Evidence for Gonadotropin-Releasing Hormone-like Peptides in a Cnidarian Nervous System

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Accepted May 25, 2000

There is increasing evidence that peptides of the gonadotropin-releasing hormone (GnRH) family, long considered a vertebrate preserve, are also present in invertebrate (molluscan) nervous systems. The possibility was examined that GnRHs are present and bioactive in cnidarians, considered to be representatives of the most primitive animals possessing a nervous system. Immunoreactive GnRH was detected in endodermal neurons of two anthozoans, the sea pansy Renilla koellikeri and the sea anemone Nematostella vectensis. In the sea pansy, immunoreactivity was detected throughout the autozooid polyps, including gamete-producing endoderm. High-performance liquid chromatography and radioimmunoassays of extracts from whole sea pansy colonies yielded two elution peaks exhibiting GnRH immunoreactivity with antisera raised against shark or mammalian GnRH. Vertebrate GnRHs as well as the two sea pansy GnRH-like factors inhibited the amplitude and frequency of peristaltic contractions in the sea pansy, and these actions were blocked by the LHRH analog [D-pGlu₁,D-Phe²,D-Trp₃,6]-LHRH. These results suggest that the GnRH family of neuropeptides is more widespread in metazoans than previously thought. Although our physiological data are preliminary, they point to a role for GnRHs as inhibitory modulators of neuromuscular transmission in the sea pansy.

Key Words: Cnidaria; GnRH; neuropeptide; sea pansy; molecular evolution.

Several of the families of structurally related neuropeptides first identified in vertebrates are also represented in invertebrates by native forms (De Loof et al., 1989), suggesting that peptide families are conserved through evolutionary lineages from invertebrates to vertebrates. This conservation may also exist in the lowest phylum (Cnidaria) in which neurons are identifiable. For example, neurons of several cnidarian species contain peptides belonging to the FMRFamide family (Grimmelikhuijzen et al., 1992; Grimmelikhuijzen and Westfall, 1995), all but one (Yasuda et al., 1993) unique to this phylum.

The gonadotropin-releasing hormones (GnRHs) constitute a peptide family whose members are remarkably conserved in length and composition of amino acid sequences and appear to have a distribution restricted to vertebrates (Sherwood, 1987). The possibility that GnRHs are also represented in invertebrates is supported by evidence showing that GnRH-like immunoreactive material is present in neurons of the gastropod mollusc Helisoma trivolvis and that extracts from its nervous system induced electrophysiological responses in Helisoma neurons and gonadotropin release from dispersed goldfish pituitary cells (Goldberg et al., 1993). In addition, GnRH-like immunoreactive material was detected in several protocordates (Georges and Dubois, 1980; Dufour et al., 1988; Kelsall et al., 1990; Mackie, 1995; Cameron et al., 1999) and two new peptides of the GnRH family were identified in the tunicate Chelyosoma productum (Powell et al., 1996), thus demonstrating that vertebrate GnRHs had ancestors in the protocordate lineages.
This paper addresses whether GnRH-like peptides occur in invertebrates other than molluscs and tunicates and, focusing on cnidarians, whether the evolutionary history of the GnRH family is traceable to the earliest invertebrates known to possess a nervous system. The primary cnidarian used to investigate these questions was the sea pansy, *Renilla koellikeri*, an octocorallian belonging to the sea pen group (Pennatulacea). The organization of the pennatulacean nervous system is reasonably well known (Buisson, 1970; Satterlie *et al.*, 1980), and the sea pansy was the focus of extensive mapping of neurotransmitter-specific neurons (Umbriaco *et al.*, 1990; Pani *et al.*, 1995; Anctil and Ngo Minh, 1997; Mechawar and Anctil, 1997). In addition to classical neurotransmitters, the sea pansy contains large amounts of the peptide Antho-RFamide which is widely distributed in its nervous system (Grimmelikhuijzen and Groeger, 1987).

A combination of immunohistochemical and chromatographic techniques was used to gain evidence for the presence of GnRH-like peptides in the sea pansy. In addition, the physiological function of GnRHs was investigated by comparing the activity of synthetic GnRHs with chromatographically purified sea pansy GnRH-like fractions on sea pansy muscle activity. Finally, confirmatory immunohistochemical data were obtained in another anthozoan species, the starlet sea anemone *Nematostella vectensis*. Together, the results from these approaches provide strong evidence for the presence and physiological relevance of GnRHs in Cnidaria. Preliminary results of this study were presented to the XIIIth International Congress of Comparative Endocrinology (Anctil, 1997).

**MATERIALS AND METHODS**

Colonies of sea pansy (*Renilla koellikeri* Pfeffer) were purchased from Marinus Inc. (Long Beach, CA) and maintained in aquaria with recirculating, filtered artificial sea water (ASW, Instant Ocean) at 12–16° and pH 8.0. The unfed colonies were exposed to a light–dark cycle according to the photoperiod length experienced in their environment before shipment and were processed within 2 weeks of shipment.

Starlet sea anemones (*Nematostella vectensis*) were maintained in culture dishes at room temperature in diluted (30%) local sea water at the Whitney Laboratory. They were fed three times weekly with brine shrimps, at which times their culture medium was changed.

**Immunohistochemistry.** Ten sea pansy colonies were prepared for whole-mount immunohistochemistry. Sea pansies were anesthetized in equal parts of 0.37 M MgCl₂ and ASW. Autozooid polyps and their recessed reproductive tissue inside the colonial mass (rachis) were excised and immersed in Zamboni’s fixative (4% paraformaldehyde and 7.5% saturated picric acid) freshly prepared in 0.1 M phosphate buffer with 2.4% NaCl (PBS) at pH 7.4. Fixation proceeded at room temperature for 1 h and at 4° for the following 24 h. After washing in PBS, tissues were incubated for 48 h at 4° in either of three different primary antisera: an antiseraum raised in rabbit against chicken GnRH-II and graciously provided by Dr. J. P. Chang, University of Alberta (Goldberg *et al.*, 1993) and two other antisera raised in rabbit against LHRH, one from a commercial source (Sigma Chemicals) and the other obtained as a courtesy from Dr. Robert Benoît (LR-1: Benoît *et al.*, 1987). All three antisera were diluted 1:1000 in PBS with 1% normal goat serum or 1% bovine serum albumin and 0.3% Triton X-100. After further washing in PBS, tissues were treated for 1 h with Cy3-conjugated goat anti-rabbit IgG (Jackson Immunocinicals) diluted 1:100 in PBS containing 0.3% Triton X-100. After a final wash, tissues were mounted in a 1:3 mixture of glycerol and PBS and examined with a Wild-Leitz Dialux 20 fluorescence microscope. Controls were performed by replacing the primary antisera with nonimmunized serum and by preabsorption of the primary antisera with 0.1 mM LHRH or chicken GnRH-II.

The double-labeling immunohistochemistry procedure of Würden and Homberg (1993) was used to determine whether FMRFamide-like immunoreactivity (Fair), reflecting the presence of native Antho-RFamide, colocalizes with GnRH-like immunoreactivity (GnRHir) in the same cells. Tissues were treated for 48 h in the LR-1 LHRH antiserum diluted 1:100 followed by the Cy3-conjugated secondary antiserum as previously described. After washing in three 1-h changes of rabbit IgG (Jackson Immunochemicals) diluted 1:100 in PBS containing 0.3% Triton X-100. After a final wash, tissues were mounted in a 1:3 mixture of glycerol and PBS and examined with a Wild-Leitz Dialux 20 fluorescence microscope. Controls were performed by replacing the primary antisera with nonimmunized serum and by preabsorption of the primary antisera with 0.1 mM LHRH or chicken GnRH-II.
method of Harlow and Lane (1986). Tissues were then incubated for 3 h in streptavidin-conjugated fluorescein isothiocyanate (FITC, Jackson Immunochemicals) diluted 1:20.

Starlet sea anemones were processed similarly to sea pansies except that anesthesia was performed in equal mixtures of 30% sea water and 0.12 M MgCl₂, and PBS was prepared with 0.8% NaCl to reflect the salinity conditions under which the animals were cultured. A dilution of 1:500 of the primary LHRH antiserum (Sigma) was used, and either Cy3 (1:100) or FITC (1:80) was used as fluorophore conjugated to secondary antibodies.

**Peptide extraction.** Whole sea pansies were extracted during the reproductive season (May–August) by the method of Goldberg et al. (1993) after modifications. Sea pansies were deflated to remove residual sea water from their gastrovascular channels, weighed, and immediately frozen in liquid nitrogen. The frozen colonies were rapidly crushed in a coffee grinder and the resulting slush was added to a mixture of acetone and 1 N HCl (100:3) on wet ice. Extraction proceeded for 3–4 h with continuous shaking at 4°. After separating the soluble extract by filtration, the residue was further extracted in a 4:1 mixture of acetone and 0.01 N HCl. The combined filtrates were washed five times by adding petroleum ether to the filtrate in the ratio 1:4 to remove hydrophobic materials. The final aqueous phase was stored frozen at −80° until used. Just before use, the extracts were concentrated in a rotary vacuum evaporator and centrifuged at 10,000 rpm and 10° for 10 min to remove particulates. A total of 120 g of tissue from 30 colonies were thus extracted to a final volume of 40 ml.

**Extract purification.** Extracts were first loaded onto a reverse-phase Brownlee RP-300 column (4.6 × 220 mm, 5-μm particles) at 2 ml/min using a Gilson 714 HPLC system. The column was loaded with an extract from the worm *Urechis* to ascertain that no immunoreactive GnRH was retained from previous loadings of GnRH standards (LHRH, chicken GnRH-II, and salmon GnRH; all from Peninsula Laboratories) or *Renilla* extracts. Fractionation of extracts was by a linear gradient of 10 to 50% acetonitrile (ACN) in 0.05% trifluoroacetic acid (TFA) over 40 min. Fractions were collected at 30-s intervals, dried in a SpeedVac concentrator (Savant), and reconstituted with 50 μl of radioimmunoassay buffer (see below). After immunoreactive GnRH (irGnRH) was detected by radioimmunoassay (RIA) in some of the fractions, more extract was subsequently processed by HPLC to select those specific fractions and pool them for further purification steps.

The selected fractions were run as above but on a smaller column (Brownlee RP-300, 2.1 × 220 mm) at 0.5 ml/min, and a stronger ion-pairing agent, heptafluoro-butyric acid (HFBA) was substituted for TFA at the same dilution. Three successive purification steps were performed with this procedure.

**Radioimmunoassay.** Iodination and RIA protocols followed Price et al. (1987). Two iodinated GnRH peptides, mammalian GnRH and salmon GnRH, were used in the RIA methods tested. Iodination was performed by mixing 2–3 μl of 125I (1–1.5 mCi) with 1 μl of peptide solution (1 mM in 0.5 M phosphate buffer, pH 7.0), followed by addition of 5 μl of 0.2% chloramine T and 100 μl of 0.5% sodium metabisulphite prepared in the same buffer. After mixing, the iodination mixture was retained in C18 SepPak cartridges (Waters) and eluted with a solution of 80% ACN and 0.1% TFA. For assays, a dilution of this trace on the order of 1:1000 was made into RIA buffer (0.01 M disodium phosphate, 0.9% NaCl, 0.73% EDTA, 0.01% Thimerosal, and 1% bovine serum albumin at pH 7.5) to yield 100,000–130,000 cpm/ml. This diluted trace was stable for at least 1 week at 4°.

Several antisera were used in attempts to optimize RIA sensitivity. Four were graciously provided by Dr. Nancy Sherwood, University of Victoria (British Columbia): FP-3, a salmon GnRH antiserum; 7CR-10, a dogfish GnRH antiserum; BLA-5, a lamprey GnRH-I antiserum (Kelsall et al., 1990); and Jasmine-6, a tunicate GnRH-I antiserum (Powell et al. 1996); all raised in rabbit. The mammalian GnRH (LHRH) antiserum was obtained from Peninsula Laboratories. The most sensitive assay method was obtained with mammalian GnRH trace antiserum (1.75000), and standard (mammalian RIA), yielding a detection limit of 12.6 pg (B/Bo = 80%). The only other workable method used salmon GnRH trace and standard with dogfish GnRH antiserum (1:10000) (fish RIA), allowing a detection limit of 51.6 pg.

The assay reaction was started by adding 100 μl of trace and 100 μl of antiserum to 50 μl of buffer (Bo), GnRH standard, or HPLC fraction and allowed to equilibrate at 4° overnight. One milliliter of charcoal slurry (0.25% activated charcoal and 0.025% Dextran with 0.01% merthiolate in phosphate buffer, pH 7.4–7.6) was then added to assay tubes and let stand for 10
min. Assay tubes were centrifuged at 3000g and 4° for 15 min and decanted in radiocounting vials.

**Bioassay.** Triangular pieces of rachis bearing several autozooids were excised from unanesthetized sea pansies, cutting from the margin of the colony to the point of emergence of the pedoncle into the rachis. The pieces were pinned on the Sylgard-coated bottom of a recording chamber and attached to a Grass FT-03C force displacement transducer at a wedge end. Isometric tension signals were transmitted to a Grass 7P122 amplifier and digitally stored for analysis with either a BIOPAC MP100WS (Goleta, CA) or a Grass Instruments PolyView (Astro-Med Inc., Longueuil, Canada) data acquisition and analysis system. The preparations were allowed to relax tension for 0.5–2 h in a fresh sea water bath before beginning experiments. Synthetic peptides or purified sea pansy extracts were delivered to the chamber with a syringe at a concentration 10 times higher than the final concentration in the bath. The same volume of ASW containing reconstituted HPLC fractions without irGnRH was similarly delivered as additional control. Baths were evacuated by gravity through an outlet at the bottom of the chamber, and fresh sea water was almost simultaneously added from the top of the chamber to maintain fluid level. All experiments were conducted at room temperature (20 – 24°).

Experiments began by recording tension activity at least 30 min after addition of normal sea water (controls). Peptides or extracts were then introduced and tension was recorded for another 30 min or more. The bath was evacuated and entirely replaced with fresh sea water, and the preparation left to stand for 1 h to let tension return to the initial baseline before measuring tension again for a minimum of 30 min (recovery). Each contraction assay preparation was used once. Both amplitude and rate of several consecutive peristaltic waves were measured for each experimental condition in time bins of 30 min. Data were averaged and normalized as percentage of controls.

**RESULTS**

**Localization of GnRH-immunoreactive neurons.** Although all three GnRH antisera used in sea pansy tissues gave positive results, the antiserum LR-1 proved to be the most sensitive and provided the most consistent staining. The immunofluorescent cells were exclusively located in the endodermal layer and were identified as neurons according to conventional morphological criteria as applied to cnidarians (Saripalli and Westfall, 1996). No GnRH-like reactivity was present in control preparations.

Specific GnRH-immunoreactive (GnRHir) neurons were detected throughout the autozooid polyps, with...
greater density in the tentacles (Fig. 1A). In the latter they were bipolar with small round somata (7–8 μm) and coarse varicose neurites ~150 μm in length. Varicosities were prominent but varied greatly in size within neurites at the endoderm/mesoglea boundary (Fig. 1B). In the trunk of the tentacles, many neurons appeared to be incompletely stained and to run in a circular orientation, whereas neurites in the pinnules branching from the trunk were better stained and could be seen to follow a course perpendicular to the long axis of the trunk (Fig. 1A). Some neurites branch off into the ectodermal layer but do not appear to reach the surface (Fig. 1B).

GnRHir neurons in the wall of the autozooid column appeared to be fewer than in tentacles. The shape and size of their somata were similar to those in tentacles. They were bi- or multipolar, with branching neurites oriented primarily along the circular axis of the column (Fig. 1C). These neurites had smaller diameters and varicosities than those in tentacles and appeared to lie over the circular muscle layer of the body wall.

The eight gastrovascular cavities of the autozooid column extend beyond the pharynx within the rachis where they form the zooecium. Numerous bipolar and multipolar GnRHir neurons were detected in the walls of these cavities (Fig. 2A). Their oblong somata, ~14–18 μm, extend from the external surface of the myoepithelium inward where they taper to a neurite bifurcating near the myoepithelium/mesoglea border (Fig. 2B) at distances up to 150 μm. At their apex they appear to bear a cilium (Fig. 2C). Neurites of the bipolar neurons tend to run parallel to each other (Fig. 2A), whereas those of multipolar neurons appear intertwined (Fig. 2C). The neurites are slightly varicose and exhibit terminal knobs (Fig. 2C, large arrow). GnRHir neurons were also present in mesenteric filaments bearing gametes (Fig. 2D). Their somata resemble those observed in the wall of the zooecium, but their neurites appear to be shorter.

FIG. 2. Whole-mounts of GnRH-immunofluorescent neurons in the zooecium of polyps, deep inside the rachis of the sea pansy. (A) Strongly fluorescent cell bodies in the zooecial wall, with their neurites running parallel to each other. (B) One neuron as in A is seen to span the endoderm layer of the zooecial wall, from the cell body bordering a gastrovascular cavity (GVC) across the wall with an axon-like neurite (arrow) that bifurcates in the circular muscle layer. (C) In septal filaments arising from the zooecial wall, neurons are polymorphous, with rugose neurites coursing jaggedly in various orientations. Their cell bodies are ciliated (small arrows). (D) Group of intertwined neurons at the distal end of a septal filament to which is attached an ovocyte (arrow).
Although Fair neurons were present throughout the endoderm, double-immunofluorescence experiments failed to reveal colocalization of Fair and GnRHir in the same cells. Figures 3A and 3B provide such an example in the wall of the zooecium.

In the starlet sea anemone, GnRHir neurons were also confined to the endoderm. They were sparsely distributed along the mesenteries of the upper region of the column, near the pharynx (Fig. 3C). Their somata had an oblong shape, were 10–12 μm in length, and appeared to be either bipolar or tripolar (Fig. 3D). Their neurites were very thin and relatively smooth, exhibiting little or no varicose swellings, and they appeared to lie over a muscle layer as in the sea pansy. GnRHir neurons, usually in clusters, were also present in mesenteric filaments bearing gametes (Fig. 3E). These differed from the stained neurons in the upper column in that their neurites were conspicuously varicose, some of which reached to the cortex of ovocytes (Fig. 3E).

**Characterization of sea pansy GnRH-like factors.** Preliminary HPLC separations of sea pansy extracts (representing 2.6 g of tissue) using the 4.6 × 220 mm column and the mammalian RIA (see Materials and Methods) resulted in the detection of irGnRH in two fraction clusters, 38–40 (19–20 min elution) and 41–43 (20.5–22.5 min elution). None of these fractions coeluted with mammalian GnRH or chicken GnRH-II, but salmon GnRH coeluted with fraction 41. Subsequent separations with larger amounts of extracts (8 g of tissue) allowed the detection of irGnRH also in fractions 38–40 using the fish RIA.

The last separation was done with an extract representing 60 g of tissue. From this extract fractions 38–39 (early GnRH-like peak; 19–19.5 min elution) and 42–44 (late GnRH-like peak; 21–22 min elution), containing the largest amount of irGnRH determined by both mammalian and fish RIAs, were selected for further purification using a 2.1 × 220 mm column, a mobile phase containing HFBA instead of TFA, and a flow rate of 0.5 ml/min (Fig. 4). The fractions containing the highest irGnRH from each peak were fractions 65–67 of early peak (32.5–33.5 min elution) and fractions 68–69 of late peak (34–34.5 min elution); these were passed again through the same HPLC column and resulted in the elution profiles shown in Fig. 5. The major peaks for the two GnRH-like factors eluted at 23.4 (factor 1) and 25.3 min (factor 2). Contrary to earlier purification steps, none coincided with the elution peak of salmon GnRH (Fig. 5). This immunoreactive material was used in the following bioactivity assays.

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**FIG. 3.** Whole-mounts of GnRH-immunoreactive (GnRHir) and FMRF-amide-immunoreactive (Fair) neurons in the endoderm of the sea pansy (A,B) and starlet sea anemone (C–E). (A,B) GnRHir neurons in the zooecial wall (A) display no Fair (B) where instead other neurons are labeled. Arrows point to corresponding area in both panels. (C) Low-power view of upper column of a starlet sea anemone in which a few sparsely distributed neurons are seen. (D) Higher magnification of neurons in the upper column of a starlet sea anemone. Note the bifurcated neurite in one such neuron (arrow). (E) Small dense groups of neurons (large arrows) are found in this mesenteric filament of the starlet sea anemone that impinges on an ovocyte. Note the varicose neurites that extend from these groupings toward the follicle epithelium of the ovocyte (small arrows).
Effects of GnRHs and sea pansy GnRH-like extracts on sea pansy peristalsis. Both LHRH and chicken GnRH-II caused a reduction of the amplitude and frequency of rachidial peristaltic waves in a dose-dependent and reversible manner (Figs. 6 and 7). LHRH was 10 times more potent than chicken GnRH-II in eliciting these responses at a threshold concentration of $10^{-7}$ M and maximal responses at $10^{-4}$ M (Fig. 7). The responses appeared 2–3 min after peptide addition to the bath and vanished 10–15 min.
after peptide removal. The LHRH peptide antagonist [D-pGlu\textsuperscript{1},D-Phe\textsuperscript{2},D-Trp\textsuperscript{3,6}]-LHRH often enhanced the amplitude of peristaltic contractions while blocking the response to LHRH (Fig. 8).

The lyophilized sea pansy factors 1 and 2 corresponding to the HPLC peaks shown in Fig. 5 were reconstituted each in 300 μl sea water (representing 0.9 and 0.6 nmol of equivalent LHRH activity, respectively) and tested with the sea pansy peristalsis bioassay. Both factors produced effects qualitatively and quantitatively similar to those of authentic GnRHs, reducing peristaltic contractions in dose-dependent and antagonist-sensitive manners (Figs. 6 and 8). In contrast to the responses to authentic GnRHs, those of the sea pansy factors were of shorter duration, waning before the extracts were removed from the bath (Fig. 6).

**DISCUSSION**

In this study evidence for the presence of GnRH-like peptides in cnidarians was obtained by visualizing
neurons in two anthozoan species with three different GnRH antisera, by separating GnRH-like factors in sea pansy extracts using HPLC–RIA methods, and by assaying the extracted GnRH-like factors in a sea pansy bioassay in which peristaltic contractions are inhibited by GnRHs. Along with reports of GnRH-like neuropeptides and GnRH-like physiological activity in mollusks (Goldberg et al., 1993; Young et al., 1999; Pazos and Mathieu, 1999) and tunicates (Powell et al., 1996; Craig et al., 1997), this evidence points not only to the existence and functional involvement of peptides of the GnRH family in invertebrates, but also to their ancestral association with representatives of the earliest nervous systems to have arisen in metazoans.

In both the sea pansy and the starlet anemone GnRH-like immunoreactivity was associated exclusively with a population of neurons that share similarities of morphology, distribution, and innervation targets. All stained neurons were either bi- or multipolar with more or less varicose neurites extending in the endoderm either over parietal muscle sheets or over mesenteric filaments associated with gonad tissues. Apart from the innervation of gonad tissues, the density of stained neurons was greater in the upper than in the lower column of the individuals: in the tentacles of the sea pansy or at the pharynx level in the starlet anemone. This is in contrast to other cnidarian peptidergic neurons such as Fair neurons, which are more widely distributed over the whole animal and in all tissue layers (Grimmelikhuijzen et al., 1996). Although Fair neurons are present in areas where the sea pansy GnRHir neurons are located, no evidence of colocalization of GnRHir and Fair in the same neurons was revealed by double-labeling. Thus, the GnRHir neurons constitute a discrete subset of neurons which appear to serve specific functions based on the association of their neurites with body wall muscles and with gamete-bearing tissues (see below).

These neurons exhibit a few intriguing morphological features. In the sea pansy, GnRHir neurons in the tentacles were observed to project a neurite in the ectoderm. As the neurite does not appear to reach the outer surface, it is doubtful that it serves a direct sensory role. However, it is possible that these neurites connect with sensory cells known to be abundant in the tentacle ectoderm (Lyke, 1965; Umbriaco et al., 1990). Many GnRHir neurons possess a cilium. The apex of the somata of these neurons reaches the gastrovascular cavity so that the cilium may assist in detecting chemical or mechanical signals in the cavity fluids. Furthermore, some of the neurons in which neurites lack conspicuous varicosities possess large

![FIG. 6.](image.png)

**FIG. 6.** Effects of GnRH peptides on peristaltic contractions of a sea pansy rachis. Arrows indicate time of addition of peptides. Factor 1 refers to reconstituted sea pansy factor 1 (see Fig. 5). Ant., LHRH antagonist [D-pGlu1,D-Phe2,D-Trp3,6]-LHRH. Note that contractions are multiphasic.

![FIG. 7.](image.png)

**FIG. 7.** Dose-response curves for the reducing effect of LHRH, chicken GnRH-II, and LHRH + antagonist (10 μM) on sea pansy peristalsis (n = 6). Data points are mean ± SE.
terminal swellings. Such an arrangement suggests an endocrine mode of neurosecretion in which neurons release large amounts of peptide at one site, from which it diffuses widely in surrounding tissues.

The characterization of two GnRH-like factors from sea pansy extracts by HPLC–RIA provides supporting evidence that the GnRHir neurons described above contain peptides of the GnRH family. The characterization was validated by developing two different RIAs which consistently detected immunoreactive GnRHs in the same elution fractions and by consistent detection of GnRHir materials upon repeated runs of elutions in different HPLC columns. None of the two characterized factors coeluted with either LHRH, chicken GnRH-II, or salmon GnRH, contrary to extracts from the gastropod Helisoma which coeluted with mammalian GnRH (Goldberg et al., 1993). The detection of two forms of GnRH-like factors in the sea pansy follows a pattern that is common in vertebrate species. In several vertebrates the highly conserved chicken GnRH-II is often the second GnRH form and where present the two forms appear to serve different functions (Sherwood et al., 1993; King and Millar, 1995).

It will be desirable to determine the molecular structure of the two sea pansy factors to assess their phylogenetic divergence from known GnRHs and gain insights into the early history of this peptide family. Although the available material was insufficient for such purposes, it allowed testing their effect in a previously developed sea pansy bioassay for synthetic GnRHs (Anctil, 1997). Sea pansy colonies are traversed by peristaltic waves of contraction every few minutes (Parker, 1920). These waves are modulated by indolamines, serotonin enhancing and melatonin reducing the amplitude of the contractions (Anctil, 1989; Anctil et al., 1991). As shown here, both sea pansy GnRH-like factors reduced the amplitude of peristaltic waves in the same manner as synthetic GnRHs (LHRH and chicken GnRH-II), including sensitivity to the blocking action of the analog [D-pGlu¹,D-Phe³,D-Trp⁶⁷]-LHRH. The only departure from the response to synthetic GnRHs is the tendency of the response to the sea pansy factors to wane faster. Although the reason for this discrepancy is unclear, one possibility is that the sea pansy factors may lack protective groups like the pyroglutamate or amidated C-terminus possessed by the vertebrate GnRHs, thereby becoming more susceptible to degradation by peptidases.

These results suggest that native sea pansy GnRHs are physiologically active and play a role in the modulation of peristalsis. GnRHs are primarily known as regulators of reproduction in vertebrates, although other roles were suggested (Sherwood et al., 1993; King and Millar, 1995). In prevertebrate chordates a role in reproduction is suggested by the stimulatory

![FIG. 8. Histogram summarizing data on the effect of LHRH and sea pansy GnRH-like factors on peristaltic contractions of the sea pansy. Bar values are mean ± SE. Asterisks indicate significant differences compared with controls at $P < 0.05$ (*) or $P < 0.01$ (**) using paired t test.](image-url)
effect of native and other GnRH forms on estradiol levels in gonads of the tunicate Chelyosoma productum (Craig et al., 1997). There is some evidence to suggest that GnRH-like peptides are also involved in control of reproduction in invertebrates. In cephalopod and gastropod molluscs GnRH immunoreactivity was reported to be associated with the innervation of either reproductive organs or their brain center (Di Cosmo and Di Cristo, 1998; Young et al., 1999) and synthetic GnRHs promoted gametogenesis in a bivalve mollusc (Pazos and Mathieu, 1999). In contrast, no GnRH immunoreactivity was found in brain extracts of an arthropod (fleshfly) by radioimmunoassay (De Loof et al., 1995).

In cnidarians the evidence for a role of GnRH-like peptides in reproduction is less compelling. Experiments with ovary extracts from the jellyfish Spirocodon saltatrix led Ikegami et al. (1978) to suggest that an unidentified peptide of low molecular weight was involved in oocyte maturation and spawning. Now the finding that GnRHir neurons innervate the mesenteric filaments bearing gametes in both the sea pansy and the starlet sea anemone provides support for such an involvement by native GnRHs and a framework for investigating their roles in reproductive functions. The present study shows that peristalsis, which is strongly enhanced during spawning in the sea pansy (Satterlie and Case, 1979), is inhibited by native and other GnRHs. It may be that sea pansy GnRHs are not involved, or are involved negatively, in spawning. Repression of peristalsis by GnRHs may serve another, as yet unknown, function, but an alternative reproductive role, such as in gametogenesis, cannot be ruled out at present.

ACKNOWLEDGMENTS

I thank Dr. Nancy Sherwood (University of Victoria, B.C., Canada) for her generous gift of the FP-3, 7CR-10, BLA-5, and Jasmine-6 antisera. Dr. Peter A. V. Anderson, Director of the Whitney Laboratory and Dr. Michael J. Greenberg kindly provided laboratory spaces and facilities. I am particularly indebted to Karen Doble for her technical assistance and advice at the Whitney Laboratory and Dr. Michael J. Greenberg kindly provided laboratory and Dr. Peter A. V. Anderson, Director of the Whitney Laboratory and Dr. Michael J. Greenberg kindly provided laboratory spaces and facilities. I am particularly indebted to Karen Doble for her technical assistance and advice at the Whitney Laboratory and for suggestions to improve the manuscript. This research was supported by a grant from the Natural Sciences and Engineering Research Council of Canada to M.A.

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