on Endocrinology (Gunma Univ., Maebashi, Japan), pp. 1–118.
7. Lacanilao, F. & Bern, H. A. (1972) *Proc. Soc. Exp. Biol. Med.* **140,** 1212–1253.
8. Pearson, D., Davis, M. L., Geschwind, I. I., Bern, H. A., Nishioka, R. S. & Gunther, R. L. (1979) in *16th Gunma Symposium on Endocrinology*, ed. Yamamoto, K. (Gunma Univ., Maebashi, Japan), pp. 111–118.
9. Zelnik, P. R. & Lederis, K. (1973) *Gen. Comp. Endocrinol.* **34,** 347–359.
10. Chan, D. K. O., Gunther, R. L. & Bern, H. A. (1978) *Gen. Comp. Endocrinol.* **34,** 347–359.
11. Liu, T.-Y & Chang, Y. H. (1971) *J. Biol. Chem.* **246,** 2842–2848.
12. Del Valle, U. & Shively, J. E. (1979) *Anal. Biochem.* **96,** 77–83.
13. Yamauchi, K., Sugimae, T. & Kinoshita, M. (1977) *Tetrahedron Lett.* **13,** 1199–1202.
14. Hirs, C. H. W. (1967) *Methods Enzymol.* **11,** 197–203.
15. Wittman-Liebold, B. (1973) *Hoppe-Seyler's Z. Physiol. Chemie* **354,** 1415–1431.
16. Wittman-Liebold, B., Graffinder, H. & Kohls, H. (1976) *Anal. Biochem.* **75,** 621–633.
17. Hunkapiller, M. W. & Hood, L. E. (1978) *Biochemistry* **17,** 2124–2133.
18. Hunkapiller, M. W. & Hood, L. E. (1980) *Science* **207,** 523–525.
19. Holladay, L. A. & Puett, D. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 1199–1202.
20. Holladay, L. A., Rivier, J. & Puett, D. (1977) *Biochemistry* **16,** 4897–4900.
21. Veber, D. F., Holly, F. W., Palaveda, W. J., Nutt, R. F., Bergstrand, S. J., Rochina, M., Glitzer, M. S. Saperstein, R. & Hirschmann, R. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 2636–2640.
22. Veber, D. F., Holly, F. W., Nutt, R. F., Bergstrand, S. J., Brady, S. F., Hirschmann, R., Glitzer, M. S. & Saperstein, R. (1979) *Nature (London)* **280,** 512–514.
23. Brown, M., Rivier, J., Vale, W. & Guillemin, R. (1975) *Biochem. Biophys. Res. Commun.* **65,** 752–756.
24. Rivier, J., Brown, M. & Vale, W. (1975) *Biochem. Biophys. Res. Commun.* **65,** 746–751.
25. Bloom, S. R., Mortimer, C. H., Thorner, M. O., Besser, G. M., Hall, R., Gomez-Pan, A., Roy, V. M., Russell, R. C. G., Loy, D. H., Kastin, A. J. & Schally, A. V. (1974) *Lancet* **ii,** 1106–1109.
26. Stoff, J. S., Rosa, R., Hallac, R., Silva, P. & Epstein, F. H. (1979) *Am. J. Physiol.* **237,** F138–F144.
27. Marshall, W. S. & Bern, H. A. (1979) *Science* **204,** 519–520.
28. Loretz, C. A. & Bern, H. A. (1980) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **39,** 493.
29. Oyama, H., Bradshaw, R. A., Bates, J. & Permutt, A. (1980) *J. Biol. Chem.* **255,** 2251–2254.