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Gonadotropin-Releasing Hormone in Mulberry Cells of *Saccoglossus* and *Ptychodera* (Hemichordata: Enteropneusta)

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Mulberry cells are epidermal gland cells bearing a long basal process resembling a neurite and are tentatively regarded as neurosecretory cells. They occur scattered through the ectoderm of the proboscis, collar, and anterior trunk regions of the acorn worms *Saccoglossus,* usually in association with concentrations of nervous tissue. They contain secretion granules that appear from electron micrographs to be released to the exterior. The granules are immunoreactive with antisera raised against mammalian and salmon gonadotropin-releasing hormone (GnRH). Similar results were obtained with another enteropneust, *Ptychodera bahamensis,* using antisera raised against tunicate-1 and mammalian GnRH. Mulberry cells were not found in either *Cephalodiscus* or *Rhabdopleura* (Hemichordata: Pterobranchia). Extracts of tissues from 4200 *Saccoglossus* contain an area of immunoreactive GnRH that is detected by an antiserum raised against lamprey GnRH when characterized by high-performance liquid chromatography and radioimmunoassay. This is the first report of the occurrence of GnRH in hemichordates, probably the most primitive group clearly belonging to the chordate lineage. The physiological function of GnRH in enteropneusts is unknown, but an exocrine function appears more likely than an endocrine or neurotransmitter role. © 1999 Academic Press

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The enteropneusts, with the pterobranchs and planktosphaeroid larvae, compose the small deuterostome phylum Hemichordata, generally regarded as an early offshoot of the chordate line of evolution (Ruppert and Barnes, 1994). Several authors have described a distinctive type of epidermal gland cell in enteropneusts termed the ''mulberry'' cell from its berry-like cluster of apical granules (Brambell and Cole, 1939; Hyman, 1959; Knight-Jones, 1952; Welsch, 1984). Such cells have been reported under other names, for instance, ''granular gland cell'' (Bullock and Horridge, 1965) and ''elongated gland cell'' (Dawydoff, 1948). Schneider (1902) called them ''protein cells'' from the reaction of the granules with haematoxylin and aniline dyes. All authors agree on distinguishing mulberry cells from the more common mucous cells, but there may be other densely staining granular cells present which could cause confusion (see Welsch, 1984). Pardos and Benito (1989), for example, describe mulberry-like cells (''coarse grain cells'') in the endoderm of the pharynx, but as these are ciliated they cannot be equated with mulberry cells, which lack cilia.

As described by Brambell and Cole (1939), in *Saccoglossus cambrensis* the cells are oval or pear-shaped, measuring 20 μm in length, and approximately 7 μm at the thickest part. The cells have a narrow basal process

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that runs down and mingles with the mass of intraepidermal nerve fibers lying over the basal lamina. This basal process resembles a neurite, and the mulberry cell may therefore be a type of neuron (Bullock, 1945), but physiological evidence of neural function is lacking. The only two electrophysiological studies so far carried out on enteropneusts (Pickens, 1970; Cameron and Mackie, 1996) were both restricted to extracellular recordings and provide no information on the activity of individual excitable units.

We show in this paper that the mulberry cell granules are immunoreactive with antisera against gonadotropin-releasing hormone (GnRH, previously termed luteinizing hormone-releasing hormone, LHRH). The best-known function of this important reproductive hormone in vertebrates is to elicit the release of the pituitary gonadotropins. GnRH has been identified by primary structure in each class of vertebrates, where the effect of GnRH release on reproduction has been investigated (see Sherwood *et al.*, 1993). Peptides of the GnRH family have N-termini modified to form a pyroglutamyl ring and conserved amino acid residues in positions 1, 2, 4, 9, and 10. This conservation of form has recently been extended to include a tunicate, the ascidian *Chelyosoma productum* (Powell *et al.*, 1996). There is one report of a form of GnRH that coelutes with mammalian GnRH in a mollusc (Goldberg *et al.*, 1993), and another report of GnRH immunoreactivity in a Cnidarian (Anctil, 1997), challenging the view that GnRH is restricted to the chordate lineage.

Immunocytochemical evidence alone cannot provide conclusive evidence of the presence of a particular peptide. In the tunicate work, the initial identification of a GnRH-like peptide (Georges and Dubois, 1980) was later extended by column chromatography (Dufour *et al.*, 1988) and high-performance liquid chromatography (HPLC) (Kelsall *et al.*, 1990), showing that GnRH was indeed present. By the same token, satisfactory demonstration of the presence of GnRH in *Saccoglossus* requires chromatographic evidence as well as data from immunocytochemistry. We therefore embarked on this project using both techniques.

MATERIALS AND METHODS

Two species of the acorn worm *Saccoglossus* occur in coastal waters of British Columbia and Washington, *Saccoglossus bromophenolosus* from Willapa Bay and Padilla Bay, Washington (King *et al.* 1994), and a species provisionally designated *Saccoglossus* species A, pending determination by a specialist, from Barkley Sound, British Columbia. They differ in size, coloration, and habitat. *S. bromophenolosus* lives intertidally in mud flats and can be collected during periods of low tide, while *S.* species A typically occurs subtidally in coarse, calcareous sediment of biogenic origin and was collected by SCUBA. Immunolabelling for GnRH was carried out on both species, but *Saccoglossus* species A provided the preparations used for the photographs in this paper and was also selected for sectioning for optical and electron microscopy. GnRH extraction requires large numbers of animals, so the more easily collected *S. bromophenolosus* was chosen for this work. Specimens of both species were collected during spring and summer 1994 and were transported to the University of Victoria, where they were maintained in their natural substrates in slowly running sea water at 12°C. More specimens of *S. bromophenolosus* were collected in the same season in 1995 but were frozen whole in liquid nitrogen at the collection site. Specimens of *Ptychodera bahamensis* (Enteropneusta: Ptychoderidae) and *Cephalodiscus gracilis* and *Rhabdopleura normani* (Pterobranchia) were obtained intertidally at Castle Beach and under the Causeway Bridge, Bermuda, and were studied at the Bermuda Biological Station for Research in March 1996.

Immunocytochemistry. After anesthesia in sea water containing 151 mM Mg^{2+}, specimens were dissected and pieces of tissue were removed and pinned out in Sylgard-lined petri dishes where they were fixed for 24 h in Zamboni's fixative (Zamboni and DeMartino, 1967) at pH 7.3. After washing in phosphate-buffered saline (PBS) the tissues were treated with the primary (rabbit) antibody diluted 1:500 in PBS + 3% Triton X-100 + 1% goat serum. Tissues were washed repeatedly in PBS containing 0.3% Triton X-100 for 18 h and treated with FITC-labeled goat anti-rabbit gamma globulin (FITC-GARGG) diluted 1:500 in PBS + 3% Triton X-100 for 60 min. Tissues were washed again in PBS and mounted as whole mounts in 50% glycerol containing 1.5% *N*-propylpyrogallate. Two primary antisera were used: rabbit anti-mammalian GnRH, sample U705-23, from Dr. Gerald Kozlowski (University of Texas) and rabbit anti-salmon GnRH, sample

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GF4, from N. M. Sherwood (University of Victoria). In addition, an antiserum against tunicate-1 GnRH was used in the experiments conducted in Bermuda. Both the primary antibodies and FITC-GARGG were used at a dilution of 1:500. Two types of controls were run. In the first, the primary antibody was omitted during the initial incubation. In the second, the preparations were run with primary antisera preabsorbed for 24 h with mammalian GnRH.

For visualizing nuclei in the mulberry cells, the fluorescent Hoechst dye No. 33342 (Sigma) was used on Zamboni-fixed whole mounts.

Sections. Specimens were fixed in Bouin's fluid for paraffin sectioning. Four specimens were serially sectioned from the proboscis through the collar to the anterior part of the trunk. The sections were stained with eriochrome cyanin.

Pieces of tissue were fixed in 2.5% glutaraldehyde in 0.2 M Millonig's phosphate buffer at pH 7.4 for 1.5 h. They were postfixed in 1% osmium tetroxide in 0.2 M Millonig's buffer for 1 h, dehydrated through a graded series of ethanol solutions followed by propylene oxide, and embedded in Epon 812. Thick (ca. 1.0 μm) sections were cut with glass knives and stained with Richardson's stain for light microscopy. Thin sections, stained in uranyl acetate and lead citrate, were examined with a Hitachi H-7000 transmission electron microscope.

Extraction of peptides. Approximately 1200 *S. bromophenolosus* were collected in 1994 from intertidal sediments of Willapa Bay West, Washington, and transported in sediment and sea water to aquaria at the University of Victoria. The probosces and collars (73.2 g) were dissected, immediately frozen on dry ice, and stored at −80°C. Frozen tissue was powdered with liquid nitrogen in a Waring Blendor. The powdered material was treated as described by Sherwood *et al.* (1986). Briefly, the material was added to 1 N HCl/acetone (3/100 v/v), stirred for 3 h, and filtered through a No. 1 Whatman filter. The solids were resuspended in 0.01 N HCl/acetone (1/5 v/v) and stirred for 3 min. Acetone, lipids, and other hydrophobic substances were removed by five successive additions of petroleum ether (20%, v/v). The final aqueous phase (800 ml) was evaporated in a vacuum centrifuge to approximately 200 ml. An additional 3000 specimens of the same species were collected at the same location in 1995. The acorn worms (575 g) were frozen whole in liquid nitrogen at Willapa Bay and then taken to the University of Victoria.

Sep-Pak HPLC. Ten Sep-Pak C18 cartridges (Waters) were connected in series and washed with 6 ml of methanol followed by 6 ml of Milli-Q water. The aqueous extract from the tissue sample was pumped through the cartridge column using a peristaltic pump at a flow rate of 1.5 ml/min. The material remaining on the column was eluted after the cartridge column was connected to a Beckman Model 125 HPLC apparatus. Initial conditions of solvent flow in the column were 95% solution A (0.05% trifluoroacetic acid (TFA) in water) and 5% solution B (0.05% TFA in 80% acetonitrile and 20% water) at a flow rate of 1 ml/min. A gradient that increased at a rate of 1% solution B per minute was applied to the cartridge column for 60 min. Fractions of 1 ml were collected for 60 min and assayed for GnRH-like immunoreactivity. Specimens collected in both years were extracted by the same method.

Purification of GnRH. Procedural steps for the purification of a GnRH-like peptide included three successive HPLC stages after Sep-Pak HPLC. A new C18 Supelco column connected to a Beckman 125/166 HPLC and detector was used. Solvents and ion-pairing agents for the HPLC steps are listed in Table 1. The last step of the purification was done with a phenyl column (Vydac) to determine if more than one GnRH-like peptide could be separated. Aliquots of 100 μl were used to determine the amount of immunoreactive GnRH (irGnRH) in each fraction collected. Fractions that contained irGnRH were selected for further purification in successive steps.

TABLE 1
Steps in the HPLC Purification of GnRH from *Saccoglossus bromophenolosus*

HPLC step	Column type	Solvent A	Solvent B
1	Sep-Pak	0.05% TFA	0.05% TFA in 80% ACN/20% H_2O
2	C18	1.2 mM TEAF	ACN
3	C18	0.05% TFA	0.05% TFA in 80% ACN/20% H_2O
4	Phenyl	0.05% TFA	0.05% TFA in 80% ACN/20% H_2O

Note. Solvent and column types are listed for each successive step. Immunoreactive areas identified by radioimmunoassay were reduced in volume, combined, and applied to the next step of purification. Abbreviations: ACN, acetonitrile; TEAF, triethylammonium formate; TFA, trifluoroacetic acid.

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In most GnRH purification schemes, some of the immunoreactive GnRH elutes early (fractions 1–10) without interacting with the column. This occurred also in the *Saccoglossus* GnRH purification. With the extract prepared in 1995 several methods were designed to improve the yield of GnRH. To disrupt any weak protein binding to carrier molecules and allow stronger interaction (retention) with the C-18 column, pooled fractions of early-eluting material were treated with a 6 M guanidine–HCL solution prior to column loading. This treatment did not alter the elution position. A stronger ion-pairing agent, 0.05% heptafluorobutyric acid (HFBA), was then used in the HPLC program and similar results were obtained. Finally, a polyhydroxyethyl aspartamide column (200 × 4.6 mm, PolyLC, Inc., Columbia, MD) was tested for use in purification of the early-eluting fractions using hydrophilic interaction chromatography (HILIC). HPLC fractions 1–10 were vacuum dried to 0.5 ml and then diluted with a 20% methanol / 80% acetonitrile solution to a final volume of 3 ml. Five consecutive 600-µl volumes were injected into the column (at 2-min intervals) and eluted using the following elution profile: 100% B for 10 min (A, 0.05% TFA; B, 0.05 TFA / 90% acetonitrile), 85.5% B for 10 min, and then 85.5 to 54% during a 40-min period.

Radioimmunoassay (RIA). Two identical RIA methods were used except that the labeled trace was mammalian GnRH in one method and lamprey GnRH-I in the other method. Iodination methods were identical for both GnRH peptides. Antisera GF-4, BLA-5, and R-42 were used in the mammalian GnRH method and antisera 36–52 and 7CR-10 were used in the lamprey GnRH-I method. The standard was mammalian GnRH for the first three antisera, lamprey GnRH-I for the 36-52 antiserum and chicken GnRH-II for the 7CR-10 antiserum. An aliquot of each HPLC fraction was assayed for irGnRH by standard RIA (Sherwood *et al.*, 1983, 1986).

The cross-reactivity of GF-4, BLA-5, and R-42 have been previously reported (Kelsall *et al.*, 1990; Sherwood *et al.*, 1991). Antiserum GF-4 (raised against salmon GnRH) was used in a dilution of 1:25,000, resulting in 22–32% binding of ^{125}I-mammalian GnRH. Antiserum BLA-5 (raised against lamprey GnRH-I) was used in a dilution of 1:10,000, resulting in 9–17% binding of ^{125}I-mammalian GnRH. Antiserum R-42 (raised against mammalian GnRH) was used in a dilution of 1:50,000, resulting in 10% binding of ^{125}I-labeled mammalian GnRH. All of the known GnRH forms are recognized by at least one of these antisera. Limits of detection (B/B_0 = 80%) for each assay averaged 10.4 pg for GF-4 and 47.6 pg for BLA-5.

Antiserum 36-52, raised against lamprey GnRH-III, was a gift from Dr. Stacia Sower (University of New Hampshire). Antiserum 36-52 was used in a dilution of 1:25,000 and resulted in 42% binding of ^{125}I-labeled lamprey GnRH-I. The limit of detection was 33 pg. The cross-reactivity of 36-52 was 100% for lamprey GnRH-III. Antiserum 7CR-10 (raised against dogfish (df) GnRH) was used in a dilution of 1:37,500 resulting in 12% binding of ^{125}I-labeled lamprey GnRH-I. The limit of detection was 22 pg. The cross-reactivity of 7CR-10 is 100% for cGnRH-II, 25% for dfGnRH, 6% for lamprey GnRH-I, and less than 0.03% for other known forms of GnRH.

RESULTS

Mulberry cells in Saccoglossus: Structure and immunolabelling. As noted above, these cells have been described by several previous workers and are readily identified by their prominent granular contents, long basal processes, and lack of cilia. The apical end of the cell forms part of the epithelial surface, while the basal process runs down into the mass of nerve fibers lying over the basal lamina. The nucleus lies just below the mass of granules in the part of the cell that tapers down to form the basal process. In paraffin sections and in thick Epon sections after staining with basic dyes the granules are strongly basiphilic. They are electron dense under the electron microscope after osmium staining. In both species examined, the granules label strongly with anti-GnRH antisera (Figs 1A and 1B). No labeling was observed in the basal processes. No GnRH-like immunofluorescence was detected in control preparations.

Seen by electron microscopy fully differentiated mulberry cells could be followed from their apical poles down to their narrow basal processes in the nerve fiber layer (Fig. 1D) close to the basal lamina. At the apical pole, granules appear to be undergoing release to the exterior (Fig. 1C). There were typically

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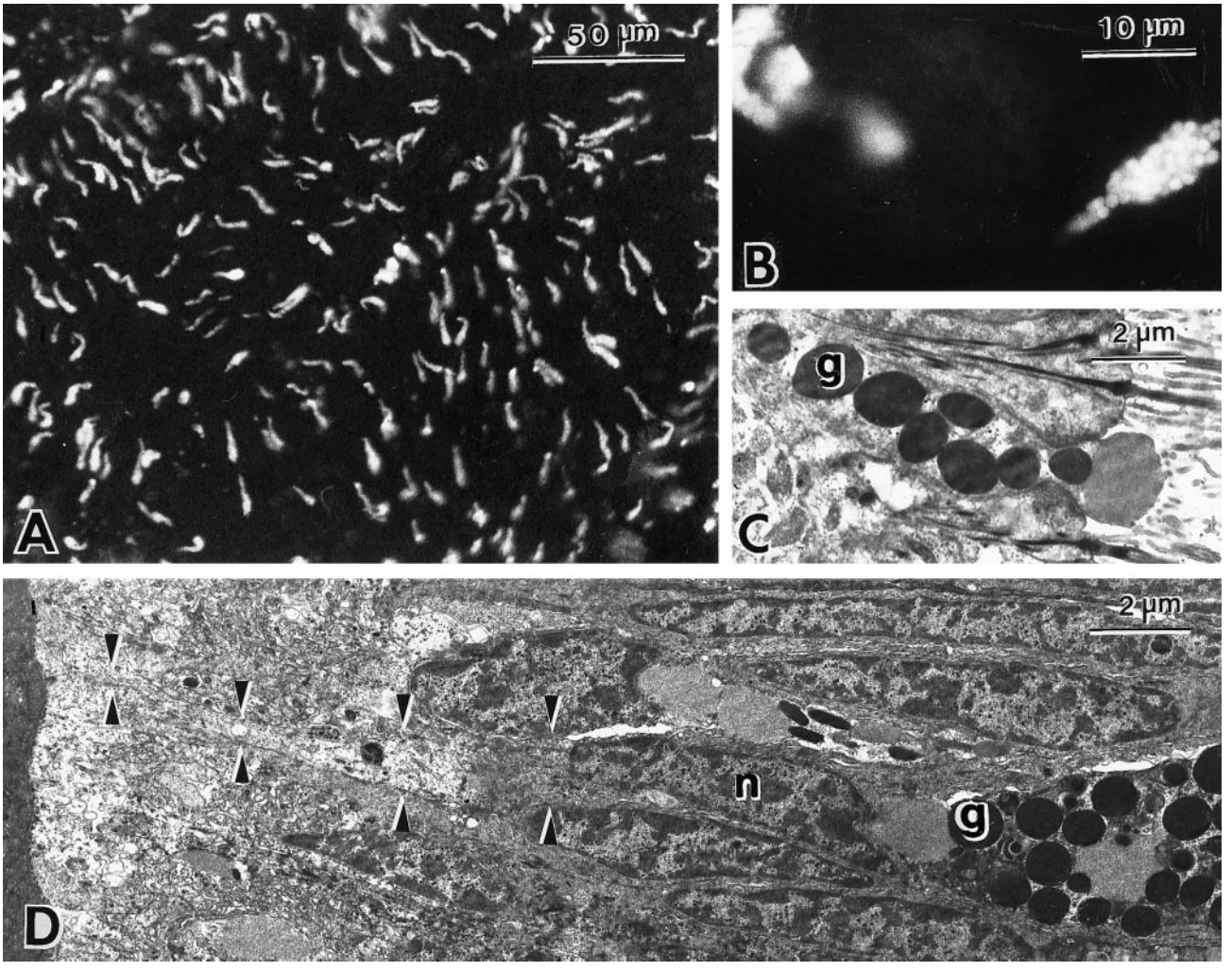

FIG. 1. Mulberry cells in *Saccoglossus* shown by immunofluorescence (A,B) and transmission electron microscopy (C,D). (A) GnRH-like immunoreactivity in mulberry cells scattered through the posterior collar epithelium, dorsal to the collar nerve cord. (B) Higher magnification, cells extending from the dorsal collar nerve cord. (C) Apical portion of a mulberry cell in the mid-collar region, with granules. (D) Transmission electron micrograph showing granules (g), nucleus (n), and the tapering base of the mulberry cell running down into nerve fiber layer (arrowheads).

about 30–45 granules per cell, the granules showing a mean diameter of 970 ± 280 nm ($n = 62$). These measurements are in agreement with measurements made on the granules in immunolabeled whole mounts. The narrow basal processes extending into the fibrous layer mingle with the processes of neurons and neurosensory cells and cannot readily be distinguished from the latter in terms of either size of cytoplasmic contents. The processes lying in the fiber layer contain numerous microtubules and mitochondria. Small clear vesicles and various sizes of dense-cored vesicles are seen in many processes (Fig. 1D). It has not been possible to trace the basal processes of mulberry cells far enough in the fibrous layer for their lengths to be estimated and it is not known if they make synaptic interconnections with other processes.

Mulberry cell distribution in Saccoglossus. Observed in immunolabeled whole mounts, mulberry cells were found widely distributed through the body wall ectoderm in the proboscis, collar, and anterior part of the trunk (Fig. 2). In the proboscis they were particularly abundant ventrally to the neural keel in

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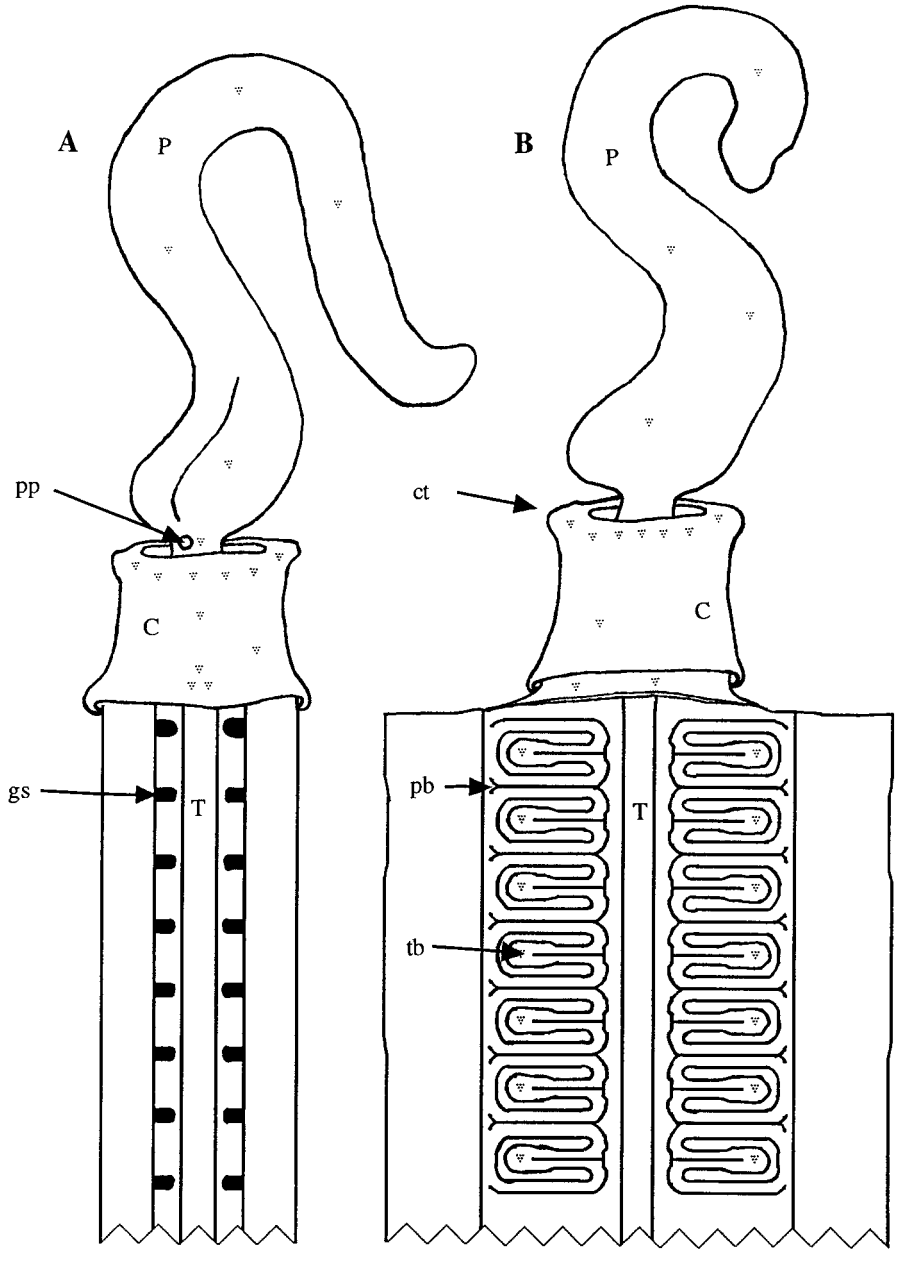

FIG. 2. The distribution of GnRH-immunoreactive mulberry cells in *Saccoglossus.* (A) Dorsal view showing mulberry cells in the general proboscis epithelia with incresing density in the region of the gill pore, collarette, and overlaying the dorsal collar cord. (B) Ventral view of the mulberry cell around the collarette, and with the trunk segment cut mid-ventrally to show the distribution in the gill bar epithelia. Not to scale. Collar (C), collarette (ct), gill slit (gs), primary gill bar (pb), proboscis (P), proboscis pore (pp), tongue gill bar (tb), trunk (T), mulberry cell

the proboscis neck and adjacent to the proboscis pore. In the collar, they were abundant in the anterior collarette epithelium and in the anterior part of the collar nerve cord, but they were rarely observed in the internalized part of the collar cord. Where the cord surfaces again at the back of the collar, mulberry cells were again found. In the trunk, mulberry cells were seen as lines of cells following the gill slit primary and tongue bars. Mulberry cells were not observed elsewhere in the trunk. In all regions where they were

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observed, the mulberry cells were associated with nervous tissue and sent their basal processes into the nerve fiber layer.

Mulberry cells in other hemichordates. Mulberry cells were located in *Ptychodera behamensis* using antisera against tunicate-1 GnRH and mammalian GnRH. The cells showed a distribution similar to that described above for *Saccoglossus.* No immunoreactive cells were found in the pterobranchs *Cephalodiscus* and *Rhabdopleura* using these antisera.

Characterization of Saccoglossus GnRH. Of the five antisera used in RIA, antiserum 36-52 combined with ^{125}I-labeled lamprey GnRH-I detected irGnRH from a C-18 column in HPLC eluates from *S. bromophenolosus* extract. In contrast, as noted above, GF-4 detected irGnRH in whole mounts of both species studied. A total of 2.7 ng irGnRH was detected in fractions 33–40 of Sep-Pak HPLC (Fig. 3A). These fractions were combined, reduced in volume, and applied to the isocratic-TEAF HPLC method where eluates in fractions 19–25 contained a total of 3.3 ng irGnRH (Fig. 3B). In further purification using the gradient TFA method a total of 0.3 ng of irGnRH was detected in fractions 22–24 (Fig. 3C). After application of these fractions to the phenyl column with TFA in the mobile phase, 0.3 ng of irGnRH was detected in fractions 22 and 23 (Fig. 3D).

Elution of the irGNRH material from the hydrophilic column resulted in several small peaks. GnRH-like material was detected using anti-lamprey (36–52) in HILIC fractions 4 (0.4 ng), 25, 31–32 (0.64 ng), and 44 (0.31 ng). Antiserum R-42 also detected immunoreactive GnRH in fractions 25, 31–32, and 44. Tunicate GnRH-I antiserum detected GnRH immunoreactivity in fractions 8, 24, 32, and 48, whereas antiserum 7CR-10 detected irGnRH only in fraction 44. The immunoreactive material was purified further, but insufficient material was available for determination of sequence or mass.

DISCUSSION

A close phylogenetic relationship between hemichordates and chordates, first suggested by Bateson (1885), is now widely accepted. Some evidence from molecular biology (Holland *et al.,* 1991) supports the idea of the hemichordates as an early offshoot from the chordate line of evolution. The finding of GnRH in enteropneusts provides further support for this view as GnRH is prototypically a chordate line hormone. Other studies link the group with the echinoderms (Wada and Satoh, 1994; Turbeville *et al.,* 1994; Halanych, 1995). It now becomes highly desirable to obtain the amino acid sequence for saccoglossan GnRH for the evidence it may provide regarding early evolution of this important molecule in the chordate lineage.

The characterization of this irGnRH-like peptide by HPLC-RIA is not unlike the HPLC elution pattern seen in the purification of other vertebrate and invertebrate GnRH peptides by this method (Sherwood *et al.,* 1986; Powell *et al.,* 1996). Indeed, the presence of at least one GnRH peptide is supported by the repeated application, elution, and detection of irGnRH from a new C18 HPLC column in repeated HPLC procedures. The detection of GnRH-like immunoreactivity with several antisera following hydrophilic interactive chromatography strengthens the hypothesis that GnRH-like material exists in *Saccoglossus* and suggests that more than one form of GnRH may exist.

Furthermore, this study parallels the work by Kelsall and co-workers (1990) who detected irGnRH by immunohistochemistry and in HPLC eluates of the ascidian *C. productum*. This material was later identified by primary structure (Powell *et al.,* 1996). The detection of only one form of irGnRH in HPLC eluates of *Saccoglossus* suggests either that additional GnRH peptides were not detected by the antisera or that there is only one form of GnRH in hemichordates. The presence of two distinct GnRH peptides in *Chelyosoma* may mean that a gene duplication occurred in ancestral tunicates after they separated from hemichordates. Until *Saccoglossus* GnRH is sequenced, such evolutionary questions cannot be answered.

In vertebrates and ascidians, GnRH is typically a secretion product of neurons. Thus, it is not surprising to find that in *Saccoglossus* and *Ptychodera* irGnRH is found only in the mulberry cells, which are probably neurons. The anatomy of the mulberry cell is suggestive of a neurosecretory cell that releases its contents to the exterior. If so, the peptide would presumably mix with the mucus covering the skin or diffuse into the surrounding sea water. In vertebrates and ascidians by contrast the cytological evidence is entirely consistent

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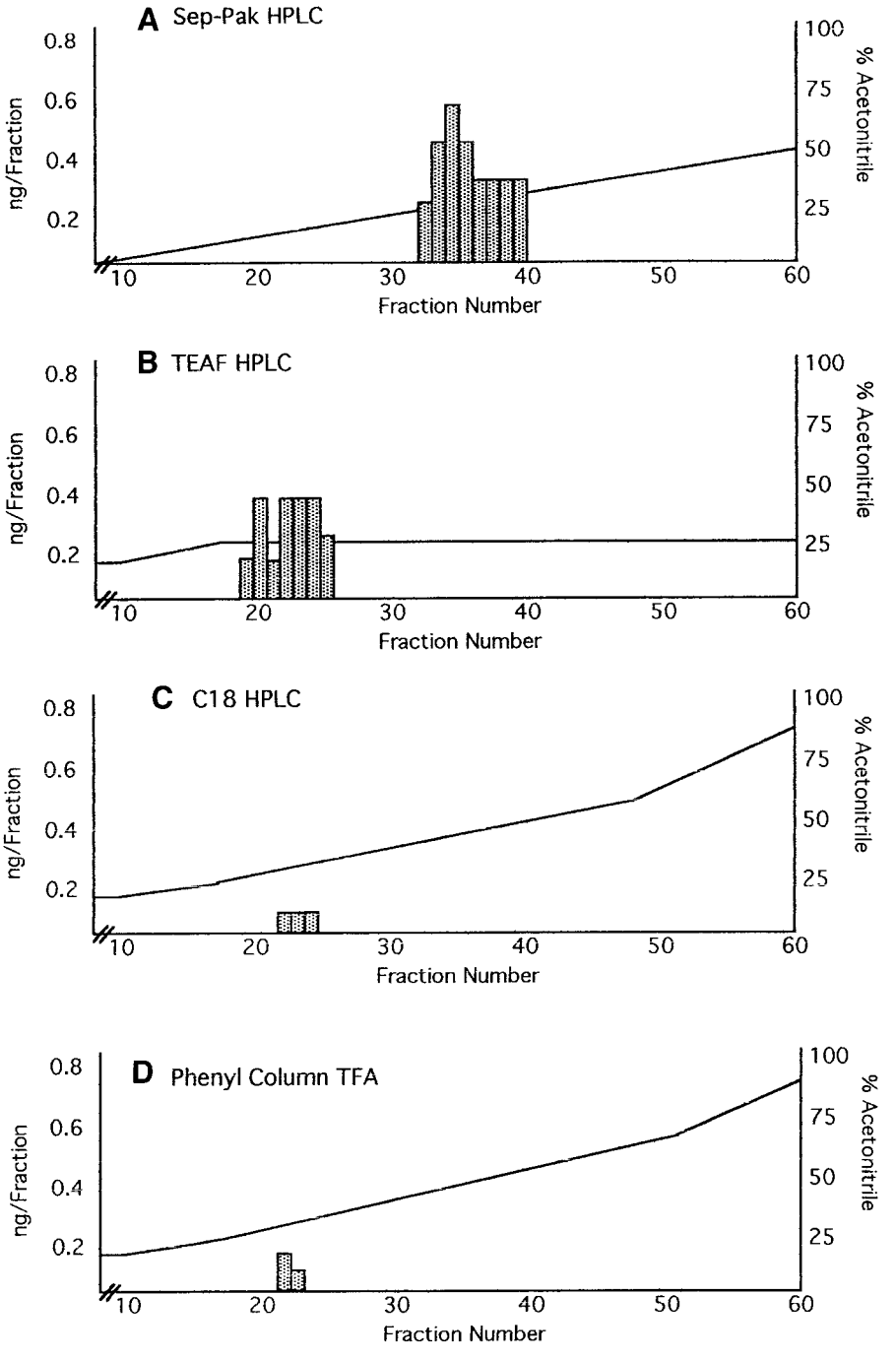

FIG. 3. HPLC analysis of immunoreactive GnRH from *S. bromophenolosus* extract. (A) irGnRH in eluates from a Sep-Pak column with TFA in the mobile phase. (B) irGnRH in eluates from a C18 column with TEAF in the mobile phase. (C) irGnRH in eluates from a C18 column with TFA in the mobile phase. (D) irGnRH in eluates from a phenyl column with TFA in the mobile phase. Solid lines indicate % acetonitrile in the mobile phase.

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with a purely endocrine role and there is no reason to suppose that GnRH is liberated to the exterior. The basal neurites of the mulberry cells show little if any irGnRH, which suggests that the peptide is not used as a transmitter or modulator at synapses in the fiber layer and is not released internally as a hormone. On the basis of present evidence we therefore propose an exocrine (possibly pheromonal) role for saccoglossan GnRH.

REFERENCES

Anctil, M. (1997). Gonadotropin releasing hormone-like factor in a primative metazoan: Immunohistochemical evidence and bioactivity. *In* ''Advances in Comparative Endocrinology'' (S. Kawashima and S. Kikuyama, Eds.), pp. 703–707. Monduzzi Editore, Bologna.

Bateson, W. (1885). The later stages in the development of *Balanoglossus kowalevskii*, with a suggestion as to the affinities of the Enteropneusta. *Q. J. Micr. Sci.* **25**(Suppl.), 81–122.

Brambell, F. W. R., and Cole, H. A. (1939). The preoral ciliary organ of the Enteropneusta: Its occurence, structure, and possible phylogenetic significance. *Proc. Zool. Soc. Lond. B* **109,** 181–193.

Bullock, T. H. (1945). The anatomical organisation of the nervous system of Enteropneusta. *Q. J. Micr. Sci.* **86,** 55–111.

Bullock, T. H., and Horridge, G. A. (1965). ''Structure and Function in the Nervous Systems of Invertebrates.'' Freeman, San Francisco.

Cameron, C. B., and Mackie, G. O. (1996). Conduction pathways in the nervous system of *Saccoglossus* (Enteropneusta). *Can. J. Zool.* **74,** 15–19.

Dawydoff, C. (1948). ''Classe des Entèropneustes: Traitè de Zoologie, Vol. XI, ''Echinodermes, Stomocordès'' (P.-P. Grassè, Ed.). Procordès. Masson, Paris.

Dufour, S., Monniot, F., Monniot, C., Baloche, S., Kerdelhue, B., and Fontaine, Y.-A. (1988). Dosage radioimmunologique, chez *Ascidiella aspersa*, d'un facteur de type gonadolibèrine (GnRH) de poids molèculaire apparent supèrieur celui du dècapeptide mammalien. *C. R. Acad. Sci. Paris* **306,** 253–256.

Georges, D., and Dubois, M. P. (1980). Mise en èvidence par les techniques d'immunofluorescence d'un antigëne de type LH-RH dans le systëme nerveux de *Ciona intestinalis* (Tunicier ascidiacè). C. R. Acad. Sci. Paris **290,** 29–31.

Goldberg, J. I., Garofalo, R., Price, C. J., and Chang, J. P. (1993). Presence and biological activity of a GnRH-like factor in the nervous system of *Helisoma trivolvis*. *J. Comp. Neurol.* **336,** 571–582.

Halanych, K. (1995). The phylogenetic position of the pterobranch hemichordates based on 18S rDNA sequence data. *Mol. Phylog. Evol.* **4,** 72–76.

Holland, P. W. H., Hacker, A. M., and Williams, N. A. (1991). A molecular analysis of the phylogenetic affinities of *Saccoglossus cambrensis* Brambell & Cole (Hemichordata). Phil. Trans R. Soc. London B **332,** 185–189.

Hyman, L. H. (1959). ''The Invertebrates,'' Vol. 5, ''Smaller coelomate groups.'' Mcgraw–Hill, New York.

Kelsall, R., Coe, I. R., and Sherwood, N. M. (1990). Phylogeny and ontogeny of gonadotropin-releasing hormone: Comparison of guinea pig, rat and a protochordate. *Gen. Comp. Endocrinol.* **78,** 479–494.

King, G. M., Giray, C., and Kornfield, I. (1994). A new hemichordate *Saccoglossus bromophenolosus* (Hemichordata: Enteropneusta: Harrimaniidae), from North America. *Proc. Biol. Soc. Wash.* **107,** 383–390.

Knight-Jones, E. W. (1952). On the nervous system of *Saccoglossus cambrensis* (Enteropneusta). *Phil. Trans. R. Soc. London B* **236,** 315–354.

Pardos, F., and Benito, J. (1989). Ultrastructure of the pharynx of *Glossobalanus minutus* (Enteropneusta). II. Secretory cells. *Z. Mikrosk. Anat. Forsch.* **103,** 748–767.

Pickens, P. E. (1970). Conduction along the ventral nerve cord of a hemichordate worm. *J. Exp. Biol.* **53,** 515–528.

Powell, J. F. F., Reska-Skinner, S. M., Prakash, M. O., Fischer, W. H., Park, M., Rivier, J. E., Craig, A. G., Mackie, G. O., and Sherwood, N. M. (1996). Two new forms of gonadotropin-releasing hormone in a protochordate and the evolutionary implications. *Proc. Natl. Acad. Sci. USA* **93,** 10461–10464.

Ruppert, E. E., and Barnes, R. D. (1994). ''Invertebrate Zoology.'' Saunders College Publishing, Fort Worth.

Schneider, K. C. (1902). ''Lehrbuch der Vergleichenden Histologie der Tiere.'' Fischer, Jena.

Sherwood, N., Eiden, L., Brownstein, M., Spiess, J., Rivier, J., and Vale, W. (1983). Characterization of a teleost gonadotropin-releasing hormone. *Proc. Natl. Acad. Sci. USA* **80,** 2794–2798.

Sherwood, N. M., Sower, S. A., Marshak, D. R., Fraser, B. A., and Brownstein, M. J. (1986). Primary structure of gonadotropin-releasing hormone from lamprey brain. *J. Biol. Chem.* **261,** 4812–4819.

Sherwood, N. M., Doroshov, S., and Lance, V. (1991). Gonadotropin-releasing hormone (GnRH) in bony fish that are phylogenetically ancient: Reedfish (*Calamoichthys calabaricus*), sturgeon (*Acipenser transmontanus*), and alligator gar (*Lepisosteus spatula*). *Gen. Comp. Endocrinol.* **84,** 44–57.

Sherwood, N. M., Lovejoy, D. A., and Coe, I. R. (1993). Origin of mammalian gonadotropin-releasing hormones. *Endocrine Rev.* **14,** 241–254.

Turberville, J. M., Schulz, J. R., and Raff, R. R. (1994). Deuterostome phylogeny and the sister group of the chordates: evidence from molecules and morphology. *Mol. Biol. Evol.* **11**(4), 648–655.

Wada, H., and Satoh, N. (1994). Details of the evolutionary history from invertebrates to vertebrates, as deduced from the sequences of 18S rDNA. *Proc. Natl. Acad. Sci. USA* **91,** 1801–1804.

Welsch, U. (1984). Hemichordata. *In* ''Biology of the Integument,'' Vol. 1, ''Invertebrates'' (J. Bereiter-Hahn, A. G. Matoltsy, and K. S., Richards, Eds.), pp. 790–799. Springer, Berlin.

Zamboni, L., and DeMartino, C. (1967). Buffered picric acid-formaldehyde: A new, rapid fixative for electron microscopy. *J. Cell. Biol.* **35,** 148a.

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