Novel fish hypothalamic neuropeptides stimulate the release of gonadotrophins and growth hormone from the pituitary of sockeye salmon

Masafumi Amano, Shunsuke Moriyama, Masayuki Iigo1, Shoji Kitamura2, Noriko Amiya, Kunio Yamamori, Kazuyoshi Ukena3 and Kazuyoshi Tsutsui3

School of Fisheries Sciences, Kitasato University, Ofunato, Iwate 022-0101, Japan
1Department of Applied Biochemistry, Faculty of Agriculture, Utsunomiya University, Utsunomiya, Tochigi 321-8505, Japan
2Freshwater Fisheries Research Division, National Research Institute of Fisheries Science, Nikko, Tochigi 321-1661, Japan
3Laboratory of Brain Science, Faculty of Integrated Arts and Sciences, Hiroshima University, Higashi-Hiroshima 739-8521, Japan
(Requests for offprints should be addressed to K Tsutsui; Email: tsutsui@hiroshima-u.ac.jp)

Abstract

We recently identified a cDNA encoding three novel fish hypothalamic neuropeptides, having LPXRF-NH2 from the goldfish brain. In this study, to clarify the physiological functions of these three LPXRFamide peptides (gfLPXRFa-1, -2, and -3), we analysed the localisation and hypophysiotrophic activity of these peptides using sockeye salmon, Oncorhynchus nerka, in which immunoassay systems for several anterior pituitary hormones have been developed. gfLPXRFa-immunoreactive cell bodies were detected in the nucleus posterioris periventricularis of the hypothalamus and immunoreactive fibres were distributed in various brain regions and the pituitary. We also detected gfLPXRFa-immunoreactivity in the pituitary by competitive enzyme-linked immunosorbent assay combined with reversed-phase HPLC. These three gfLPXRFamide peptides stimulated the release of FSH, LH and GH, but did not affect the release of prolactin (PRL) and somatolactin (SL) from cultured pituitary cells. These results suggest that novel fish hypothalamic LPXRFamide peptides exist in the brain and pituitary of sockeye salmon and stimulate the release of gonadotrophins and GH from the pituitary.


Introduction

Neuropeptides containing a C-terminal -Arg-Phe-NH2 sequence (RFamide peptides) have been identified in the brains of several vertebrates. RFamide peptides have been shown to have important physiological roles in neuroendocrine, behavioural, sensory and autonomic functions (Panula et al. 1996, 1999, Iba et al. 2000).

We previously identified a novel hypothalamic dodecapeptide, Ser-Ile-Lys-Pro-Ser-Ala-Tyr-Leu-Pro-Leu-Arg-Phe-NH2, in the brain of Japanese quail Coturnix japonica (Tsutsui et al. 2000). This dodecapeptide was shown to be located in the hypothalamo-hypophysial system and to inhibit gonadotrophin (GTH) release, it was therefore dubbed gonadotrophin-inhibiting hormone (GnIH; Tsutsui et al. 2000). GnIH-immunoreactive (ir) cell bodies and terminals were localized in the paraventricular nucleus and median eminence, respectively, indicating that GnIH acts directly on the pituitary (Tsutsui et al. 2000, Ubuka et al. 2003, Ukena et al. 2003a). GnIH-ir fibres were further observed in extremely close proximity to gonadotrophin-releasing hormone (GnRH) neurons in the preoptic area in birds (Bentley et al. 2003, Ukena et al. 2003a). It is therefore suggested that GnIH acts at the level of the hypothalamus to regulate GTH release, as well as at the pituitary. We also characterized a cDNA encoding the GnIH precursor in the brain of Japanese quail (Satake et al. 2001) and Gambel’s white-crowned sparrow Zonotrichia leucophrys gambelii (Osugi et al. 2004). The GnIH precursor encodes one GnIH and two GnIH-related peptides (GnIH-RP-1 and GnIH-RP-2) that include Leu-Pro-Xaa-Arg-Phe-NH2 (Xaa=Leu or Gln) at their C-terminals (Satake et al. 2001, Osugi et al. 2004). Based on this structural feature, GnIH and GnIH-RPs are considered to be LPXRFamide peptides (where X=L or Q) as a new member of the RFamide peptide family (see Ukena and Tsutsui 2005).

We further identified LPLRFamide peptide from the bullfrog hypothalamus which possessed growth hormone (GH) releasing activity, and was designated as frog GH-releasing peptide (fGRP) (Koda et al. 2002). The fGRP precursor also encodes one fGRP and three related peptides

0022–0795/06/0188–417 © 2006 Society for Endocrinology Printed in Great Britain
DOI: 10.1677/joe.1.06494
Online version via http://www.endocrinology-journals.org
Downloaded from Bioscientifica.com at 11/03/2018 08:47:30PM via free access
(fGRP-RP-1, -2, and -3; Sawada et al. 2002a), which were identified as mature LPXR-Famide peptides (Ukena et al. 2003b). Among them, fGRP-RP-2 stimulated not only GH, but also prolactin (PRL) release (Ukena et al. 2003b).

cDNAs that encode novel RFamide peptides similar to GnlH and fGRP have been detected in mammalian brains with a gene database search (Hinuma et al. 2000). The cDNAs of human and bovine peptides encode three peptides, which were dubbed RFamide-related peptide-1, -2, and -3 (RFRP-1, -2, and -3). RFRP-1 and -3 are both mammalian LPXR-Famide peptides. Intracerebroventricular administration of the deduced human LPXR-Famide peptide, hRFRP-1, increased PRL release in the rat (Hinuma et al. 2000). Thus, to establish that LPXR-Famide peptides generally contribute to the regulation of pituitary hormone release in vertebrates, we need to identify fish LPXR-Famide peptides and clarify their hypophysiotrophic activities in fish.

Recently, a cDNA that encoded three novel fish LPXR-Famide peptides (gfLPXRFa-1, -2, and -3) was characterised from the goldfish Carassius auratus brain, and gfLPXRFa-3 was identified as a mature peptide (Sawada et al. 2002b). Distribution of gfLPXRFamide peptides in the brain of goldfish was further examined by immunohistochemistry. Immunoreactive cell bodies were restricted to the nucleus posterioris periventricularis (NPPv) and the nervous terminalis (NT), and immunoreactive fibres were distributed in several brain regions, including the nucleus lateralis tuberis pars posterioris (NLTp) and pituitary (Sawada et al. 2002b). In light of previous reports in other vertebrates (Tsutsui et al. 2000, Hinuma et al. 2000, Koda et al. 2002, Ukena et al. 2003b, Osugi et al. 2004), and considering that gfLPXRFamide peptides innervated the pituitary of the goldfish, it is hypothesised that gfLPXRFamide peptides act on the pituitary to regulate pituitary hormone secretion.

Therefore, in this study, we examined whether gfLPXRFamide peptides, newly identified fish hypothalamic LPXR-Famide peptides, have releasing activities on anterior pituitary hormones, i.e. follicle-stimulating hormone (FSH), luteinizing hormone (LH), GH, PRL and somatolactin (SL), using cell cultures of sockeye salmon Oncorhynchus nerka pituitaries, in which immunoreassays for all these hormones have been developed. Prior to the culture study, we confirmed that gfLPXRFa-like substances are present in the brain and pituitary of sockeye salmon by immunohistochemistry and competitive ELISA combined with reversed-phase HPLC.

Materials and Methods

Animals

Sockeye salmon reared in well water of constant temperature (9–10 °C) at the Freshwater Fisheries Research Division, National Research Institute of Fisheries Science (Nikko, Tochigi Prefecture, Japan) were used. The experimental protocol was approved in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the Kitasato University, Utsunomiya University and the National Research Institute of Fisheries Science, Japan.

Immunohistochemistry

We used 13 fish for immunohistochemistry. Fish were anaesthetised by immersion in 0.05% 2-phenoxyethanol. Immunohistochemistry was conducted using paraplast sections. Brains were fixed with Bouin’s fluid for 48 h at 4 °C and subsequently rinsed in cold 70% ethanol, dehydrated through a graded series of ethanol concentrations and embedded in paraplast. Serial sagittal or frontal sections were cut at 5 or 8 μm, separated into four groups every two sections, and mounted on gelatinized slides. The antibody against fGRP was diluted 1000-fold with 0.1 M phosphate buffer (pH 7.4) raised in Tsutsui’s laboratory (Koda et al. 2002), and contained 0.75% NaCl and 0.3% Triton X-100. The specificity of the antibody was checked by a competitive ELISA in a previous study (Sawada et al. 2002b). The IC₅₀ values (concentrations yielding 50% displacement) in the competitive ELISA were estimated as follows: 0.46 pmol for gfLPXRFa-1, 3.43 pmol for gfLPXRFa-2, 1.13 pmol for gfLPXRFa-3, 0.74 pmol for fGRP, 20.96 pmol for chicken RFamide (LPLRFamide) and more than 1000 pmol for other RFamide peptides, e.g., Carassius RFamide (SPEIDPFWYVGRGVRP) and molluscan RFamide (FMRFamide). For immunohistochemical reactions, a Histofine immunostaining kit (Nichirei, Tokyo, Japan) was used for all immunohistochemical reactions detailed. To test the specificity of immunoreactions, the antiserum was pre-adsorbed overnight at 4 °C with an excess amount of gfLPXRFa-1, -2 or -3 (50 μg gfLPXRFa/ml). The subsequent procedure was as described above.

Reversed-phase HPLC

Pituitary glands from 140 precocious males (approximately 250 g body weight) were boiled for 7 min and homogenised in 5% acetic acid as described previously (Tsutsui et al. 2000, Sawada et al. 2002b). The homogenate was centrifuged at 15000 g for 20 min at 4 °C and the supernatant was collected. The supernatant was passed through a disposable C-18 cartridge column (Sep-pak; Waters, Milford, MA, USA) and the retained material, eluted with 60% methanol, was loaded onto a reversed-phase HPLC column (ODS-80TM; Tosoh, Tokyo, Japan) with a linear gradient of 15–31% acetonitrile (CH₃CN) containing 0.1% trifluoroacetic acid for 40 min at a flow rate of 0.5 ml/min. The fractions (1 ml each/tube) were then subjected to a competitive ELISA using the antibody raised against fGRP.
Pituitary cell culture

100 precocious males (approximately 100 g body weight) were anaesthetised in 0·05% 2-phenoxyethanol and decapitated. Pituitaries were immediately dissected out, kept in ice-cold Minimum Essential Medium (MEM; Gibco, Grand Island, NY, USA) containing 0·1% bovine serum albumin (BSA; Sigma, St Louis, MO, USA) and 0·01% antibiotic-antimycotic solution (Gibco), and immediately transported to Utsunomiya University (Utsunomiya, Tochigi Prefecture, Japan) where the dissociation procedure was carried out. Pituitaries were washed with Hank’s balanced salt solution without Ca²⁺ and Mg²⁺ (HBSS), minced with a tissue slicer (Narishige, Tokyo, Japan), and treated with 0·2% collagenase type V (Sigma) and 0·005% DNase (Deoxyribonuclease Grade II; Boehringer Mannheim Biochemicals, Indianapolis, IN, USA) in HBSS in a spinner flask (Wheaton Science Products, Millville, NJ, USA) at 80 r.p.m. for 60 min at 18 °C. Partially digested tissues were separated by centrifugation at 80 g for 5 min at 18°C, and pituitary cells were dispersed by pipetting in the culture medium (see below). The viability of the cells tested with a trypan-blue exclusion test was >90%. Cells were plated on poly-L-lysine-coated 24-well dishes (Iwaki, Tokyo, Japan) at a density of 5 × 10⁴ cells/well in 1 ml MEM containing 25 mM Hepes (Dojin, Kumamoto, Japan), 1·4 mM L-glutamine (Gibco), 10% fetal bovine serum (ICN Biomedicals, Aurora, OH, USA), and 0·01% antibiotic-antimycotic solution and preincubated at 18°C for 72 h under a humidified atmosphere in 100% air. Cells were plated into 52 wells: seven wells for the control group and the remaining 45 wells for the gfLPXRFamide peptide treatment groups (nine treatment groups of three peptides each). To examine the effects of gfLPXRFamide peptides on pituitary hormone release, cells were washed twice with 1 ml MEM containing 0·1% BSA and 0·01% antibiotic-antimycotic solution and then incubated with or without gfLPXRFa-1, -2, or -3 (10⁻⁹, 10⁻⁷, or 10⁻⁵ M) in the same medium for an additional 2 h at 18°C. Culture media were collected at the end of incubation and immediately frozen and kept at -80°C until amounts of pituitary hormones released could be determined by time-resolved fluoroimmunoassay (TR-FIA) or RIA.

Immunocells

Released GTH levels were measured by TR-FIA for salmonid FSH and LH (Amano et al. 2000). Released GH, PRL and SL levels were measured by RIAs (Swanson 1995).

Statistics

Results of immunocells were expressed as the mean ± S.E. The effects of gfLPXRFa-1, -2 and -3 on the release of FSH, LH, GH, PRL and SL from cultured pituitary cells were analysed for significance by one-way ANOVA followed by Dunnett’s test.

Results

Distribution of novel fish LPXRFamide peptides in the brain and pituitary

We first investigated the localisation of the novel fish LPXRFamide peptides in the sockeye salmon brain and pituitary by immunohistochemistry. Pre-adsorption of the antibody with synthetic gfLPXRFa-1, -2, or -3 resulted in the disappearance of the reaction product in the brain (Fig. 1B, C, D) and the neurohypophysis of the pituitary (Fig. 1H for gfLPXRFa-3, data not shown for gfLPXRFa-1 and -2), indicating that the antibody recognises these three peptides. The distribution of gfLPXRFa-ir cell bodies and fibres is summarised in Figure 2. Immunoreactive cell bodies were localized in the NPPv of the hypothalamus (Fig. 1A, E). Immunoreactive fibres were distributed in various brain regions from the olfactory bulb (OB) to the spinal cord, except for the cerebellum. In addition, some immunoreactive fibres projected to the pituitary (Fig. 1F, G).

Detection of novel fish LPXRFamide peptides in the pituitary by reversed-phase HPLC and ELISA

To detect gfLPXRFa-immunoreactivity in the pituitary gland, we carried out the competitive ELISA in conjunction with reversed-phase HPLC. Three gfLPXRFa-immunoreactive peaks were detected in fractions around 12, 16 and 18, exhibiting similar retention times to gfLPXRFa-2, -3 and -1, respectively (Fig. 3). The immunoreactive peak corresponding to gfLPXRFa-3 seemed to be much smaller than the other two peaks.

Effects of novel fish LPXRFamide peptides on the release of FSH, LH, GH, PRL and SL from pituitary cells

Using primary cultures of sockeye salmon pituitaries, we then conducted experiments to ascertain whether gfLPXRFamide peptides have any influence on the release of anterior pituitary hormones such as FSH, LH, GH, PRL and SL. As shown in Figure 4A, gfLPXRFa-1, -2 and -3 significantly stimulated FSH release. The stimulatory effect of these peptides tended to be dose-dependent. The threshold concentrations of gfLPXRFa-1, -2 and -3 ranged less than 10⁻⁹ M, between 10⁻⁹ and 10⁻⁷ M, and between 10⁻⁹ and 10⁻⁷ M, respectively (Fig. 4A). Similarly, these three peptides significantly stimulated LH release (Fig. 4B). The stimulatory effect also tended to be dose-dependent and the threshold concentrations of gfLPXRFa-1, -2 and -3 ranged between 10⁻⁹ and 10⁻⁷ M,
between $10^{-9}$ and $10^{-7}$ M, and less than $10^{-9}$ M, respectively (Fig. 4B). In addition to FSH and LH, gfLPXRFa-1, -2 and -3 significantly stimulated GH release in a dose-dependent manner. The threshold concentrations of gfLPXRFa-1, -2 and -3 ranged from $10^{-9}$ and $10^{-7}$ M, $10^{-7}$ and $10^{-5}$ M, and $10^{-5}$ and $10^{-7}$ M, respectively (Fig. 4C). On the other hand, none of the three gfLPXRFamide peptides had a significant effect on the release of PRL (Fig. 4D) and SL (Fig. 4E).

**Discussion**

Pre-adsorption of the antibody with the synthetic gfLPXRFa-1, -2, or -3 resulted in disappearance of the reaction product in the brain and the neurohypophysis of the pituitary in the sockeye salmon, indicating that the antibody recognises these three peptides. gfLPXRFa-ir cell bodies were localised in the NPPv of the hypothalamus. Unlike goldfish (Sawada et al. 2002b),

![Figure 1](image-url)
immunoreactive cell bodies were not detected in the NT of the sockeye salmon. In sockeye salmon, immunoreactive fibres are distributed in various brain regions from the OB to the spinal cord, except for the cerebellum. Interestingly, some immunoreactive fibres further projected to the pituitary gland. In addition, we could detect gfLPXRFa-immunoreactivity in the pituitary by the competitive ELISA combined with reversed-phase HPLC. This analysis suggested that plural LPXRFamide peptide-like substances are present in the pituitary of sockeye salmon, as immunoreactive peaks corresponding to gfLPXRFa-1, -2 and -3 were detected. This is supported by the fact that the three peptides, gfLPXRFa-1, -2 and -3, were encoded in identical precursor cDNA (Sawada et al. 2002b). These immunochemical results suggest that novel fish LPXRFamide peptide-like substances act directly on the pituitary to regulate pituitary hormone secretion. Thus, we examined whether these three peptides regulate pituitary hormone release in vitro.

All three fish LPXRFamide peptides, gfLPXRFa-1, -2 and -3, stimulated the release of FSH and LH from cultured pituitary cells of sockeye salmon, and these effects on GTHs may be dose-dependent. Moreover, gfLPXRFa-1, -2 and -3 also stimulated GH release in a dose-dependent manner. On the other hand, gfLPXRFa-1, -2 and -3 did not affect the release of PRL and SL. The stimulatory effects of these three peptides on the release of GTHs and GH may be taken as physiological actions, because threshold concentrations ranged from less than $10^{-5}$ M (GH), $10^{-7}$ M (FSH) to $10^{-9}$ M (LH).

**Figure 2** Schematic illustration of the distribution of gfLPXRFa-ir cell bodies (large dots) and gfLPXRFa-ir fibres in a parasagittal section (A) and a frontal section (B) of sockeye salmon brain. Bars indicate 1 mm. C, cerebellum; DF, nucleus diffuses of the inferior lobe; M, medulla oblongata; NLT, nucleus lateralis tuberis; NPGl, nucleus pregglomerulosus lateralis; NPPv, nucleus posterioris periventricularis; OB, olfactory bulb; Olf, olfactory nerve; ON, optic nerve; OT, optic tectum; PIT, pituitary; T, telencephalon.
Thus, gfLPXRFamide peptides may be a novel factor regulating pituitary hormone secretion in fish. It has been reported that fGRP-RP-2 stimulates not only GH, but also PRL release both in vitro and in vivo in the bullfrog (Ukena et al. 2003b). Therefore, it is considered that LPXRFamide peptides regulate the release of plural pituitary hormones in frogs as well as fish.

Considering that GnIH, an avian LPXRFamide peptide, inhibits the release of GTHs (Tsutsui et al. 2000, Osugi et al. 2004), and fGRP and fGRP-RP-2, frog LPXRFamide peptides, stimulate the release of GH and GH/PRL, respectively (Ukena et al. 2003b), it is likely that gfLPXRFa-1, -2 and -3, fish LPXRFamide peptides, are also involved in the regulation of pituitary hormone secretion. The present study indicates that gfLPXRFa-1, -2 and -3 stimulate the release of GTHs and GH, suggesting that these fish LPXRFamide peptides function like GnRH and GH-releasing hormone (GHRH). In sockeye salmon, salmon GnRH (sGnRH), which is distributed from the olfactory nerve through the hypothalamus, is involved in the release of GTH (Amano et al. 1998). Furthermore, salmon GHRH (sGHRH) was identified in sockeye salmon (Parker et al. 1993) and a stimulatory effect of sGHRH on GH release from the pituitary of coho salmon (Oncorhynchus kisutch) was reported (Parker et al. 1997). There is no report indicating physiological changes in plasma FSH, LH and GH levels in sockeye salmon. To understand the physiological roles of gfLPXRFamide peptides in fish reproduction and growth, we need to examine physiological changes in circulating FSH, LH and GH levels and the effects of
in vivo administration of gfLPXR-Famide peptides on these hormone levels in sockeye salmon. Moreover, it will be interesting to examine the relationship between sGnRH, GHRH and gfLPXR-Famide peptides in the future.

In conclusion, we have shown that gfLPXR-Famide peptides, a new member of the hypothalamic RFamide peptide family, contribute to the multifactorial regulation of pituitary hormone release in vertebrates from fish to mammals. Moreover, judging from the wide distribution of immunoreactive fibres in the brain of sockeye salmon, it is suggested that gfLPXR-Famide peptides function as neuromodulators and/or neurotransmitters in the brain. Further study is needed to clarify these functions of novel fish LPXR-Famide peptides.

Acknowledgements

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan (13210101, 15207007 and 16086206 to KT; 15770040 to KU). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References


Parker DB, Coe IR, Dixon GH & Sherwood NM 1993 Two salmon neuropeptides encoded by one brain cDNA are structurally related to members of the glucagon superfamily. European Journal of Biochemistry 215 439–448.


Received 17 November 2005
Accepted 5 December 2005