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Two new forms of gonadotropin-releasing hormone in a protochordate and the evolutionary implications

(neuropeptides/reproduction/evolution/ascidian)

JAMES F. F. POWELL*, SABINA M. RESKA-SKINNER*, MANISH OM PRAKASH*, WOLFGANG H. FISCHER†, MINKYU PARK†, JEAN E. RIVIER†, A. GREY CRAIG†, GEORGE O. MACKIE*, AND NANCY M. SHERWOOD*‡

*Department of Biology, University of Victoria, Victoria, BC Canada V8W 2Y2; and †The Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, 10010 North Torrey Pines Road, La Jolla, CA 92037

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ABSTRACT The neuropeptide gonadotropin-releasing hormone (GnRH) is the major regulator of reproduction in vertebrates. Our goal was to determine whether GnRH could be isolated and identified by primary structure in a protochordate and to examine its location by immunocytochemistry. The primary structure of two novel decapeptides from the tunicate *Chelyosoma productum* (class Ascidiacea) was determined. Both show significant identity with vertebrate GnRH. Tunicate GnRH-I (pGlu-His-Trp-Ser-Asp-Tyr-Phe-Lys-Pro-Gly-NH_2) has 60% of its residues conserved, compared with mammalian GnRH, whereas tunicate GnRH-II (pGlu-His-Trp-Ser-Leu-Cys-His-Ala-Pro-Gly-NH_2) is unusual in that it was isolated as a disulfide-linked dimer. Numerous immunoreactive GnRH neurons lie within blood sinuses close to the gonoducts and gonads in both juveniles and adults, implying that the neuropeptide is released into the bloodstream. It is suggested that in ancestral chordates, before the evolution of the pituitary, the hormone was released into the bloodstream and acted directly on the gonads.

The origins of the vertebrate forebrain and pituitary gland are enigmatic. In the tunicates, a protochordate group representing an early offshoot from the main chordate lineage (1, 2), the brain is a single, simple ganglion, and there is no pituitary gland. As in amphioxus and the vertebrates, the nervous system develops from the neural tube, formed as an infolding of the dorsal embryonic ectoderm. Although not all the adult derivatives of the tunicate neural tube have homologs in vertebrates, the tunicate central nervous system can be said to "foreshadow" that of vertebrates (3). An important physiological function of the vertebrate central nervous system is the regulation of reproduction. In all classes of vertebrates, reproduction is mediated by the neuropeptide, gonadotropin-releasing hormone (GnRH; ref. 4). In some invertebrates, the nervous system controls reproduction (5), but GnRH-like molecules have not hitherto been implicated in their reproductive physiology. As shown here, however, GnRH is present in tunicates and is located in nerve cells along the dorsal strand.

MATERIALS AND METHODS

Tissue Collection. GnRH was extracted from 1200 adult ascidians (*Chelyosoma productum*) that were obtained by divers at ≈60 feet in the Strait of Juan de Fuca near Victoria, BC, Canada. The dorsal portion of the animal, including the cerebral ganglion, the neural gland, and the dorsal strand was dissected after removal of the tunic. The tissue was frozen and stored at −80°C.

Peptide Extraction and Purification. The frozen tissue was powdered, extracted in an acetone/HCl mixture, and defatted with petroleum ether as described (6). Sequential HPLC procedures in which the ion pairing agents varied were used to purify the two GnRH peptides. Detection of GnRH in each step of the procedure was based on antisera raised against lamprey GnRH (Bla-5) or salmon GnRH (GF-4) in our laboratory.

Characterization of the Primary Structure. The primary structure of the purified peptides was determined by matrix-assisted laser desorption (MALD) mass spectrometry using post-source decay (PSD) and by chemical sequence analysis after treatment with pyroglutamate aminopeptidase.

The PSD spectrum was measured using MALD on a Bruker reflex time-of-flight instrument (Bruker Instruments, Billerica, MA; ref. 7) for native tunicate GnRH (tGnRH)-I and tGnRH-II, the latter as a disulfide-linked dimer and after reduction with tris(2-carboxyethyl) phosphine. The sequence assignments were based on a series of seven b-type ions and other fragment ions, including y-type ions. The isotope distribution of the intact protonated molecules ion ($[M+H]^+$) was measured on a JEOL HX110 magnetic sector instrument equipped with MALD. The sequence assignments were confirmed by chemical sequence analysis using an Applied Biosystems 470A protein sequencer after treatment of the peptide with pyroglutamyl aminopeptidase (8). To ascertain the presence of C-terminal amidation, the accurate mass of both peptides was determined.

Immunocytochemistry. Tissue was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer or in Zamboni's fixative (9). After pieces were washed in PBS, some pieces were cryostat-sectioned and others were processed as whole mounts. The sections or pieces were incubated in antiserum raised against lamprey GnRH diluted 1:100 in PBS containing 0.3% Triton X-100 and 2% goat serum. After they were washed in PBS, they were transferred to one of two secondary antibodies: fluorescein isothiocyanate-conjugated goat anti-rabbit gamma globulin for fluorescence microscopy or biotinylated goat anti-rabbit antibody for streptavidin peroxidase processing following standard procedures. Preabsorption controls with lamprey GnRH and controls omitting the primary antibody were carried out.

Peptide Synthesis. Solid-phase synthesis of tGnRH-I and tGnRH-II was carried out on a methylbenzhydrylamine resin (Boc strategy) using previously established methods (10) and the following protecting groups: pyro-Glu(carbobenzoxy), Boc-His(tosyl), Boc-Ser(benzyl), and Boc-Tyr(2-bromocarbobenzoxy). tGnRHs were deprotected and cleaved from the solid support with hydrofluoric acid. After purification with

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Abbreviations: GnRH, gonadotropin-releasing hormone; MALD, matrix-assisted laser desorption; PSD, post-source decay; tGnRH, tunicate GnRH.

Data deposition: The sequences reported in this paper have been deposited in the GenBank data base (accession nos. P80677 and P80678).

‡To whom reprint requests should be addressed.

reverse-phase HPLC (>97% pure) in two solvent systems (11), the structure was confirmed by mass spectral and amino acid composition analyses.

Antibody Production. Synthetic tGnRH-I was conjugated to bovine thyroglobulin using the carbodiimide method described by Skowsky and Fisher (12). The rabbit was injected subcutaneously and intramuscularly with the conjugated tGnRH-I combined initially with Freund's complete adjuvant and every 4 weeks thereafter, combined only with incomplete adjuvant. Serial blood samples were taken 2 weeks after each injection. The third blood sample resulted in the same pattern of staining as shown in Fig. 3. Attempts were also made to conjugate tGnRH-II, but antiserum with crossreactivity to this peptide did not result.

RESULTS AND DISCUSSION

The structures for two forms of GnRH from *Chelyosoma* are shown in Figs. 1 and 2. Both are novel. The sequence assignment is based on both MALD time-of-flight mass spectrometry and chemical sequencing. The mass of the intact protonated molecule ion ($[M+H]^+$) of tGnRH-I was measured as m/z 1246.56 (cf. the calculated monoisotopic $[M+H]^+$ for <EHWSDYFKPG-NH_2 of 1246.564 Da). tGnRH-I is identified by its conservation of length (it is a decapeptide) and its sequence identity, in that five amino acids match those of all known forms of GnRH and six amino acids match mammalian GnRH. The relationship of tGnRH-I to vertebrate GnRH peptides is supported also by preliminary physiological data; the addition of tGnRH-I to a final concentration of 100 ng/ml in a frog pituitary preparation resulted in a 4-fold increase in the concentration of luteinizing hormone in the medium (P. S. Tsai and P. Licht, personal communication). However, tGnRH-I has little structural kinship to yeast α-mating factor (13), a peptide that has been shown to release luteinizing hormone from rat pituitary cells (ref. 14; Fig. 2). The closest structural tie exists between tGnRH-I and the lamprey forms of GnRH (6, 15), in that a lysine is present in position 8 and Gly^6 is absent. Another remarkable feature of tGnRH-I is that it has a potential salt bridge between the negatively charged aspartic acid in position 5 and the positively charged lysine in position 8. This formation straddling the proposed β-turn, important for biological activity in the vertebrates, may serve to stabilize a bioactive conformation. This structural feature has been investigated and led to the design of potent, cyclic, vertebrate GnRH antagonists (16).

The unique feature of tGnRH-II is the presence of a cysteine residue, which has not been found in any other GnRH. The homodimeric nature of tGnRH-II was established by measurement of the mass of the peptide before and after reduction (Fig. 1*B*). The sequence of the homodimer is distinct from tGnRH-I and all other forms of GnRH (Fig. 2). The mass of the intact $[M+H]^+$ was measured as m/z 2232.14 (cf. the calculated monoisotopic $[M+H]^+$ for the disulfide-linked <EHWSLCHAPG-NH_2 dimer of 2231.977 Da). After reduction, the mass of the intact $[M+H]^+$ was measured as m/z 1117.52 (cf. the calculated monoisotopic $[M+H]^+$ for <EH-WSLCHAPG-NH_2 of 1117.50 Da). Dimerization may render the molecule resistant to degrading enzymes due to spatial inhibition. It can be speculated that each monomeric subunit of the dimer binds to a receptor molecule, enhancing dimerization of the receptors. In vertebrates, Gregory *et al.* (17) found that dimerization of occupied GnRH receptors was sufficient without further aggregation to activate the release of luteinizing hormone from porcine pituitary cells (17). tGnRH-II may illustrate an ancestral mechanism to ensure receptor dimerization.

Large numbers of GnRH-rich neurons, recognized by immune labeling techniques, form a plexus surrounding the dorsal strand and lining the walls of the blood sinus in which it lies. The dorsal strand is an epithelial structure derived from the embryonic neural tube (18–20). It is thought to retain a neurogenic role in adult ascidians, serving as a source of

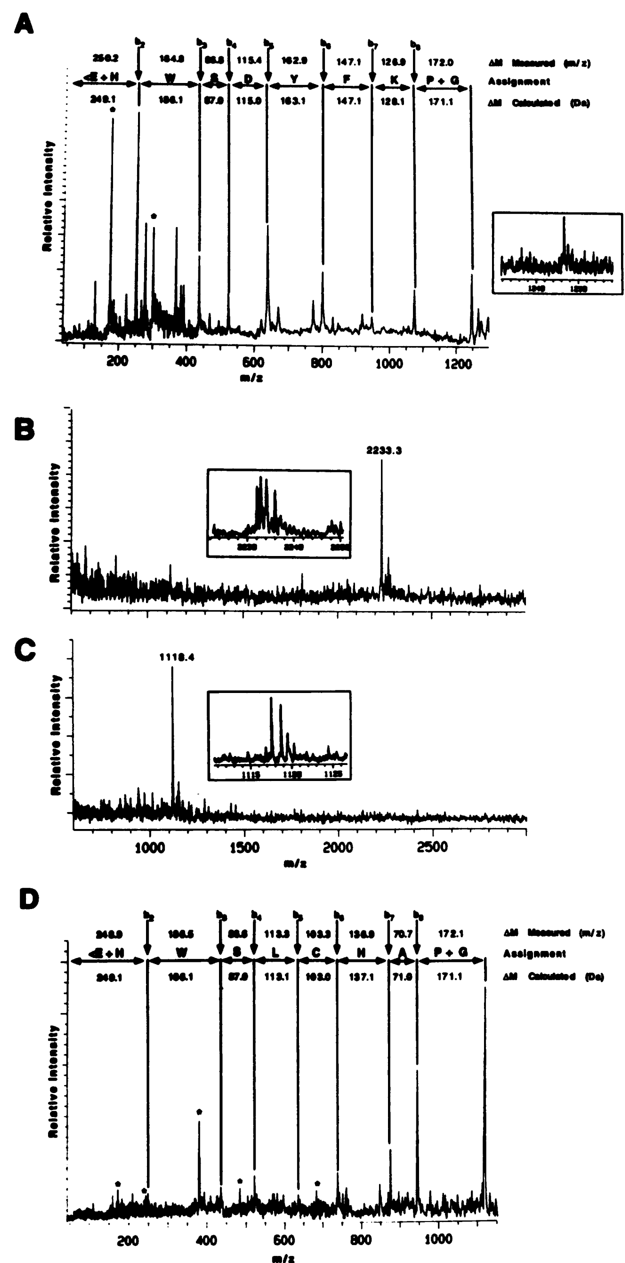

FIG. 1. Characterization of the primary structure of tGnRH-I and -II. (*A*) PSD spectrum of tGnRH-I measured using MALD on a Bruker reflex time-of-flight instrument. The sequence assignment is based on a series of seven b-type ions (indicated) and other fragment ions including y-type ions marked by an asterisk (*). The insert shows the resolved isotope distribution of the intact protonated molecule ion ($[M+H]^+$) (measured m/z 1246.56, cf. calculated monoisotopic $[M+H]^+$ for <EHWSDYFKPG-NH_2 of 1246.564 Da). The sequence assignment was confirmed by chemical sequence. (*B*) MALD time-of-flight spectrum of native tGnRH-II. The insert shows the resolved isotope distribution of the intact $[M+H]^+$ (measured m/z 2232.14, cf. calculated monoisotopic $[M+H]^+$ for the disulfide-linked <EHWS-LCHAPG-NH_2 dimer of 2231.977 Da). (*C*) MALD time-of-flight spectrum of tGnRH-II after reduction with tris(2-carboxyethyl) phosphine. The insert shows the resolved isotope distribution of the intact $[M+H]^+$ (measured m/z 1117.52, cf. calculated monoisotopic $[M+H]^+$ for <EHWSLCHAPG-NH_2 of 1117.50 Da). (*D*) PSD spectrum of reduced tGnRH-II. The sequence was determined also by chemical sequence.

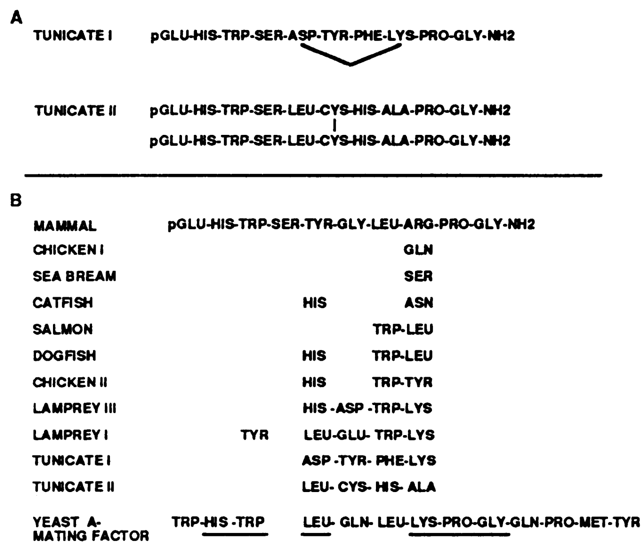

FIG. 2. The primary structure of GnRH peptides. (*A*) Two novel tGnRH peptides are shown. Solid line in tGnRH-I shows the site of a potential salt bridge (Asp[5]-Lys[8]). tGnRH-II was isolated as a dimer as shown. (*B*) Eleven known forms of GnRH are shown together with yeast α-mating factor. Residues that are identical with mammalian GnRH are omitted. Amino acids in yeast α-mating factor that are identical to those in at least one GnRH peptide are underlined. For maximal alignment, one space is inserted in the mating factor.

neurons in brain regeneration (21) and possibly giving rise to the GnRH neurons of the dorsal strand plexus (22, 23). The plexus (Fig. 3) follows the dorsal strand from its origin at the back of the neural gland to its termination in the visceral sinus. In the region of the gonoducts, neurites run out in the blood spaces surrounding the gonoducts. GnRH fibers run within the walls of the visceral blood sinus close to the gonads and are especially abundant here in younger animals. Some GnRH-positive neurites are seen in other regions, but their cell bodies can usually be traced to cell bodies lying within the dorsal strand plexus. As seen in cryostat sections, the neurites always run within blood spaces or in the proteinaceous material lining these spaces, except for those that enter posterior nerve roots leading into the cerebral ganglion. These mingle with the nonimmunoreactive processes that form the bulk of the central nervous system. No GnRH cell bodies have been observed in the central nervous system. GnRH-positive neurites have not been seen to penetrate the dorsal strand itself, nor its anterior swelling, the neural gland. The above studies were carried out using antisera raised against lamprey GnRH and provide no information regarding possible differences in the distribution of the two forms of tGnRH. However, the staining was repeated with an antiserum that we raised to tGnRH-I, for which crossreactivity with tGnRH-I was 100%, but with tGnRH-II, it was only 0.4%. Staining with this specific antiserum against tGnRH-I resulted in the same pattern as with the antiserum against lamprey GnRH. The rabbit injected with tGnRH-II did not generate antibodies, and hence the distribution remains to be determined.

Immunoreactive GnRH cells were reported previously in tunicates, both in *Ciona* (24) and *Chelyosoma* (25). These earlier studies showed immunoreactive neurites in and around the brain but gave no hint of the richness of the population of GnRH cells in the dorsal and visceral sinuses. Their abundance in these regions, now seen in both *Ciona* (23) and *Chelyosoma* (this paper), and their proximity to the gonoducts and gonads suggest that the peptide may be released into the blood in the immediate vicinity of the reproductive organs and may act directly upon them. The lack of GnRH cell bodies in the cerebral ganglion and their concentration peripherally along the dorsal strand are consistent with the observation that gonadogenesis and spawning continue unaltered for over a year after removal of the cerebral ganglion and neural gland (26). This operation would leave the bulk of the GnRH system intact. The dorsal strand has long been thought to play a role in the development of the gonads as the gonad primordia start to develop only after making contact with the dorsal strand

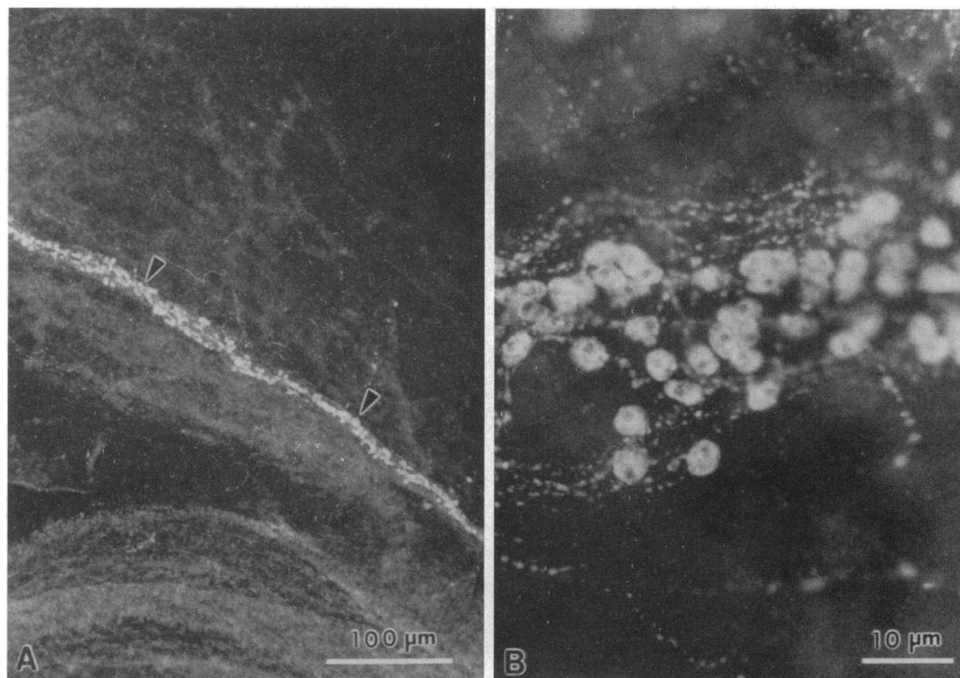

FIG. 3. Immune labeling of cells containing immunoreactive GnRH in *C. productum*. (*A*) Whole mount preparation showing a long stretch of the dorsal strand plexus (arrowheads) containing a large number of neurons that crossreact with antiserum Bla-4 raised against lamprey GnRH. (*B*) An enlarged section of the dorsal strand plexus, showing individual immunoreactive neurons.

(27, 28) but it now seems likely that the critical factor in initiation of gonadal development may be the proximity of the dorsal strand plexus (as a source of GnRH) rather than of the dorsal strand itself. Further experiments are needed to determine if GnRH alone is sufficient for induction of gonadal differentiation. In mammals, the concentration of centrally generated GnRH in the systemic blood is thought to be too low to act directly on the gonads (29). The synthesis of GnRH in testis is still arguable, but the ability of the ovary to express GnRH mRNA (30) and the presence of GnRH receptors in the rat ovary at 10 days of age and testis at 40 days of age (31) may be indications that GnRH has a role in the gonad before sexual maturation.

The findings reported here suggest that, before the evolution of the pituitary, GnRH neurons may have released their secretion directly into the bloodstream, with the possibility that the hormone acted directly on the gonads. The presence of specific receptors for GnRH in the gonads of vertebrates, including mammals, supports this concept. The intense immunoreactivity of the GnRH plexus in the dorsal and posterior blood sinuses argue for an important GnRH function in reproduction.

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