Evidence that gonadotropin-releasing hormone (GnRH) functions as a prolactin-releasing factor in a teleost fish (*Oreochromis mossambicus*) and primary structures for three native GnRH molecules

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Abstract

Three forms of gonadotropin-releasing hormone (GnRH) are isolated and identified here by chemical sequence analysis for one species of tilapia, *Oreochromis niloticus*, and by HPLC elution position for a second species of tilapia, *O. mossambicus*. Of the three GnRH forms in *O. mossambicus*, chicken GnRH-II (cGnRH-II) and sea bream GnRH (sbGnRH) are present in greater abundance in the brain and pituitary than salmon GnRH (sGnRH). These three native forms of GnRH are shown to stimulate the release of prolactin (PRL) from the rostral pars distalis (RPD) of the pituitary of *O. mossambicus* in vitro with the following order of potency: cGnRH-II > sGnRH > sbGnRH. In addition, a mammalian GnRH analog stimulated the release of PRL from the pituitary RPD incubated in either iso-osmotic (320 mosmol/l) or hyperosmotic (355 mosmol/l) medium, the latter normally inhibiting PRL release. The response of the pituitary RPD to GnRH was augmented by co-incubation with testosterone or 17β-estradiol. The effects of GnRH on PRL release appear to be direct effects on PRL cells because the RPD of tilapia contains a nearly homogeneous mass of PRL cells without intermixing of gonadotrophs. Our data suggest that GnRH plays a broad role in fish, depending on the species, by affecting not only gonadotropins and growth hormone, but also PRL.

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Introduction

Teleosts, like most vertebrates other than placental mammals, have multiple forms of gonadotropin-releasing hormone (GnRH) that differ in their spatial distribution throughout the brain (Sherwood et al. 1994). The presence of multiple forms of GnRH together with multifarious distributions of these forms has led to speculation that separate functions may be subserved by distinct GnRH peptides. These functions may extend beyond the regulation of gonadotropins (GtH) since GnRH has also been shown to function as a regulator of growth hormone (GH) in several teleosts, including tilapia (Marchant et al. 1989, Melamed et al. 1995).

Growth hormone and prolactin (PRL) are part of the same polypeptide family and are thought to be derived from a common ancestral gene (Niall et al. 1971). Not surprisingly, PRL and GH are regulated by many of the same factors: somatostatin, cortisol and changes in osmotic pressure (Nishioka et al. 1988). Despite these commonalities between GH and PRL, and the knowledge that GnRH regulates GH in some species, the effects of GnRH on PRL release in teleosts has received little attention.

There is little direct evidence to support or oppose a role for GnRH as a regulator of PRL in teleosts. In the Atlantic salmon, injections of GnRH have been shown to increase PRL cell activity, based on light and electron microscopical evidence (Ekenengren et al. 1978). It was thought, however, that this effect was mediated via GtH and in turn 17β-estradiol (E2). GnRH did not bind to PRL cells of the goldfish, suggesting that it may not be a direct regulator of PRL cell function in this species (Cook et al. 1991).

In the rat, GnRH stimulates PRL release indirectly via paracrine secretions of GtH cells (Denef & Andries 1983,
Lamberts et al. (1989). There is strong evidence that angiotensin II from gonadotrophs is a paracrine mediator in this interaction (Saavedra 1992). PRL cells are found in very close association with GtH cells in mammals (Sato 1980). In contrast, the PRL cells in the tilapia are segregated into the anterior most region of the anterior pituitary, the rostral pars distalis (RPD), as a nearly homogeneous mass, whereas the GtH cells are located in the posterior region of the anterior pituitary, the proximal pars distalis (Bern et al. 1975). This anatomical arrangement of the tilapia pituitary gland makes a paracrine-mediated effect through GtH cells less likely.

The objectives of the present study were to: (1) identify GnRH peptides native to tilapia, based on primary structure; (2) determine which forms of GnRH reach the pituitary; (3) determine whether GnRH injections affect serum concentrations of PRL; (4) determine whether GnRH alters the release of PRL from tilapia RPD and characterize the effect of medium osmolality on this release; (5) compare potencies of the native GnRH forms in releasing PRL; and (6) determine whether the steroid hormones, testosterone and E2, alter the effect of GnRH on PRL cells. The RPD of the tilapia secretes two distinct PRL molecules, one composed of 177 amino acid residues (tPRL177) and a second composed of 188 amino acid residues (tPRL188) (Specker et al. 1985, Yamaguchi et al. 1988). Effects of GnRH on both PRLs were examined.

Materials and Methods

Extraction of GnRH peptides from tissues

Frozen tissues were powdered using a cold mortar and pestle or a Waring Blender cooled with liquid nitrogen. Extraction of peptides with HCl and acetone, and subsequent removal of substances soluble in petroleum ether were performed as detailed previously (Ngamvongchon et al. 1992). The volume of the extracts was reduced in a vacuum centrifuge before application to a HPLC column.

Purification of GnRH peptides

The brains and pituitaries of 7000 (1-25 kg) O. niloticus of mixed sex were removed and frozen on dry ice. Tissues were shipped on dry ice from the National Aquaculture Genetics Research Institute, Bangkok, Thailand to the University of Victoria and stored at -80 °C until extracted for peptides. Ten Sep-Pak cartridges were assembled in series to form an HPLC column. The tilapia brain extract was divided and loaded on to two Sep-Pak columns using a peristaltic pump. The Sep-Pak columns were attached to a Beckman model 166 HPLC apparatus. A flow rate of 1 ml/min was used for the program described in Table 1, step 1. Fractions of 1 ml were collected.

An aliquot of 10 µl from each fraction was assayed for immunoreactive GnRH (irGnRH) and the fractions with irGnRH combined from both Sep-Pak HPLC eluates. These fractions were reduced in volume and applied to a C18 column not previously exposed to standards. Aliquots of 800 µl were applied to the column in six repeated injections at a flow rate of 1 ml/min using solvents and a program described in Table 1, for step 2. Fractions of 1 ml were collected for 60 min and an aliquot of 10 µl assayed for irGnRH. Four areas of irGnRH were identified in fractions and were treated separately thereafter.

Pooled fractions of each irGnRH region were combined, reduced in volume and applied to the HPLC.
column (step 3, Table 1; Rivier 1978). Fractions (1 ml) were collected for 50 min and assayed for irGnRH. All four previously identified groups of irGnRH were applied to the column in succession before the solvents were changed in the next step of the purification.

Fractions containing irGnRH were combined, reduced in volume and applied to a phenyl column with a 1 ml/min flow rate (step 4, Table 1). Fractions (1 ml) were collected for 50 min and assayed for irGnRH. An 800 µl portion of solution A was injected on to the column in advance of sample application and run as a blank to determine residual contamination. The fractions were dried and assayed for irGnRH.

The first two areas of irGnRH to be identified in the triethylammonium formate (TEAF) method were further purified by individual application to the phenyl column using an isocratic program. Fractions with irGnRH were combined, reduced in volume and applied to the column (step 5, Table 1). Fractions (1 ml) were collected for 60 min and assayed for irGnRH.

Fractions that contained partially purified GnRH were reduced in volume, frozen on dry ice and shipped to the Salk Institute, La Jolla, CA. Fractions were thawed and aliquots (10%) were applied to a narrow-bore HPLC column using a linear gradient with increasing concentrations of acetonitrile in 0·05% aqueous trifluoroacetic acid. Fractions were collected on the basis of their absorbance at 210 nm. Another aliquot of partially purified preparations was subjected to digestion with pyroglutamyl aminopeptidase followed by HPLC separation under the same conditions as above (Fischer & Park 1992). Material that shifted its elution position was then analyzed by automated Edman degradation on an Applied Biosystems protein sequencer (470A).

**RIA for GnRH**

For the purification of GnRH in *O. niloticus* extracts, 10 µl aliquots were used in RIA, whereas 100 µl aliquots were dried before determination of irGnRH for *O. mossambicus* HPLC samples. Methods for detection of irGnRH in fractions were as described (Sherwood et al. 1984, 1986). Antiserum GF-4 (1:25 000 final dilution) was used to detect irGnRH with iodinated mammal GnRH (mGnRH) used as tracer and mGnRH used as the standard. The cross-reactivity of the antiserum for several known forms of GnRH has been reported (Kelsall et al. 1990, Sherwood et al. 1991) and is summarized in Results.

This assay was selected because we found previously that the assay detects many forms of GnRH because of the broad cross-reactivity of antiserum GF-4 with many GnRH forms (Kelsall et al. 1990, Sherwood et al. 1991). This strategy is important in examining brains in which the forms of GnRH cannot be predicted before the study. In this assay, the sbGnRH and mGnRH standard curves are nearly parallel. However, the cross-reactivity is greater for sbGnRH and less for sGnRH and cGnRH-II as discussed in Results.

**Brain and pituitary content of GnRH forms in *O. mossambicus***

A sample of 100 brains and a sample of 50 pituitaries were collected from reproductively mature *O. mossambicus* of mixed sex at the University of Hawaii. The tissues were frozen in liquid nitrogen, shipped to the University of Victoria and stored at −80 °C. The peptides were extracted separately from the two tissues and the volume reduced. Aliquots of 800 µl were applied to a C18 HPLC column in two injections at 5% solution B (0·1 M heptfluorobutyric acid (HFBA) in 75% acetonitrile–25% water) and 95% solution A (0·1 M HFBA in water) and a flow rate of 1 ml/min. After 10 min, a gradient increase of 1% solution B per minute was initiated and continued for 65 min. Fractions of 1 ml were collected and an aliquot of 100 µl assayed for irGnRH. After the column was again equilibrated to 5% solution B, synthetic standards were applied to the column using the same method as for brain extract. A mixture of seven known synthetic GnRH peptides (catfish, dogfish, lamprey, chicken-I, chicken-II, salmon and mammalian) was injected at a concentration of 200 ng each. Synthetic sbGnRH was not available to apply to the column. Fractions (1 ml) were collected for 60 min and the elution positions of the standards were determined by the assay of 10 µl aliquots for irGnRH.

**Synthesis of peptides**

sGnRH, cGnRH-II and sbGnRH were synthesized using a solid phase method on a methylbenzylamine resin as described (Rivier et al. 1992, Powell et al. 1994).

**Animals for physiology studies**

Tilapia (*O. mossambicus*) were collected from brackish water streams and maintained in fresh water in outdoor tanks at a temperature of 22–25 °C for at least 2 months before use. The fish were fed to satiation twice daily with Purina trout chow (Purina Mills, Inc., St Louis, MO, USA). Fish were taken directly from these tanks for use in *in vitro* studies. All fish were reproductively mature adults and ranged in size from 70 to 120 g body weight.

**Injection study**

Mature male and female tilapia were moved to indoor oval fiberglass tanks (60 liters) supplied with a continuous flow of fresh water and constant aeration. The photoperiod was maintained at 14 h light:10 h darkness. The fish were placed eight individuals per tank and acclimated to these conditions for at least 2 weeks before the injections. The
fish were not fed on the day of sampling. Fish were anesthetized with 2-phenoxethanol (Sigma, St Louis, MO, USA) at a concentration of 1 ml/l for 1 min before being weighed and given intraperitoneal injections of mGnRH analog (mGnRHα; des-Gly10, D-Ala5]-mGnRH) dissolved in 0.9% saline or saline for injected controls. Non-injected controls were also anesthetized. Fish received 0.1 µg/g body weight of mGnRHα in an injection volume of 1 µl/g body weight. Fish sampled at 11 h after injection were injected 9 h before the other treatments so that all treatments were sampled between 1500 and 1800 h. Fish were also anesthetized for blood collection.

**Static incubations**

Mature male tilapia were decapitated and the pituitary removed. Each pituitary was dissected and the RPD containing a nearly homogeneous (95–99%) population of PRL cells was placed individually in wells of 96-well culture plates with 100 µl Krebs bicarbonate–Ringer solution containing glucose, L-glutamine and minimal essential medium (Wigham et al. 1977). Osmolality of the incubation medium was adjusted by varying the concentration of NaCl and measured using a vapor pressure osmometer. The tissues were incubated at 28 °C under a humidified atmosphere of 95% O₂/5% CO₂ and placed on a gyratory platform (80 r.p.m.). The GnRHs were dissolved in hyperosmotic medium to 1m M and the steroid hormones (Sigma) were dissolved in absolute ethanol to 1m M before dilution in medium. In studies that included steroid hormones dissolved in ethanol, all treatments received equal volumes of ethanol. The cGnRH-I, sGnRH and cGnRH-II used in the study comparing these three forms were purchased from Peninsula Laboratories, San Carlos, CA, USA; the mGnRHα was purchased from Sigma. The sbGnRH, sGnRH and cGnRH-II used in all other studies were synthesized at the Salk Institute.

**Perifusion incubations**

For perifusion, the pituitary RPD regions from mature males were placed eight per chamber in six parallel chambers. The perifusion apparatus has been described (Grau et al. 1986, Weber 1995). The tissues were pre-incubated in control medium (355 mosmol/l) overnight. sbGnRH was introduced as 5 min pulses in graded doses of 0, 0.1, 1, 10, 100 and 1000 nM with 2 h between pulses. Fractions were collected every 10 min. Half of the chambers received increasing concentrations of GnRH and the other half received decreasing concentrations.

**Quantification of PRL forms**

The PRL forms within tissue and medium for the 18–20 h incubations were measured by separating the PRLs using SDS slab gel electrophoresis and quantifying hormone content by densitometry of Coomassie brilliant blue R–250-stained bands (Kelley et al. 1988). PRLs in serum, tissue and medium from 3 h incubations and perifusion incubations were measured using the homologous RIAs (Ayson et al. 1993). For all static incubations, the release of PRLs into the incubation medium was normalized as a percentage of the total hormone in the tissue and the medium. For perifusion incubations, the data are expressed as percentage of baseline release, which is the sum of the concentrations of hormone in each of the three fractions following and including the introduction of the GnRH, divided by the sum of the hormone in the three fractions preceding the introduction of the GnRH, expressed as a percentage.

**Statistical analysis**

Differences among groups were determined using analysis of variance and the least significant difference test for a priori pairwise comparisons (LSD) analysis. Owing to the greater response to GnRH than to the steroid hormones, the mean sum of squares used in the LSD analysis was derived using only values from groups being compared (Snedecor & Cochran 1980). Experiments with only two groups were analyzed using the unpaired Student’s t-test. Data are expressed as means ± s.e.m.

**Results**

**Purification of GnRH from O. niloticus**

RIA after Sep-Pak HPLC detected a total of 10.0 µg irGnRH. Values reported for the purification procedure are not corrected for cross-reactivity because the peaks may overlap in some HPLC programs and the purpose is only to obtain sufficient material for identification. Further separation using TEAF HPLC resulted in four areas of irGnRH that were called tilapia (T) I–IV corresponding to their elution times (Fig. 1). T-I had 3.5 µg irGnRH in fractions 21 and 22. T-II had 1.9 µg irGnRH in fractions 25–27. T-III had 0.35 µg in fractions 38–40 and T-IV had 2.4 µg irGnRH in fractions 46–49. In the next step, application of the individual areas to TEAF HPLC resulted in detection of irGnRH as shown in Table 2. Application of the irGnRH areas to a phenyl column resulted in the detection of irGnRH as also shown in Table 2. Finally, isocratic HPLC using a phenyl column for further purification yielded irGnRH in fractions 17–20 for both T-I (1.5 µg) and T-II (1.1 µg).

**Characterization of the primary structure**

Partially purified tilapia GnRH peptides (T-I–IV) were further purified by narrow-bore HPLC. Chemical
sequence analysis was performed on samples after enzymatic removal of the N-terminal pyroglutamic acid residue. Based on the data obtained, the primary structures were deduced as follows. Sequence data from the irGnRH areas were determined to be: T-I, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Ser-Pro-Gly; T-II, pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly; T-IV, pGlu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly.

These sequences correspond to the known GnRH peptides of sbGnRH, cGnRH-II and sGnRH for T-I, T-II and T-IV respectively. T-III, although low in amount, was sequenced and found to be identical with sGnRH.

Brain and pituitary content of GnRH in O. mossambicus

Three areas of irGnRH were identified in the initial HPLC profile of the brain extract of O. mossambicus (Fig. 2). These three areas coincided with the elution positions of the synthetic standards cGnRH-I (21·6 ng), cGnRH-II (3·2 ng) and sGnRH (4·8 ng). However, we have demonstrated that cGnRH-I and sbGnRH are coeluted using this HPLC method (Powell et al. 1995). Furthermore, using the same assay as in the present study in which mGnRH is the tracer and standard, we determined the cross-reactivity of GF-4 with different GnRH peptides at 50% binding. The measured concentration of each peptide at 50% binding compared with that of mGnRH produced the following results: mGnRH, 100%; sbGnRH, 120%; cGnRH-II, 4%; and sGnRH, 69%. If the amount of GnRH detected in our HPLC fractions is adjusted for percentage cross-reactivity, the amounts are 18·0 ng sbGnRH, 80·0 ng cGnRH-II and 7·0 ng sGnRH. Fifty pituitaries from O. mossambicus contained 40 ng sbGnRH, 125 ng cGnRH-II and 0·4 ng sGnRH (values before

![Figure 1](https://example.com/figure1.png)

Figure 1 Step 2 of the HPLC purification of GnRH from brain extract of O. niloticus using an isocratic method with TEAF. Numbers at the top of the bars represent ng irGnRH detected in each fraction. The areas of high irGnRH levels are designated T-I (solid bars), T-II (cross-hatched bars), T-III (striped bars) and T-IV (hatched bars). Solid line indicates acetonitrile.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>irGnRH detected in eluates of tilapia brain extract in the purification procedure. Amounts indicated and the fractions containing irGnRH were combined for further purification in subsequent steps. Tilapia GnRH T-III and T-IV were not further purified by the isocratic method using the phenyl column</th>
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<tr>
<td>T-I</td>
<td>T-II</td>
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<tr>
<td>Amount (µg)</td>
<td>Fraction(s)</td>
</tr>
<tr>
<td>TEAP</td>
<td>3·6</td>
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<tr>
<td>Phenyl TFA</td>
<td>3·4</td>
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<tr>
<td>Isocratic TFA</td>
<td>1·5</td>
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TFA=trifluoroacetic acid.
correcting for cross-reactivity are: 48 ng sbGnRH, 5 ng cGnRH-II and 0.3 ng sGnRH). Hence cGnRH-II and sbGnRH are more abundant in the brain and pituitary than sGnRH.

Effects of mGnRHa injections on serum concentrations of PRL

Intraperitoneal injections of 0.1 µg mGnRHa/g body weight elevated serum tPRL188 and tPRL177 concentrations in mature male and female tilapia at 1 h after injection over levels observed in vehicle-injected and non-injected controls (Fig. 3). Serum hormone concentrations were not significantly different from controls at 11 h after injection. Injections did not alter GH serum levels in any group (data not shown).

Effects of mGnRHa on the in vitro release of PRL from RPD incubated in iso-osmotic medium

To determine the response of PRL cells to GnRH, RPD were incubated for 18–20 h in isosmotic medium (320 mosmol/l) containing graded concentrations of mGnRHa ranging from 0.01 nM to 1 µM. Tissues were incubated in isosmotic medium (320 mosmol/l) which elicits moderate baseline PRL release so that either a stimulatory or inhibitory effect of GnRH on PRL release could be observed. Incubation with mGnRHa stimulated the release of tPRL177 and tPRL188 with the greatest response elicited at a concentration of 10 nM mGnRHa (Fig. 4). Release is variable with isosmotic pressure. For this reason, data from each of two replicate experiments were normalized as a percentage of controls before being combined.

Effects of medium osmolality on mGnRHa-stimulated release of PRL from RPD

One purpose of this study was to determine whether GnRH can overcome the effects of medium osmolality on PRL release. Pituitary RPD were incubated for 18–20 h in hyposmotic medium (300 mosmol/l), isosmotic medium (320 mosmol/l) or hyperosmotic medium (355 mosmol/l) with graded concentrations of mGnRHa. All osmolalities used in this study are within the physiological range of the animals. PRL release of both PRL forms showed a typical inverse correlation with medium osmolality (Fig. 5). Release of both PRLs was stimulated by mGnRHa at a concentration of 100 nM when incubated in isosmotic and hyperosmotic medium (P<0.01 and P<0.001 respectively). There was no significant difference among treatments displaying the greatest stimulation.
for each osmolality, possibly because stimulation was near-maximal under each condition. Better separation of treatment effects is observable with hyperosmotic medium because of a lower baseline release. For this reason, subsequent experiments were conducted using hyperosmotic medium.

**Relative potency of GnRH forms on the release of PRL from RPD**

The potency of the GnRH forms was compared using 18–20 h static incubations of RPD in hyperosmotic medium. These incubations differ from the previous incubations in that the tissues were preincubated in hyperosmotic control medium overnight and the medium was changed and tissues rinsed 3 times before the treatment medium was added. This long preincubation reduced baseline release and provided a better separation of responses. In both sets of experiments, similar responses were observed for tPRL$_{188}$ and tPRL$_{177}$. In the first set of experiments (Fig. 6), cGnRH-I, a form not found in the tilapia, was the least potent form of GnRH tested, not eliciting a significant response until a concentration of 1000 nM. Both native forms of GnRH, sGnRH and cGnRH-II, were more potent than cGnRH-I. A significant response with sGnRH was observed with 1 nM sGnRH. cGnRH-II was found to be the most potent of the three forms tested. A significant response was observed with the lowest concentration tested, 0.01 nM cGnRH-II. All three forms evoked the maximal response observed. When the three forms of GnRH that are native to the tilapia were compared (Fig. 7), cGnRH-II was again found to be the most potent form of the native GnRHs, followed by sGnRH. sbGnRH was the least potent for stimulating the release of both PRLs.

**Effects of sbGnRH on PRL release in perfusion**

Single short exposures (5 min pulse) of RPD tissue to sbGnRH stimulated the release of both PRLs in a dose-dependent manner at concentrations similar to that observed with the long-term static incubations (Fig. 8). A stimulation was first observed at a concentration of 10 nM sbGnRH.
Effects of testosterone and E2 on sGnRH-induced PRL release

Treatment with sGnRH (100 nM), testosterone (10 nM) and E2 (10 nM) stimulated the release of both PRLs from RPD during 3 h incubations in hyperosmotic medium, after an overnight incubation in control medium (Fig. 9). Combined treatments resulted in greater release of both PRLs than either sGnRH or steroid treatments alone.

Discussion

This is the first report of the isolation and peptide sequencing of three distinct forms of GnRH from any species. Therefore this is the first study to demonstrate that tissues are producing the three proteins and cleaving the precursor into the peptides. Previous studies have suggested that the three forms of tilapia GnRH are present in the brain of other fishes in the order Perciformes, including sea bream, Sparus aurata, pumpkinseed, Lepomis gibbosus, and another species of cichlid, Haplochromis burtoni (Powell et al. 1994, 1995). In previous studies the primary sequence was determined for two forms in sea bream and for one form in H. burtoni, whereas HPLC elution position was used to identify the other forms.

The identification by primary structure of the third and fourth HPLC eluted regions from tilapia brain extracts as sGnRH confirms our earlier observation on another species that sGnRH can be eluted in two positions. We found that chum salmon brain extracts produced three HPLC regions; two of these regions were identical in peptide sequence, including the C-terminal amidation, with sGnRH (J F F Powell, unpublished observation). It is possible that the T-III fraction contains a carrier protein that shifts the elution position of part of the sGnRH.

Although pituitary peptide content does not distinguish between synthesis and release, the content does indicate that all three GnRH peptides are present, presumably in axon endings, in the tilapia pituitary. In the tilapia O. mossambicus and the cichlid H. burtoni, in situ hybridization studies show that neuronal cell bodies synthesizing sGnRH are in the olfactory region, the cGnRH-II-producing cells are in the midbrain and the sbGnRH-producing cells are in the preoptic region (White et al.
Each set of neurons has axons that are widely distributed in the brain, as shown in several species of fish including _O. mossambicus_ (Kah et al. 1986, Amano et al. 1991, Leprette et al. 1993, Parhar & Iwata 1994). However, the immunocytochemical studies do not distinguish the specific type of GnRH axons that enter the pituitary. Here, the tilapia GnRH terminals in the pituitary are shown by indirect means using HPLC analysis of pituitary tissue to contain all three forms of GnRH with greater storage of sbGnRH and cGnRH-II.

Our studies provide evidence that two native forms of GnRH that are abundant in the pituitary gland of the tilapia, sbGnRH and cGnRH-II, function as PRL-releasing factors. Incubation of pituitary RPD in static culture and in perifusion with sbGnRH and cGnRH-II increased the release of tPRL177 and tPRL188 from PRL cells. The dosages used in these studies are consistent with receptor-mediated action and concentrations shown in previous studies to stimulate GtH and GH release from pituitary fragments of teleosts including tilapia (Levavi-Sivan & Yaron 1989, Peter et al. 1990, Melamed et al. 1995). The large differences in potencies among GnRH molecules and inhibitory effects of somatostatin and urotensin II on PRL release from RPD of _O. mossambicus_ (Grau et al. 1982) suggest that PRL-induced release by GnRH is not a non-specific effect of small peptides. Likewise, thyrotropin-releasing hormone does not alter PRL release from RPD unless the tissues are exposed to E2 (Barry & Grau 1986).

sbGnRH and cGnRH-II were able to stimulate PRL release when tissues were incubated in hyperosmotic medium, demonstrating that GnRH is a potent stimulator of PRL release. Consistent with the role of PRL in osmoregulation, PRL cells are sensitive to changes in the osmolality of the medium (Grau et al. 1994). PRL release from RPD is decreased during incubation in hyperosmotic medium and increased when incubated in hyposmotic medium (Wigham et al. 1977). We found that physiological doses of GnRH were able to overcome this inhibition and increase PRL release to levels observed with tissues incubated in hyposmotic medium.

Injections of mGnRHa increased serum PRL concentrations, demonstrating GnRH activity _in vivo_. We concentrated our efforts on _in vitro_ methods because injections of GnRH may act indirectly to stimulate PRL release. Injections of GnRH almost certainly lead to an increase in GtH release which in turn lead to increased release of gonadal steroids. We present data that confirm earlier findings that testosterone and E2 stimulate PRL release in the tilapia (Barry & Grau 1986).

The stimulation of PRL release from RPD shows that the pathway of GnRH action on PRL cells of the tilapia...
clearly differs from that of the rat and may differ from that of the goldfish. In the rat, GnRH has been shown to stimulate PRL release via paracrine action involving GtH cells (Lamberts et al. 1989). The RPD of the tilapia does not contain either GtH cells or GH cells. Thus the effects of GnRH on PRL release in our incubation studies cannot be mediated via GtH or GH cells. Cook and colleagues (1991) found negligible binding of an sGnRH analog to PRL cells of the goldfish compared with binding to GtH and GH cells. Either the PRL cells of the goldfish do not respond to GnRH or the receptors are not always expressed. The direct regulation of PRL cells by GnRH may be a late development in the evolution of teleosts. Direct regulation of PRL cells by GnRH may be a characteristic of highly derived teleosts such as the tilapia but not of lower teleosts such as the goldfish or of mammals. Investigations of GnRH receptors on PRL cells of tilapia and of the effects of GnRH on PRL release in other fishes, including goldfish, need to be conducted.

The abundance of sbGnRH and cGnRH-II in tilapia pituitaries suggests that these GnRH forms may be the ones that regulate GtH, GH and PRL. It is not known whether there is disparate delivery of the GnRH forms to the pituitary cell types or if there are differences in potency of the GnRH forms among the pituitary cells which may allow differential regulation of GtH, GH and PRL. Schulz and colleagues (1993) found catfish GnRH to be in greater abundance than cGnRH-II in the pituitaries of the African catfish, Clarias gariepinus, with cGnRH-II being the more potent stimulator of GtH-II release in vivo and in vitro. The researchers present the possibility that catfish GnRH may be the regulator of moderate levels of circulating GtH, and the more potent cGnRH-II may be the regulator of GtH surges, such as those associated with spawning.

The regulation of pituitary cells by circulating GnRH must also be considered. Whether circulating GnRH has biological significance in most teleosts has yet to be determined. GnRH and a GnRH-binding protein have been identified in the circulation of the goldfish. Furthermore, the concentrations of GnRH in the circulation were within a range found to stimulate GtH and GH release in vitro in the same species (Huang & Peter 1988, Peter et al. 1990).

Each of the three forms of GnRH that are native to the tilapia was active in releasing PRL from RPD in vitro. Interestingly, the order of potency is the same for in vitro stimulation of PRL release in tilapia and in vivo GtH-II release in sea bream (Zohar et al. 1995). sbGnRH is the least potent and cGnRH-II is the most potent in both circumstances. sbGnRH and cGnRH-II are the most abundant forms of GnRH in the pituitaries of the tilapia, whereas cGnRH-II was not detected in sea bream pituitaries (Powell et al. 1994). Reasons for the differing potencies of the various GnRH molecules include receptor affinity, signal transduction pathway and the enzymatic degradation rates of GnRH molecules at the pituitary.

A single type of GnRH receptor has been found to date in the pituitary of most fish examined: African catfish (de Leeuw et al. 1988), winter flounder (Crim et al. 1988), stickleback (Andersson et al. 1989) and sea bream (Pagelson & Zohar 1992). More than one type of receptor has been identified in the goldfish (Habibi et al. 1987). This suggests that, in most fishes, the different forms of GnRH are competing for the same receptor and the different cell types that respond to GnRH express the same receptor type. The native GnRH peptide with the greater GtH-II-releasing activity has been found to have greater receptor affinity in African catfish (cGnRH-II>catfish GnRH (Schulz et al. 1993)) and goldfish (cGnRH-II>sGnRH (Peter et al. 1990, Habibi 1991)). Nevertheless, in the same study in the goldfish, sGnRH was more active in releasing GH.

Chang et al. (1993) have shown that different native GnRH forms activate different signal transduction pathways in GtH cells of the goldfish. They suggest that binding of different GnRH molecules to the same receptor leads to distinct receptor-conformation changes and alternative G-protein linkages. These alternative pathways may result in different potencies for the different GnRH molecules.

Differences in peripheral degradation of GnRH molecules by specific peptidases at the liver or kidney was suggested as a contributing factor to the same order of potency for stimulating GtH in sea bream; this cannot be a factor in our in vitro studies. Potency in stimulating PRL release is determined at the pituitary level. GnRH is degraded by peptidases at pituitary cells of teleosts (Goren et al. 1990, Zohar et al. 1990). Differences in biological activity of various GnRH molecules have been attributed to their disparate susceptibility to degradation by these peptidases (Goren et al. 1990, Zohar et al. 1990).

The order of potency appears to parallel the evolution of the GnRH peptides. The cGnRH-II peptide is present in cartilaginous fishes; sGnRH first appears in early teleosts; and sbGnRH appears in late teleosts (Sherwood et al. 1994). The presence of a single GnRH receptor in most teleosts suggests that the peptides may have evolved independently of the receptor or receptors and therefore the older peptides may have a greater affinity for the receptor or receptors.

PRL secretion in teleosts is thought to be predominantly under inhibitory control, but evidence has suggested that a hypothalamic PRL-releasing factor was involved in the control of PRL secretion in fish (see reviews, Clarke & Bern 1980, Ball 1981). Barry & Grau (1986) have shown that thyrotropin-releasing hormone stimulates PRL release in the tilapia but only after E2 preincubation. Until the present study, thyrotropin-releasing hormone was the only hypothalamic factor shown to have the capability to stimulate PRL release in the tilapia. Furthermore, the steroid hormones, E2 and testosterone, are able to enhance the response of PRL cells.
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to GnRH, increasing percentage release over 3-fold. Thus steroids may regulate the sensitivity of the PRL cells to GnRH stimulation during the reproductive cycle or alternatively GnRH molecules may regulate the sensitivity of PRL cells to testosterone and E2 stimulation. The potentiation of the response of PRL cells by GnRH and thyrotropin-releasing hormone by sex steroids supports the notion that there may be a shift in the control of PRL secretion with changes in the reproductive state of the tilapia.

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