LPXRFa, the Piscine Ortholog of GnIH, and LPXRF Receptor Positively Regulate Gonadotropin Secretion in Tilapia (*Oreochromis niloticus*)

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LPXRFamide (LPXRFa) peptides have been characterized for their ability to inhibit gonadotropin (GTH) release in birds and stimulate growth hormone (GH) release in frogs. However, their involvement in regulating the reproductive hypothalamo-pituitary-gonadal axis in mammals and fish is inconclusive. To study the role of LPXRFa peptides in the regulation of GTH secretion, we cloned tilapia LPXRFa and LPXRF receptor (LPXRF-R). Processing of the tilapia preproLPXRFa liberated three mature LPXRFa peptides that varied in size and post-translational modifications. Phylogenetic analysis of LPXRFa and the closely related RFamide peptide PQRFa showed clear clustering of each peptide sequence with its orthologs from various vertebrates. Signal-transduction analysis of the tilapia LPXRF-R in COS-7 cells showed clear stimulation of CRE-dependent luciferase activity, whereas the human NPFFR1 showed suppression of forskolin-induced CRE-dependent activity in this system. Administration of the tilapia pyroglutaminated LPXRFa-2 peptide to primary cell culture of tilapia pituitaries, or to reproductive female tilapia by ip injection, positively regulated both LH and FSH release in vivo and in vitro. Using double-labeled fluorescent in-situ hybridization and immunofluorescence, 

No coexpression of tilapia *lpxrf-r* mRNA was identified in GH-positive cells. These findings suggest that the LPXRFa system is a potent positive regulator of the reproductive neuroendocrine axis of tilapia. (*Endocrinology* 155: 4391–4401, 2014)

The discovery of the molluscan cardioexcitatory tetrapeptide FMRFamide triggered the discovery of various neuropeptides sharing a common C-terminal Arg-Phe-NH$_2$ (RFamide) moiety. RFamide peptides have been characterized for their involvement in the regulation of feeding, analgesia, stress, and reproductive behavior. Moreover, several vertebrate RFamide peptides have also been found to regulate pituitary hormone secretion [reviewed by (1–3)].

To date, five groups of the RFamide peptide family have been documented: the LPXRFamide peptide (LPXRFa) group, including gonadotropin-inhibitory hormone (GnIH) and RFamide-related peptide (RFRP); the PQRFamide peptide group, including neuropeptide FF (NPFF); the prolactin-releasing peptide (PrRP) group; the kisspeptin group; and the pyroglutamylated RFamide peptide (QRFP)/26RFamide group (4). Two of these peptide groups have been found to play key roles in positive (kisspeptin) and negative (LPXRFa) regulation of the reproductive hypothalamo-pituitary-gonadal axis (2). However, whereas positive regulation of gonadotropin (GTH) secretion by kisspeptin is now commonly accepted (2, 5), the role of LPXRFa peptides in this regard is...
inconclusive and varies among the vertebrate classes. Although the avian LPXRFa was found to inhibit LH release in vivo and in vitro, and was therefore designated GnIH (6, 7), the amphibian LPXRFa was found to increase growth hormone (GH) and prolactin (PRL) but not GTH release, and was therefore designated frog GH-releasing peptide [fGRP; (8, 9)]. Mammalian LPXRFa peptide (also designated RF-amide-related peptide; RFRP) was detected in the plasma of the ewe hypophysial portal system (10) and the expression of both LPXRFa and LPXRF receptor (LPXRF-R) was found to significantly increase at metestrus in the pig pituitary (11). In the rat, however, LPXRFa peptides were found to inhibit the release of LH (12) and also to increase the release of PRL (13) and GH (12). Although the administration of LPXRFa suppressed GnRH-induced LH release, no immunoreactivity to LPXRFa was identified within the external zone of the rat median eminence (14). In the Siberian hamster, LPXRFa inversely regulates the release of LH according to photoperiod (15). Taken together, these findings would suggest that LPXRFa’s ability to directly down-regulate GTH release in mammals is less pronounced.

In fish, contradictory effects of LPXRFa on LH release have been reported. Three goldfish LPXRFa forms increased the release of LH, FSH, and GH, but did not affect the release of PRL or somatotatin from cultured pituitary cells of sockeye salmon (16). However, ip injection of zebrafish LPXRFa-3 into goldfish decreased their plasma LH levels (17) and inversely regulated GTH release and synthesis (18). The hypophysiotropic role(s) of LPXRFa varies among species, and data regarding the release of FSH is lacking in some of the reports, possibly due to a lack of specific assays.

The effects of LPXRFa peptides are mediated by their receptors, which belong to the membrane-bound G protein–coupled receptor family. In mammals, two types of receptors for RFRP have been identified: GPR147, also known as NPFF1, is the endogenous receptor for RFRP, and the other, GPR74, also known as NPFF2, is a NPFF receptor and the candidate for RFRP (19). It was previously suggested that GPR147 (also known as LPXRF-R, NPFF1, OTT7T022, and GnIH-R) can inhibit cAMP-pathway activation by coupling with Gαi protein (13, 19). Nevertheless, this type of activity cannot explain the positive regulation of GTH release in hamster (15) and goldfish (18). Moreover, although LPXRF receptor (LPXRF-R) mRNA and protein have been identified in the pituitary of several mammals, avians, and fish (3), the identity of the endocrine cells expressing LPXRF-R remains elusive in most species.

Nile tilapia (Oreochromis niloticus) is an important species in aquaculture and an emerging model system for laboratory studies in many fields, including neuroendocrinology, physiology, genomic biology, and molecular genetics (20). This is even truer now, following completion of the Nile tilapia genome project in 2012.

To clarify the functional significance of the LPXRFa system and its role as a key neuropeptide involved in fish reproduction, we first cloned tilapia (ti) LPXRFa and its cognate receptor, GPR147. We then further analyzed signal transduction of the tiLPXRF-R. We also investigated the possible involvement of the tilapia pyroglutamated-LPXRF-amide-2 peptide (ti-pLPXRFa-2) in the regulation of GTHs and GH in the reproductive system of female tilapia. Finally, we identified which pituitary cell population expresses tilpxrfα and tilpxrf-β mRNA.

Materials and Methods

Animals

Sexually mature Nile tilapia (O. niloticus) were kept and bred in the fish facility unit at the Hebrew University in 500-L tanks at 26°C and 14L:10D photoperiod. Fish were fed daily ad libitum with commercial fish pellets (Raanan Fish Feed). All experimental procedures were approved by the Hebrew University administrative panel for laboratory animal care.

Bioinformatics analysis and molecular cloning

In search of the putative LPXRFa gene sequence in tilapia, we used the zebrafish (Danio rerio) GnIH peptide sequence (NP_001076418). Using the BLASTP program (http://blast.ncbi.nlm.nih.gov), we identified a potential homologous sequence for LPXRFa as well as an additional FMRFamide-related peptide sequence (XP_003449851 and XP_003451891, respectively). To identify a putative tiLPXRF-R, a similar search was performed using the three previously characterized sequences of zebrafish GnIH receptors as template [GnIHR1–ADB43133; GnIHR2–ADB43134, GnIHR3–ADB43135; (17)]. This search resulted in four putative tiLPXRFa and NPFF receptors (GPR147 and GPR74 respectively; XP_003448662, XP_003445255, XP_003449675, and XP_003441955) which were further characterized using phylogenetic trees. Phylogenetic analysis of LPXRFa prepeptide as well as the LPXRFa-R sequences was performed using Mega5 software (21). Sequences were aligned by Muscle, and neighbor-joining trees were constructed using the Poisson model with 500 bootstrap replicates.

Total RNA was extracted from sexually mature female tilapia using TRIZOL reagent (Life Technologies), and 5 μg was used as template for cDNA synthesis using Smart MMLV reverse transcriptase (Clontech). Gene-specific primers were designed according to the putative tiLPXRFa and its receptor sequences (Supplemental Table 1). PCRs were performed with Advantage2 polymerase mix (Clontech) according to the manufacturer’s instructions. PCR products were cloned into pCR1-TOPO vector and sequenced with T7 and SP6 primers. LPXRFa and LPXRF-R sequences were submitted to GenBank under accession numbers KF444208 and KF444209, respectively.
TilPXRFa signal peptide was predicted using SignalP 4.1 server (22). LXRFa prepropeptide cleavage prediction was performed using the NeuroPred application (http://neuroproteomics.scs.illinois.edu/cgi-bin/neuropred.py). The prepropeptide sequence was analyzed using the known motif model, K and R amino acids were removed from the predicted sequences, and common post-translational modifications were predicted.

**Tissue distribution of LPXRFa and LPXRF-R mRNAs in tilapia by real-time PCR**

Sexually mature male and female tilapia (body weight [BW] 128.4 ± 10.8 g and gonadosomatic index [GSI] — the percentage of gonad weight to BW — 0.29 ± 0.09%, and BW = 92.7 ± 7.06 g and GSI = 2.63 ± 1.20%, respectively) were collected and tissue samples were placed into TRIZOL reagent. Total RNA was extracted and 0.55 μg was used as a template to code for cDNA synthesis using Verso cDNA kit (Thermo Scientific). Real-time PCR analysis was performed according to Biran et al (23, 24). Primer-pair sequences with their corresponding efficiency and R² values are listed in Supplemental Table 1. The specificity of the amplification was tested at the end of the PCR by melting-curve analysis, and product purity was confirmed by sequencing.

**Peptide synthesis**

Ti-LPXRFa-2 (p-QSDERTPNSPILPQRFR-NH₂) and amidated human (hu) RFRP-3 (VPNLPQRF-NH₂) were synthesized by GL Biochem. Peptides were synthesized by the automated solid-phase method applying Fmoc active-ester chemistry, purified by HPLC to greater than 95% purity, and the C-terminus of each peptide was amidated. The peptides were dissolved to the desired concentration in fish saline (0.9% NaCl in double distilled water) for in vivo experiments and in culture media for in vitro experiments.

**Receptor transactivation assay**

To investigate the signaling pathways of tilPXR-R, its entire coding region was inserted into pcDNA3.1 (Invitrogen) expression vector (pc-lpxrfr). The pcDNA3.1 clone expressing the huNPFF1 receptor (pc-NPFF1R) was obtained from the Missouri S&T cDNA Resource Center (www.cdna.org). Transient transfection, cell procedures, and stimulation protocols were generally according to Biran et al (23) and Levavi-Sivan et al (25). Cotransfection of either pc-lpxrfr or pc-NPFF1R with a reporter plasmid was carried out using TransIT-LT1 transfection reagent (Mirus Bio). Transfection experiments were performed in triplicate with three independently isolated sets. We previously showed the specificity of the reporter receptor-responsive element (SRE) — Luc and cAMP-responsive element (CRE) — Luc to activation of protein kinase C (PKC)/Ca²⁺ and protein kinase A (PKA)/cAMP signal-transduction pathways, respectively, as well as the ability of the CRE-Luc plasmid to report the inhibition of forskolin (FSK)-induced PKA/cAMP activity (23, 25). The EC50 values were calculated from concentration-response curves by means of computerized nonlinear curve fitting with Prism 6 software (GraphPad).

**Immunofluorescence and in situ hybridization**

Tilapia pituitaries were collected from sexually mature male and female fish (BW = 74 ± 15 g, 54 ± 13 g; and GSI = 0.19 ± 0.05%, 4.57 ± 1.34%, respectively) and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS). Samples were cryoprotected in 20% (w/v) sucrose and 30% (v/v) Tissue-Tek OCT compound (Sakura, Alphen). Sagittal sections were analyzed for mRNA expression by in situ hybridization (ISH). The hybridization was generally performed using either sense or antisense probe as described in Biran et al (24) with slight modifications. Color signal was developed using Fast-Red, which allows visualization of labeled cells by light and fluorescence microscopy. After confirmation of hybridization signals, immunofluorescence labeling was performed. Sections were blocked in 5% (v/v) normal goat serum with 0.3% (v/v) Triton X-100 for 1 hour at room temperature and incubated with specific antibodies raised in rabbit against recombinant tilapia (rt)βLH (26), rtβFSH (27), or rtGH (28), diluted 1:500 in antibody dilution buffer (1% w/v BSA; 0.3% Triton X-100 in PBS) overnight at 4°C. Samples were stained using secondary antirabbit antibodies conjugated to Alexa fluorescent dyes (Invitrogen) diluted 1:300 and incubated for 2 hours at room temperature. Following staining, slides were washed and mounted using antiadhesive solution (2% w/v propyl-gallate, 75% v/v glycerol in PBS). LH and FSH antibody specificity was validated by preadsorption assay. Sections of pituitaries from adult male tilapia (BW = 122 ± 23 g; GSI = 0.68 ± 0.2%) were used for the antibody-validation trial. For preadsorption, antibodies were incubated overnight at 4°C with (preabsorbed) or without rtGTHβ in antibody dilution buffer (1% BSA; 0.3% Triton X-100 in PBS). The molar ratio of GTH:antibody was 10:1. Standard immunofluorescence was carried out as described and sections were imaged by confocal microscopy (Supplemental Figure 1). Anti-GH preadsorption assay was performed previously by Melamed et al (28).

**Primary pituitary cell culture**

Primary cell culture of tilapia pituitaries was performed as described by Levavi-Sivan and Yaron (29). Briefly, pituitary glands collected from sexually mature tilapia males (BW = 96.5 ± 9.5 g; GSI = 0.39 ± 0.12%) were placed in culture media, trypsinized, and cells were plated on a 48-well plate (250 000 cells/well per 0.5 mL). The cells were allowed to recover for 4 days at 28°C under an atmosphere of 5% CO₂ and then challenged for 6 hours with ti-LPXRFa-2 at concentrations ranging from 1nM to 1μM. Each experiment was performed in triplicate and repeated three times (separate cell preparations).

**In vivo effect of ti-LPXRFa-2**

For all in vivo experiments, we used sexually mature female tilapia (BW = 113.48 ± 25.4 g; GSI = 3.14 ± 1.1%; n = 9 per treatment). Fish were weighed and injected twice ip with ti-LPXRFa-2 peptide (100 μg/kg BW at time 0 and 4 h later). Controls were injected with fish saline or salmon-GnRH analog (sGnRHα; 10 μg/kg BW). This protocol was chosen based on previous in vivo studies with goldfish (17). Blood samples were collected from the caudal vasculature and centrifuged at 3000 rpm for 20 minutes to obtain plasma samples, which were stored at −20°C until assayed.

**ELISAs for the measurement of tilapia FSH, LH, and GH**

Levels of GTHs or GH in the plasma and primary culture media were measured by specific competitive ELISAs developed for tilapia (30), based on rtGTHs or rtGH. For the ELISA, we...
used primary antibody antisera against rtLHβ, rtFSHβ, or rtGH (28), and rtLHβα (26), rtFSHβα (27) or rtGH for the standard curves. Sensitivity for the plasma measurements was 15.84 pg/mL for LH, 0.24 pg/mL for FSH, and 35.0 pg/mL for GH. For the culture media, the sensitivity was 2.43 ng/mL and 1.52 ng/mL for LH and FSH, respectively. Interassay coefficient of variation was 14.8%, 12.5%, and 13%, whereas intra-assay coefficient of variation was 7.2%, 8%, and 8% for LH, FSH, and GH, respectively.

Statistical analysis
The results are presented as mean ± SEM. One-way ANOVA was used to compare mean values, followed by an a posteriori Tukey test (P < .05) when ANOVA revealed the presence of statistically significant differences between groups, using PRISM 6 software. Three independent experiments were carried out for the in vitro signal-transduction experiments and pituitary primary cell culture studies. Two-way ANOVA followed by all pairs Tukey Kramer HSD (honest significant difference) test was used to analyze in vivo assays using JMP 7.0. Four experiments were carried out for the in vivo studies.

Results
Cloning, identification, and phylogenetic analysis of tilPXRFα and tilPXRF-R
The tilPXRFα sequence was sought using the zebrafish GnIH peptide sequence (accession no. NP_001076418) as a template. This search retrieved two predicted peptides containing the PXRFα signature sequence of PQRFα and LPXRFα peptides. The tilPXRFα was cloned from the tilapia pituitary cDNA library using gene-specific primers (Supplemental Table 1). The tilPXRFα open reading frame contained 594 nucleotides encoding 197 amino acids (Supplemental Figure 2A). A phylogenetic tree of the tilapia peptides with previously identified LPXRFα and PQRFα peptide sequences was constructed (Figure 1A), demonstrating that the tilPXRFα peptide clusters together with other LPXRFα, GnIH, and RFRP peptide precursors, whereas the predicted PQRFα peptide clusters with other vertebrate NPFF and PQRFα peptide precursors. At the protein level, the resulting tilPXRFα hormone precursor displayed a rather low degree of identity (23–25%) with mammalian RFRP precursors, 26.1% with chicken GnIH, 21.6%; with the bullfrog tGRF precursor, and slightly higher identity with other fish GnIH precursors (29.7% with zebrafish and goldfish; Supplemental Table 2).

The ti-preproLPXRFα sequence included three LPXRFα-encoding sequences, a hallmark of the GnIH peptide family. As illustrated in Figure 1C, we suggest that the tilPXRFα precursor encodes three RFRP peptides: LPXRFα-1 is an N-terminus-acetylated, C-terminus-amidated 17-aa peptide, and the N-terminus-acetylated LPXRF-3 11-aa peptide has glutamate (E) instead of the glycine (G) required for amidation (1). Due to the length of tilPXRFα-1 and the lack of amidation of tilPXRF-3, further investigation was carried out using synthetic ti-pLPXRFα-2 peptide.

Similar searches were performed for the identification of the tilPXRF-R using the zebrafish GnIH receptor sequences as templates. This search retrieved one predicted RFRP-R and three predicted NPFF-R sequences. The tilPXRF-R was cloned from the tilapia pituitary cDNA library using gene-specific primers (Supplemental Table 1). The tilPXRF-R open reading frame contained 1449 nucleotides encoding 482 aa and a stop codon (Supplemental Figure 2B). A phylogenetic tree of the tilapia receptor sequences with previously identified GPR147 and GPR74 protein sequences was constructed (Figure 1B); it showed that the tilPXRF-R clusters together with other GPR147 proteins, whereas the other sequences clustered with other GPR74 proteins (Figure 1B). At the protein level, the tilPXRF-R displayed an average identity with placental mammalian GPR147 sequences (45%) and with chicken GnIHR (46.4%), and higher identity with other piscine receptors (43–65%; Supplemental Table 3). Sequence analysis of the tilPXRF-R identified distinct potential sites for N-glycosylation, phosphorylation by protein kinase C, protein kinase A, casein kinase II, and N-myristoylation (Supplemental Figure 2B).

To identify the anatomical spread of the LPXRFα system, we next examined the tissue distribution of lpxrf and lpxrf-r mRNAs in tilapia by real-time PCR (Supplemental Figure 3). The tilapia brain was dissected into three parts: the anterior part containing the telencephalon and olfactory bulbs, the midbrain containing the optic tectum, diencephalon, and hypothalamus, and the posterior brain containing the medulla oblongata and cerebellum. The highest levels of lpxrf mRNA were found in the anterior brain, midbrain, and retina. Moderate levels were present in the hindbrain and intestine, and low levels were evident in the pituitary and gonads (Supplemental Figure 3). Although lpxrf-r mRNA expression was detected in all brain parts, the highest expression was found in the anterior brain followed by the midbrain, and low expression levels were detected in the hind brain. Moderate expression of lpxrf-r mRNA was also identified in the pituitary and gonads, with low mRNA levels in the fat tissue.

Signal-transduction activity of tilPXRF-R
Functional expression analysis was performed in COS-7 cells to evaluate the response and signal-transduction pathway activity of the tilPXRF-R cloned in this study to ti-pLPXRFα-2, using the huNPFF1R and...
huRFRP-3 amidated peptide as reference. As shown by the constructed best-fit dose-response curves (Figure 2), exposure to increasing concentrations of either huRFRP-3 or ti-pLPXRFa-2 (0.01nM–1000nM) in H926 cells expressing either tiLPXRF-R or huNPFFR1, with corresponding EC50 values estimated at 30.16 and 29.13nM (for ti-pLPXRFa-2 and huRFRP-3 with tiLPXRF-R, respectively; Figure 2A) and 176.3 and 405.0nM (for ti-pLPXRFa-2 and huRFRP-3 with huNPFF1R, respectively; Figure 2C).

HuNPFF1R has been reported to exert its effect through inhibition of cAMP production (13). CRE-Luc activity was tested in the absence or presence of 50nM FSK (an adenylyl cyclase activator) to clarify whether the novel tiLPXRF-R is an inhibitory or stimulatory receptor. The FSK-stimulated Luc activities in COS-7 cells expressing either tiLPXRF-R or huNPFFR1 increased 1.5–1.8-fold over basal levels (Figure 2, B and D). However, whereas FSK-stimulated Luc activities in COS-7 cells expressing huNPFFR1 were significantly inhibited by both huRFRP-3 and ti-pLPXRFa-2 (Figure 2D), FSK-stimulated Luc activity in cells expressing tiLPXRF-R were unchanged or even slightly increased (Figure 2B). These results suggest that although the human receptor is coupled to the Gi proteins and therefore capable of inhibiting the intracellular cAMP-signaling pathway upon ligand bind-
sGnRHa (100 nM), FSK (20 μM), cells were exposed to saline (basal) or various concentrations of tilapia pLPXRFa-2 or huRFRP-3. In the CRE-Luc analysis, cells were exposed to the peptides with or without FSK (50 μM). The data are expressed as the change in Luc activity over basal activity and are from a single experiment, representative of a total of three such experiments. FSK basal activity is expressed relative to untreated basal activity in the same experiment. Each point was determined in triplicate and is given as mean ± SEM.

**In vitro effect of ti-pLPXRFa-2 on GTH release from primary culture of tilapia pituitary cells**

Because we showed the presence of tiLPXRF-R in the pituitary, we next aimed to study the direct in vitro effect of ti-pLPXRFa-2 on GTH release. Primary cultures of tilapia pituitary cells were exposed to saline (basal) or sGnRHa (100 nM), FSK (20 μM) or ti-pLPXRFa-2 (1 nM–1 μM). FSK significantly increased the release of LH and FSH to the culture medium whereas sGnRHa only significantly elicited the release of LH (Figure 3). Ti-pLPXRFa-2 significantly increased both FSH release (at 1000 nM; Figure 3A) and LH release (at 100 and 1000 nM; Figure 3B).

**Physiological effect of ti-pLPXRFa-2 peptide on GTH and GH release**

We next aimed to gain insight into the physiological role of the tiLPXRFa system in the regulation of GTHs and GH release in vivo. Ip injection of ti-pLPXRFa-2 peptide (100 ng/g BW) to sexually mature female tilapia induced a significant increase in both FSH and LH secretion as early as 2 hours post first injection (Figure 4, A and B). LH and FSH increased in a similar pattern in response to sGnRH injection. A second ip injection of ti-pLPXRFa-2, 4 hours later, caused no additional change in the release of either GTH (Figure 4, A and B). No significant change in GH release was detected (Figure 4C).

**Cellular localization of LPXRFa and LPXRF-R in the tilapia pituitary**

Given that we found expression of both tiLPXRFa and its receptor in the pituitary, it was interesting to determine their precise cellular localization within the gland, and whether this localization is in line with the above physiological effect. We employed ISH coupled with fluorescent immunohistochemistry (IF) methodologies. First, ISH was performed using NBT/BCIP (nitro-blue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate p-toluidine salt) substrate for chromogenic signal development (Figure 5). A signal for tilapia lpxrf and tilapia lpxrf-r mRNA expression was detected in the proximal pars distalis of tilapia pituitary sections (Figure 5, A and C). We then sought to specifically characterize the proximal pars distalis cell populations expressing tilapia lpxrf and tilapia lpxrf-r mRNAs. Hence, the ISH color signal was developed using Fast-Red substrate, which allows fluorescent (as well as chromogenic) signal visualization, followed by IF using specific homogeneous antisera generated against rtβLH, rtβFSH, and rtGH. As seen in the representative photomicrographs (Figure 6, E–G), most βLH-positive cells coexpressed tilapia lpxrf mRNA, whereas there was no colocalization of GH- or βFSH-positive cells and tilapia lpxrf-expressing ones (Figure 6, A–C and I–K). A similar analysis performed with a specific probe for tilapia lpxrf-r mRNA showed that almost all of the βLH-positive and some of the βFSH-positive cells coexpress tilapia lpxrf-r mRNA (Figure 7, E–G and A–C, respectively).
No coexpression of tilapia lpxrf-r mRNA was identified in GH-immunoreactive cells (Figure 7I–K). No sex-related differences were identified in the mRNA expression patterns of tilapia lpxrf or tilapia lpxrf-r in the examined male and female fish.

Discussion

Previous data have shown that LPXRFa and its homologs are present in vertebrates and play a significant role in the regulation of GTH secretion, with most of the experiments conducted in avian and mammalian species [for review (31)]. However, little is known about the involvement of LPXRFa in the hypothalamo-pituitary-gonadal axis of teleosts, except for several studies in less evolved fish such as salmonids (16), goldfish (17, 18, 32), and lamprey (33). We show here that tilLPXRFa-2 positively regulates GTH secretion both in vitro and in vivo. Moreover, we show that tilLPXRFa and its receptor are direct regulators of tilapia GTH cells. Finally, tilLPXRF-R was shown to regulate its activity through both cAMP/PKA and Ca$^{2+}$/PKC pathways.

LPXRFa was originally identified as a GnIH in the Japanese quail (7). Later, in bullfrog, LPXRFa was found to stimulate GH release but had no effect on GTH secretion (8). The mammalian LPXRFa ortholog RFRP was found to down-regulate plasma LH levels in Syrian hamster and rat (12, 34) and to increase GH plasma levels in the latter (12). Finally, the piscine LPXRFa, originally identified in goldfish (35), was found to stimulate GH and GTH secretion from primary cultures of sockeye salmon pituitaries (16) and to increase the expression of GTH mRNA in primary cultures of grass puffer pituitaries (36), whereas in goldfish it had variable effects on LH secretion and GTH mRNA expression, depending on gonadal recrudescence stage (18, 32). These wide physiological effects of LPXRFa on GTH and GH synthesis and release suggest a species-specific role for this peptide.

In this study, we used the zebrafish GnIH sequence to search the tilapia genetic databases for putative LPXRFa and LPXRF-R gene orthologs, which were then cloned and sequenced. We also characterized
the identified genes for their phylogenetic origins and structure. It has been recently suggested that LPXRFa and PQRFa peptide genes diverged from a common ancestral gene (33). Furthermore, huNPFF2 (GPR74) was identified in GenBank databases by using the huNPFF1 (GPR147) sequence (19). Hence, we also bioinformatically identified putative sequences for tiPQRFa and its cognate receptor. Phylogenetic analysis showed that tiLPXRFa clusters with other LPXRFa peptides, whereas tiPQRFa clusters with other PQRFa peptides. Phylogenetic analysis of the LPXRFa and PQRFa receptor protein sequences showed that tilapia has a single LPXRF receptor and three putative PQRFa receptors.

Processing prediction for ti-preproLPXRFa suggested its cleavage into three mature LPXRF peptides, each possessing specific post-translational modifications. Although the mature tiLPXRFa-1 and tiLPXRF-3 were predicted to possess N-terminus acetylation, tiLPXRFa-2 was predicted to possess N-terminus pyroglutamination. Furthermore, tiLPXRFa-1 and tiLPXRFa-2 were predicted to possess an amide group at their C terminus whereas tiLPXRF-3 possessed glutamate in its C terminus instead of the characteristic RFamide glycine required for C-terminal amidation (1). Taken together with the potential complexity in synthesizing the 70aa-long tiLPXRFa-1, we chose to limit the functional characterization of tiLPXRFa to the activity of ti-pLPXRFa-2.

Tissue-distribution analysis showed that tilapia lpxrf and tilapia lpxrf-r mRNAs are widely expressed in various tilapia tissues, suggesting involvement of the LPXRFa system in the activity of additional physiological processes in tilapia apart from its effect on the pituitary. Multiple functions of a single RFamide peptide have been characterized for the mammalian PrRP, QRFP, and the mammalian ortholog of LPXRFa, RFRP [reviewed by (1)]. However, further investigation is still required for the characterization of similar multiple roles for fish LPXRFa.

We showed that stimulation of tiLPXRF-R by both tiLPXRFa-2 and huRFRP-3 increases Luc activity via both cAMP/PKA (CRE) and Ca\(^{2+}\)/PKC (SRE) pathways. Nevertheless, whereas the huNPFFR1 showed similar positive activation of the SRE pathway, stimulation of the huNPFFR1 with either huRFRP-3 or tiLPXRFa-2 suppressed the FSK-induced CRE-dependent Luc activity, suggesting an inhibitory role for this receptor under these conditions. Previous work with huNPFFR1 could not detect any inhibitory or stimulatory effect on cAMP formation using COS-7 cells (19). Possible reasons for these differences are the time of ligand stimulation, the type of assay (cAMP accumulation vs CRE transcription), or different FSK levels. Bonini et al (19) also showed that despite its more effective coupling to the cAMP inhibitory class of G proteins, huNPFFR1 can also couple to the cAMP-
activating $\alpha$ protein, and it was later shown that huNPFFR1 can suppress FSK-induced cAMP formation in CHO cells (37). Similarly, it was recently shown that GPR147 is naturally expressed in the mouse LBT2 gonadotroph cell line, and can suppress the FSK-induced CRE-dependent Luc activity. Moreover, it was shown to suppress GnRH-induced cAMP levels and GTH transcription and release from these cells (38). Our current data regarding huNPFFR1 signal transduction is in line with previous findings of G pathway activation. This supports the stimulatory effects elicited by tiLPXRF-R in the cAMP-dependent translational pathway.

Our current findings suggest that ti-pLPXRFa-2 positively regulates GTH release from primary cell cultures of tilapia pituitaries. This is in agreement with our signal-transduction analysis for tiLPXRF-R. Similarly, goldfish LPXRFa was found to increase GTH as well as GH release from primary cell cultures of sockeye salmon pituitaries (16), and to increase GTH mRNA levels in cultured grass puffer pituitaries (36). In the goldfish, LPXRFa stimulated or inhibited GTH transcription and release from primary cell cultures of pituitaries, depending on the stage of gonadal maturation (18). Although the salmon pituitaries were collected from precocious males (16), the grass puffer and tilapia pituitaries were collected from sexually mature males and females (36 and current data). It has been suggested that goldfish LPXRFa’s ability to elicit LH release from goldfish pituitary cells in vitro is due to its interaction with other neuroendocrine regulatory mechanisms (18). This suggestion is also supported by the finding that the mammalian NPFFR2, which has a wider neuroanatomical distribution, is also activated by LPXRFa (RFRP) peptides (37). Nonetheless, the tiLPXRF-R signal-transduction analysis suggests that in tilapia, the stimulation of GTH secretion by ti-pLPXRFa-2 is at least partially directly stimulatory in nature.

The physiological effects of LPXRFa peptides vary greatly depending on the organism, physiological status, and route of administration. In rats, intracerebroventricular administration of huRFRP-1 resulted in a 6-fold increase in plasma PRL levels but did not affect other pituitary hormones (13). Conversely, rats that were intracerebroventricularly injected with RFRP-3 presented increased serum levels of GH and decreased levels of LH, with no effect on plasma FSH levels (12). In the Japanese quail, continuous ip administration of GnIH peptide suppressed LH plasma levels as well as common $\beta$GTH and $\beta$LH mRNAs in the pituitary (39), and iv injection of sparrow GnIH to white-crowned sparrows decreased the release of LH to the plasma (6). In frogs, ip injection of GRP-related peptide 2 was found to increase the release of GH and PRL to juvenile frog plasma but had no effect on GTH release (9). In fish, data regarding the physiological effects of LPXRFa in vivo is limited to goldfish, where the administration of zebrafish (17) or goldfish (18, 40) LPXRFa decreased LH plasma levels as well as $\beta$FSH and $\beta$LH mRNA levels in the pituitary. However, our data show that ip administration of ti-pLPXRFa-2 increases the release of both GTHs to the fish plasma with no significant effect on GH release. It has been recently shown that the mRNA expression patterns of grass puffer LPXRFa and its receptor are seasonal and circadian in nature (36). Hence, further characterization of the effect(s) of tiLPXRFa on GTH and GH release under different physiological and environmental conditions is required.

Specific expression patterns of tilapia lpxrf and tilapia lpxrf-r mRNA were identified in the tilapia pituitary and are in line with previous identification of LPXRFa fibers or lpxrf mRNA in the pituitary of goldfish (35), grass puffer (36), salmon (16), pig (11) and quail (41), and in the median eminence of bullfrog (8) and quail (7). Furthermore, lpxrf-r mRNA was also identified in the pituitary of goldfish (40), zebrafish (17), grass puffer (36), pig (11), rat (13), humans (42), quail, and sparrow (41). This suggests that in the tilapia, LPXRFa and its cognate receptor may serve as an autocrine/paracrine regulator of pituitary functions. Further characterization of some of these cells showed a strong interaction between the LPXRFa system and GTH-positive, but not GH-positive cells. It is worth mentioning that while most of the $\beta$LH-positive cells co-express both LPXRFa and LPXRF-R mRNAs, only a small number of $\beta$FSH-positive cells were found to exclusively express LPXRF-R mRNA, suggesting that in tilapia, the two GTHs are differentially regulated by the LPXRFa system. Together with our current results from the signal-transduction analysis and both in vivo and in vitro physiological assays, it is suggested that the LPXRFa system is a positive regulator of the reproductive axis in tilapia. Although both GTHs were positively regulated by LPXRFa, the differential LPXRF-R expression and differential regulation of GTHs in vitro vs their coregulation in vivo suggest that additional mechanisms are involved in this activation. We recently found that hypothalamic GnRH neurons possess LPXRF-Rs, explaining some of these effects (Ogawa et al, unpublished).

Avian LPXRFa peptides are well established as negative regulators of GTH secretion and operate as mediators between photoperiodic effects and the reproductive axis [reviewed by (2, 43)]. Amphibian LPXRFa peptides have been characterized by their GH- and PRL-releasing activities (8, 9). In mammals, the hypothalamic GnRH system is rather controversial. LPXRFa peptides have been found to be released into the hypothalamic portal...
circulation and regulate LH secretion in ewes (10). However, in rats, no hypophysiotropic LPXRFa neurons were identified by Fluoro-Gold retrograde tracing and immunohistochemistry (14) whereas in Siberian hamsters, LPXRFa was found to inversely regulate LH release in vivo, depending on the photoperiod (15). Findings regarding the hypophysiotropic role of the piscine LPXRFa system are also inconclusive. Goldfish LPXRFa has both somatotropic and gonadotropic effects on sockeye salmon pituitary cell culture in vitro (16), and only gonadotropic effects on grass puffer pituitaries (36). However, when applied to goldfish, it either stimulated or suppressed LH secretion; moreover, the mRNA levels of GTHβ-subunits varied with season and physiological environment, as determined by circulating estradiol, whereas consistently in varied with season and physiological environment, as determined by circulating estradiol, whereas consistently in varied with season and physiological environment, as determined by circulating estradiol, whereas consistently in varied with season and physiological environment, as determined by circulating estradiol.

In the present study, we used the tilapia genome database to clone and identify the tilapia LPXRFa system. We further characterized LPXRF-R’s cAMP/PKA- and Ca²⁺/PKC-dependent activity. Using the huNPFFR1 as a reference, the stimulatory CRE-dependent Luc activity of LPXRF-R was established. This finding was further supported by physiological assays demonstrating the tilapia LPXRFa system’s ability to enhance the release of both GTHs in vivo and in vitro. In addition, we elucidated the identity of the endocrine cell populations expressing lpxrf and lpxrf-r mRNA as LH and FSH cells, suggesting a hypophysiotropic effect of the LPXRFa system in tilapia. The effects of tilapia LPXRFa peptides at different stages of the reproductive cycle have yet to be determined.

Acknowledgments

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The nucleotide sequences reported in this article have been deposited in the GenBank database under accession Nos. KF444208 and KF444209 for tilapia LPXRFa peptide and LPXRF receptor, respectively.

Competing Financial Interests: The authors declare no competing financial interests.

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Disclosure Summary: The authors have nothing to disclose.

References


Supplemental Figure 2

A.

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LPXRFa-2

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**Supplemental Table 1.** Primers used for cloning, in situ hybridization and quantitative real-time PCR

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**Supplemental Table 2.** Percent amino acid sequence identities (black) and similarities (red) among LPXRFa (RFRP/GnIH/fGRP) of different species as determined by EMBOSS* needle alignment tool.

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Supplemental Table 3. Percent amino acid sequence identities (black) and similarities (red) among LPXRF-receptor (NPFFR1/RFRPR/GnIHR/GPR147) of different species as determined by EMBOSS* needle alignment tool.

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*http://www.ebi.ac.uk/Tools/psa/emboss_needle/