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RFamide Peptides Inhibit the Expression of Melanotropin and Growth Hormone Genes in the Pituitary of an Agnathan, the Sea Lamprey, *Petromyzon marinus*

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Neuropeptides with the Arg-Phe-amide motif at their C termini (RFamide peptides) were identified in the brains of several vertebrates, and shown to have important physiological roles in neuroendocrine, behavioral, sensory, and autonomic functions. The present study identified RFamide peptides, which are teleost prolactin-releasing peptide (PrRP) homologs, in the sea lamprey, *Petromyzon marinus* and characterized their effect on the release of pituitary hormones *in vitro*. Two RFamide peptides (RFa-A and RFa-B) were isolated from an acid extract of sea lamprey brain, including hypothalamus by Sep-Pak C18 cartridge, affinity chromatography using anti-salmon PrRP serum, and reverse-phase HPLC on an ODS-120T column. Amino acid (aa) sequences and mass spectrometric analyses revealed that RFa-A and RFa-B consist of 25 and 20 aa, respectively, and have 75% sequence identity within the C-terminal 20 aa. The RFa-B cDNA encoding a preprohormone of 142 aa was cloned from the lamprey brain, and the deduced aa sequence from positions 48–67 was identical to the sequence of RFa-B. However, the preprohormone does not include an aa sequence similar to the RFa-A sequence. Cell bodies, which were immunoreactive to anti-salmon PrRP serum, were located in the periventricular arcuate nucleus, ventral part of the hypothalamus, and immunoreactive fibers were abundant from the hypothalamus to the brain. A small number of immunoreactive fibers were detected in the dorsal half of the rostral pars distalis of the pituitary, close to the GH-producing cells. In addition, anti-salmon PrRP immunoreactivities were observed in the pars intermedia, corresponding to melanotropin cells. Likewise, signal of RFa-B mRNA was detected not only in the brain but also in the pars intermedia. The synthetic RFa-A and -B inhibited GH mRNA expression in a dose-dependent fashion *in vitro*, which is comparable to the inhibitory effect of teleost PrRP on GH release. Both RFa-A and -B also inhibited the expression of proopiomelanotropin mRNA, but no effects were observed in the expression of proopiocortin and gonadotropin β mRNAs. The results indicate that RFamide peptides, which are teleost PrRP homologs, are present in the hypothalamus and pituitary of sea lamprey, and may be physiologically involved in the inhibition of GH and melanotropin release in the sea lamprey pituitary. (*Endocrinology* 148: 3740–3749, 2007)

NEUROPEPTIDES WITH Arg-Phe-amide (RFamide) motif at their C termini, which were originally found in the ganglia of the venus clam, (FMRFamide) (1), have been identified in the brains of several vertebrates and referred to as RFamide peptide(s). A chicken pentapeptide (LPLRFP-amide) has also been isolated from its brain (2). Two pain modulatory neuropeptides [FF and AF (3)], prolactin-releasing peptide (PrRP) (4), gonadotropin-inhibitory hormone (5), and GH-releasing peptide (6, 7) are also RFamide peptides. To date, these RFamide-peptides have had important physiological roles in neuroendocrine, behavioral, sensory, and autonomic functions (8–10).

Two PrRPs consisting of 31 amino acids (aas) (PrRP31) and 20 aa (PrRP20) from bovine hypothalamus extract were potent stimulators of prolactin (PRL) release as an endogenous ligand of an orphan G protein-coupled receptor (hGR3) (4). Immunocytochemical studies showed that, in rat, PrRP cell bodies were located in the brain and hypothalamus, and that their nerve fibers projected into a wide range of areas in the brain (11–14). However, no immunoreactive fibers were observed in the external layer of the median eminence, which is known to be the release site of the classical hypophysiotropic hormones (11, 14). Moreover, in recent physiological studies in mammals, many functions other than PRL stimulation have been reported for PrRP, and these include regulation of secretion of ACTH, oxytocin, FSH, LH and GH, cardiovascular regulation, stress responses, metabolic homeostasis, sleep regulation, and food intake (15–18). These indicate that PrRP may be involved not only in stimulation of PRL release but also in the regulation of other physiological processes.

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Abbreviations: aa, Amino acid(s); C_t, cycle threshold; GTHβ, gonadotropin β-subunit; HBSS, Hank's balanced salt solution; MALDI-TOF, Matrix Assisted Laser Desorption/Ionization Time-of-Flight; MIF, melanotropins-release-inhibiting factor; MSH, melanotropin; NAPv, periventricular arcuate nucleus, ventral part; NH, neurohypophysis; PI, pars intermedia; POC, proopiocortin; POM, proopiomelanotropin; PPD, proximal pars distalis; PRL, prolactin; PrRP, prolactin-releasing peptide; RPD, rostral pars distalis; SL, somatolactin; TFA, trifluoroacetic acid; UPM, universal primer mix.

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In teleost, a homolog of mammalian PrRP20 was first isolated from the brain of Japanese crucian carp, *Carassius auratus langsdorfii* (19). To clarify whether or not the mammalian PrRP homolog is also involved in the regulation of PRL release from the pituitary, we identified RFamide peptides in chum salmon, *Oncorhynchus keta*, and tilapia, *Oreochromis mossambicus*, by cDNA cloning and peptide isolation from the brain/hypothalamus (20, 21). Salmon and tilapia RFamide peptides were identical to the crucian carp RFamide peptide. By immunocytochemical analysis, salmon RFamide peptide cell bodies were observed in the posterior part of hypothalamus, and its fibers were abundant from the hypothalamus to the brain, as in the case in mammals (18, 20). However, unlike in mammals, a few RFamide peptide fibers were projected to the pituitary, and terminated close to PRL-producing cells in the rostral pars distalis (RPD) and to the somatolactin (SL)-producing cells in the pars intermedia (PI) in rainbow trout (20). On the basis of the localization of salmon RFamide peptide, we compared its hypophysiotropic effects on the release of three evolutionarily related hormones, PRL, GH, and SL, in the rainbow trout. Salmon RFamide peptide stimulated PRL release from the pituitary both *in vivo* and *in vitro*, as well as in tilapia (20–22). Salmon RFamide peptide also affected SL and GH releases from the pituitary (20, 22). These results indicate that RFamide peptide is a major hypothalamic peptide involved in the regulation of PRL release and that this peptide may exist throughout vertebrate evolution. However, there is no evidence for the presence of PrRP and/or its homolog in the condrichthyes and agnathans.

Lampreys and hagfish of the class Agnatha are of particular importance in understanding endocrinological relationships of the brain-pituitary axis because they represent the oldest lineages of extant vertebrates (23). In lampreys, six brain/hypothalamic peptides (GnRH-I, GnRH-III, somatostatin-14, peptide methionine-tyrosine, tachykinin, and neuropeptide Y) have been identified (24), and recently, PQRFamide peptide has also been identified (25). On the other hand, the pituitary hormones in the lamprey were an enigma until we identified arginine vasotocin, melanotropins (MSHs), corticotropin (ACTH), and GH (25–31). Recently, we also identified gonadotropin β-subunit (GTHβ) from the sea lamprey pituitary (32). However, the endocrinological relationships between brain/hypothalamus and pituitary systems are still uncertain in this species. We demonstrated that GTHβ mRNA expression in the sea lamprey pituitary was stimulated after intraperitoneal injections of GnRH-I and III (32). The present study describes the identification and tissue distribution of RFamide peptides, which are homologs of teleost PrRP, in the sea lamprey, and their effects on release of pituitary hormones *in vitro*.

Materials and Methods

Tissues

Sampling and tissue collection were done in accordance with the University of New Hampshire Institutional Animal Care and Use Committee animal care guidelines. The brain, including hypothalamus and pituitary, of 4600 adult landlocked sea lampreys, *Petromyzon marinus*, in their upstream migration, were extirpated and immediately frozen on dry ice in June 2000 at Hammond Bay Biological Station, in Michigan. Brain was used for isolation of RFamide peptide and cloning of RFamide peptide cDNA. Adult sea-run sea lampreys were collected in a trap at the Cocheco River in Dover, NH, in May and June 2000 and 2005, during their upstream spawning migration from the ocean. The pituitaries dissected from freshly killed lampreys were used for incubation with lamprey RFamide peptides to measure the expression of pituitary hormones.

Isolation and structure of lamprey RFamide peptides

Pulverized frozen brain, including hypothalamus (90 g), of adult landlocked sea lamprey were boiled for 10 min and homogenized in 3% acetic acid as described by Moriyama *et al.* (20) and Seale *et al.* (21) with some modifications. The resulting supernatant was passed through three Sep-Pak C18 cartridge columns (Waters Corp., Milford, MA) and the retained material eluted with 60% acetonitrile in 0.1% trifluoroacetic acid (TFA), then loaded onto an immunoaffinity column coupled with antisynthetic salmon PrRP serum (lot no. 9807) (20) and eluted with 0.1 N acetic acid. The eluted fraction was loaded onto a reverse-phase HPLC column (ODS-120T column, 0.46 × 25 cm, 5 μm particle size; TOSOH, Tokyo, Japan), and eluted with a linear gradient of 20–50% acetonitrile in 0.1% TFA for 60 min at a flow rate of 1 ml/min and a column temperature of 40 C. Absorbance was monitored at 220 nm. Two immunoreactive peaks were detected with anti-synthetic salmon PrRP serum (20), named RFa-A and -B, and were subjected to aa sequence analysis by an automated protein sequencer (Shimadzu PPSQ-10; Shimadzu Biotech, Kyoto, Japan). Molecular weights of RFa-A and -B were estimated by Matrix Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) mass spectrometer using AXIMA CFR-plus V2.3.2 (Shimadzu Biotech). After determination of the aa sequences, RFa-A and -B were synthesized using an automated solid-phase peptide synthesizer (PSSM-8; Shimadzu Biotech) according to the manufacturer's protocols, and these peptides were purified by HPLC on a reverse-phase ODS-120T column as described previously. The characterized RFa-A and -B were compared with those of synthetic peptides by HPLC and MALDI-TOF mass spectrometry.

Cloning of lamprey RFamide peptide cDNAs

Total RNA from adult landlocked sea lamprey brain, including hypothalamus (200 mg), was prepared using ISOGEN (Nippon Gene, Tokyo, Japan) as described by Moriyama *et al.* (20). Poly(A)$^+$ RNA was prepared using Oligotex-dT30 super (Takara, Tokyo, Japan) according to the manufacturer's protocols. The concentration of total RNA and poly(A)$^+$ RNA was estimated by measuring the absorbance at 260 nm (conversion factor: 1 OD = 40-μg RNA/ml), and the purity was determined from the ratio of absorbance at 260:280 nm. First-strand cDNA was reverse transcribed from poly(A)$^+$ RNA using a SMART RACE cDNA Amplification Kit (BD Biosciences Clontech, Palo Alto, CA) according to the manufacturer's protocols. Two degenerate antisense primers and universal primer mix (UPM) provided in the kit were used to clone of 5′ region of putative RFamide peptide cDNA. Primers were designed based on the conserved regions of teleost PrRPs as follows:

ASP-1: 5′-GGGAAA(Ag)Cg(Cg)CC(Ag)AT(Tg)gg(Tg)C(Tg)(TC)AC-3′

ASP-2: 5′- CTCTTCCCA AA(Ag)Cg(Cg)CC-3′.

Primers were synthesized by Nihon Gene Research Laboratories, Inc. (Sendai, Japan). During PCR, a 50-μl reaction mixture [2 μl first-strand cDNA, 2 μl each of antisense and UPM (final concentration, 0.4 μM), 25 μl HotStar*Taq* Master Mix (QIAGEN, Hilden, Germany), and 19 μl RNase free water] was subjected to 35 cycles of amplification by PCR. After activation of *Taq* at 94 C for 15 min, each cycle consisted of 1 min denaturation at 94 C, 1 min primer annealing at 50 C, and 1 min primer extension at 72 C. The final extension was 7 min at 72 C.

For the amplification of the 3′ partial region of cDNA encoding RFa-B, three forward primers were synthesized based on the nucleotide sequence of the 5′ partial cDNA fragment as follows:

RFa-B-1: 5′-TACGCTCATAACGCGGCGGATCAACACGG-3′

RFa-B-2: 5′-TCCCCACCATGCTCCTACCGCTATTGAAA-3′

RFa-B-3: 5′-GAGAGCAGGCGACCACAAC-3′.

PCR conditions were as those described previously.

PCR-amplified cDNA products were electrophoresed on agarose gels (Nippon Gene) and visualized by ethidium bromide staining (Nippon

Gene). The cDNAs were extracted and purified from agarose gels using a QIAEX II Gel Extraction Kit (QIAGEN), ligated into pT7 Blue T-Vector (Novagen, Madison, WI), and transformed into JM109 competent cells (Nippon Gene) according to the manufacturer's protocols. Recombinant plasmid DNAs were prepared by the alkaline-sodium dodecyl sulfate method and sequenced on both strands with a capillary DNA sequencer (ABI PRISM 3100 genetic analyzer; PE Applied Biosystems, Foster City, CA) using a BigDye Terminator Cycle Sequencing Kit Ver. 1.1 (PE Applied Biosystems). To compensate for the errors associated with PCR, at least three clones from three independent PCRs were sequenced. DNASIS-Mac (Hitachi Software Engineering Co. Ltd., Yokohama, Japan) was used for processing the sequence, calculating sequence identity, and sequence alignment.

Immunohistochemistry

Brains with and pituitaries from female adult sea-run sea lamprey were used. Immunohistochemistry was conducted basically as described by Nozaki *et al.* (33), Moriyama *et al.* (20), and Kawauchi *et al.* (30) with slight modifications. Immunohistochemical staining of RFamide peptide neurons and fibers was performed using an antisynthetic salmon PrRP serum using a Histofine immunostaining kit (Nichirei, Tokyo, Japan). Adjacent sections were also stained with anti-lamprey nasohypophysial factor (lot no. 9207) (34), synthetic GH fragment (8-23) (lot no. 9901) (30), synthetic GTHβ fragment (52-68) (lot no. 0401) (32), and synthetic MSH-B (lot no. 9311) (27) sera. The anti-synthetic salmon PrRP and lamprey nasohypophysial factor sera were diluted 1:10,000, while the antisynthetic GH fragment, synthetic GTHβ fragment, and synthetic MSH-B sera were diluted 1:5,000, 1:2,500, and 1:8,000, respectively. To test the specificity of the immunoreactions, the control sections were incubated with anti-synthetic salmon PrRP and synthetic MSH-B sera that were preabsorbed overnight at 4 C with an excess amount of synthetic salmon PrRP and MSH-B (10 μg peptide in 1 ml antiserum), respectively.

Detection of RFa-B mRNA in the brain and pituitary

The brain and pituitary of adult landlocked sea lamprey were used. The pituitaries were separated into the RPD, the proximal pars distalis (PPD), and the PI. Total RNA of brain, RPD, PPD, and PI were individually extracted with 0.25 ml ISOGEN according to the methods described previously. The cDNA fragment for sea lamprey RFa-B, GH (30), GTHβ (32), proopiomelanotropin (POM), and proopiocortin (POC) (28) were amplified using the OneStep RT-PCR Kit (QIAGEN). RFa-B, GH, GTHβ, POM, and POC gene-specific sense and antisense primers were synthesized based on the nucleotide sequences as follows:

RFa-B BSP: 5′-TCCCCACCATGCTCCTACCGCTATTGAAA-3′
ASP: 5′-ACAACAAACACACCCATAAACAAAC-3′
GH SP: 5′-CGCCCTGCCGCGCGGGACAATGATC-3′
ASP: 5′-TCAGGGCTTGCTGCAGTCATG-3′
GTHβ SP: 5′-ACTGGCTCTGTGGCTCGAGGTG-3′
ASP: 5′-TAAACTCGAGGGATGTGATCGACTGG-3′
POC SP: 5′-ATGATGGGAAACTGCTCTCGACTGC-3′
ASP: 5′-CCCTCGGACTTCCACCACTCTCGCC-3′
POM SP: 5′-ACTACGAGCAGTGCTCCAACCCGGA-3′
ASP: 5′-CTCCTCCTCCAAGGAGCACAATCTC-3′
β-Actin SP: 5′- TACCCCATCGAGCACGGCATCATC-3′
ASP: 5′- TTGGGGTTGAGGGGGGCCTCTGT-3′.

During PCR, 25-μl reaction mixes [2 μl total RNA (100 ng), 5 μl 5xQIAGEN OneStep RT-PCR buffer, 1 μl dNTP Mix (10 mM each), 2 μl gene specific sense and antisense primers (10 μM), 2 μl β-actin sense and antisense primers (5 μM), 0.5 μl RT-PCR Enzyme Mix, and 8.5 μl RNase free water] were subjected to 30 cycles of amplification by RT-PCR. After reverse transcription at 50 C for 30 min and activation of *Taq* at 94 C for 15 min, each cycle consisted of 1 min denaturation at 94 C, 1 min primer annealing, and 1 min primer extension at 72 C. The final extension was 7 min at 72 C. The final PCR conditions were determined in preliminary examinations using 26–36 cycles. PCR products were analyzed by 3% agarose gel electrophoresis (Nippon Gene). The amplified DNAs were visualized with 0.025% ethidium bromide (Nippon Gene), and the area of the visualized DNA was measured using Densitograph (Atto, Tokyo, Japan). The amplified internal fragment of β-actin was also used as a standard. The relative amount of pituitary hormone cDNAs and β-actin cDNA was determined.

Effect of RFa-A and -B on the expression of pituitary hormone genes

Pituitaries from both sexes of adult sea-run sea lamprey were used. After dissection, pituitaries were washed twice with 1 ml Hank's balanced salt solution (HBSS) containing 25 mM HEPES (pH 7.0). They were then preincubated individually in a well of a 24-well multiple plate containing 500 μl HBSS with 25 mM HEPES at 20 C for 24 h. After removing the culture media, pituitaries (n = 6) were incubated with 500 μl culture media containing synthetic RFa-A and -B at 0, 10, 100, and 1000 pM at 20 C for 24 h. Pituitaries were then collected and stored at −80 C until used.

Quantitative real-time PCR assay

Total RNA from the RFa-A and -B incubated pituitaries was extracted individually with 0.25 ml ISOGEN, and single-strand cDNA was reverse transcribed using an Omniscript RT Kit (QIAGEN) according to the manufacturer's protocols. Primers and TaqMan probes specific for lamprey GH, GTHβ, POC, POM, and β-actin were designed with Primer-Express software (PE Applied Biosystems) according to the manufacturer's protocols. The following primers were used:

GH forward primer: 5′-CAGACACTCTGTTGCCAAAAGC-3′
Reverse primer: 5′-CGACCCCACGCGTCTCT-3′
TaqMan probe: 5′-CTACAATGAAAGGAGGCTCTCTCGCGC-3′
GTHβ forward primer: 5′-CGCCGAGTGTCGTTACATCA-3′
Reverse primer: 5′-ACCTCCTGGGCAATCTTCCT-3′
TaqMan probe: 5′-CTACACCTGGCAACTGATCGGGCAC-3′
POC forward primer: 5′-TGCTGGAATGATGGGAAACTG-3′
Reverse primer: 5′-GCCCGTGTCCCATTGCT-3′
TaqMan probe: 5′-ACGGCTGGACCAGGGGTGCTTC-3′
POM forward primer: 5′-GGCGTGCGAGAGCTGTCT-3′
Reverse primer: 5′-CCCTCTGGCGCCTCATCT-3′
TaqMan probe: 5′-CCCAGCCTGAGCCGCTCTGCT-3′
β-actin forward primer: 5′-GACCTCACCGACTACCTGATGAA-3′
Reverse primer: 5′-TGATGTCGCGCACGATCT-3′
TaqMan probe: 5′-CGTTCACCACGACGGCCGAGC-3′.

Real-time PCR was performed in 25-μl reaction mixture consisting of 1 × TaqMan Universal PCR Master Mix (PE Applied Biosystems), 900 nM each sense and antisense primers, 250 nM TaqMan probes, and 2.5-ng first-strand cDNA as a template by using ABI PRISM 7000 (PE Applied Biosystems). PCR conditions were 50 C for 2 min and 95 C for 10 min, followed by 40 reaction cycles of 95 C for 15 sec and 60 C for 1 min each. For each reaction, the cycle threshold (C_t) was determined, *i.e.* the cycle number at which fluorescence was detected above an arbitrary threshold (1.0). At this threshold, C_t values are within the exponential phase of the amplification. To estimate the relative amounts of GTHβ, GH, POC, and POM mRNA in pituitaries from RFamide peptide-treated and control fish, C_t values were normalized to those of the internal fragment of β-actin and compared.

Statistical analysis

All data are presented as mean ± SE. Group comparisons were performed using two-way ANOVA, followed by Fisher's least significant difference test. Differences at $P < 0.05$ and $P < 0.01$ were considered significant.

Results

Isolation of two RFamide peptides

Figure 1 shows a reverse-phase HPLC chromatogram of the adsorbed peptide from immunoaffinity chromatography coupled with anti-synthetic salmon PrRP serum. Two immunoreactive peaks were observed that eluted at 22 min (31%) and 24 min (32%), and named RFa-A and RFa-B, respectively. N-terminal sequences of RFa-A and -B were determined to be SASNAGSDINPEWYFGRGVRPIGR and

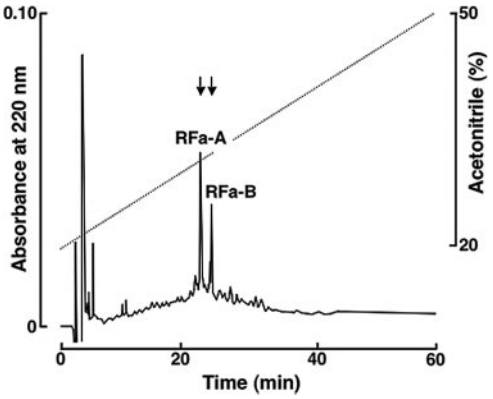

FIG. 1. HPLC of an adsorbed fraction from an immunoaffinity column on a reverse-phase ODS-120T column (0.46 × 25 cm; 10-μm particle size) at a temperature of 40 C and a flow rate of 1 ml/min. The *dashed line* represents a gradient of acetonitrile in 0.1% TFA. *Arrows* indicate elution positions of synthetic RFa-A and -B.

GREVNPLWYVGRGVRP, respectively. The total yields of RFa-A and -B were 25 and 10 pmol, respectively, from 90 g lamprey brain based on the yield of aa sequence analysis. By MALDI-TOF Mass spectrometry, the molecular ion peaks in the spectrum of RFa-A and -B were 2753.84 and 2328.78 mass (m/z), respectively (Fig. 2). These values were close to the mass numbers 2752.37 and 2327.30 calculated for SASNAGSDINPEWYFGRGVRPIGRF-NH_2 and GREVNPLWYVGRGVRPIGRF-NH_2 (Fig. 3). To confirm these sequences, these peptides were synthesized and compared with the natural peptides with regard to their retention times on HPLC and the mass number. Both natural and synthetic peptides showed identical elution times on reverse-phase HPLC (Fig. 1). Thus, we concluded that RFa-A and -B consist of 25 and 20 aa, respectively. RFa-A is five aa longer than RFa-B at the N terminus, and differed only at five aa positions within the C terminal 20 aa. RFa-A is 14 aa identical with those of teleost PrRPs and putative chicken PrRP (35), and 15 aa identical with those of putative frog PrRP (36), putative chicken C-RFa (35), and bovine PrRP31 (Fig. 3). RFa-B is 15 aa identical with those of teleost PrRPs, putative fog PrRP, and putative chicken C-RFa, and 12 aa and 11 aa identical with those of bovine PrRP31 and putative chicken PrRP.

Cloning of RFa-B cDNAs

A RFa-B cDNA fragment of 263 bp at the 5′ region was amplified in the first PCR using the degenerate reverse primer ASP-1 and UPM (Fig. 4). The second PCR using a 3′ RACE forward primer and UPM yielded a product of 1324 bp that spanned from the beginning of the 3′ end and overlapped with the known sequence of the 5′region. Excluding the poly(A) tail, the RFa-B cDNA consisted of 1400 nucleotides. The RFa-B cDNA open reading frame encodes 142 aa. The deduced aa sequence between positions 48 and 67 of preproRFa-B was identical to the sequence of the purified RFa-B. The typical proteolytic cleavage sequence, Lys and Arg, were located at positions 47 and 69–70; the amidation motif (Gly-Lys-Arg) was identified at positions 68–70.

Immunohistochemistry

The distribution of cell bodies and fibers, which were immunoreactive to anti-salmon PrRP serum, is summarized in Fig. 5. The immunoreactive RFamide cell bodies were observed in the periventricular arcuate nucleus, ventral part (NAPv) of the hypothalamus (Fig. 6A, a and b) (37). Immunoreactive fibers were abundant from the hypothalamus to the brain (Fig. 6A). A small number of immunoreactive fibers were projected to the dorsal half of the PPD and terminated close to GH-producing cells (Fig. 7A). Cells immunoreactive to anti-salmon PrRP serum were also found in the PI (Fig. 7C) corresponding to the MSH-producing cells (Fig. 7D) and neurohypophysis (NH) (Fig. 7C). No immunoreactive cells were observed in tissues incubated with antibody preabsorbed with an excess amount of synthetic salmon PrRP (data not shown).

Detection of RFa-B mRNAs in the brain and pituitary

RFa-B mRNA was detected in the brain of the landlocked lamprey by RT-PCR (Fig. 8). RFa-B mRNA was also detected in the PI, but not in the RPD and PPD. On the other hand, POC, GH, GTHβ, and POM mRNA were detected in the RPD, dorsal and ventral half of the PPD, and PI, respectively.

Effects of RFa-A and -B on the expression of GH, GTHβ, POC, and POM genes

Pituitaries from sea lamprey were incubated with the synthetic RFa-A and -B at concentrations of 0, 10, 100, and 1000 pM, respectively, for 24 h at 20 C. Incubation of sea lamprey pituitary with the synthetic RFa-A and -B at concentrations of 100 and 1000 pM for 24 h resulted in a significant decrease in GH mRNA levels measured by real-time PCR (Figs. 9A and 10A) compared with the controls. No change was observed in GTHβ mRNA levels in pituitaries incubated with

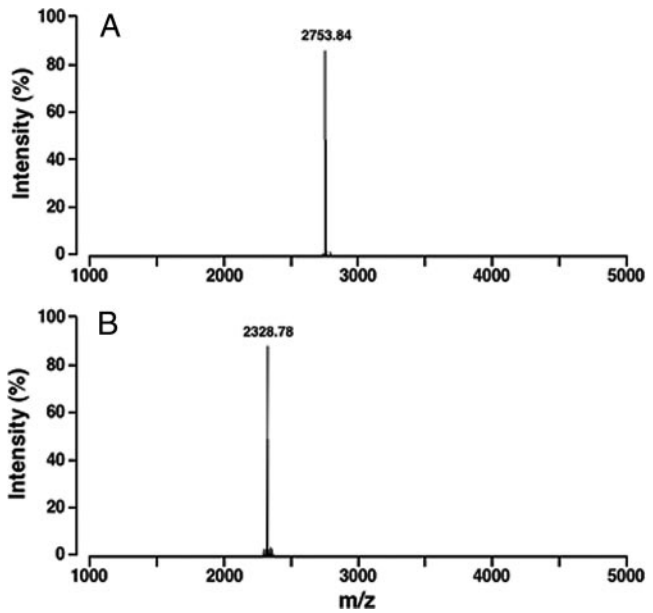

FIG. 2. MALDI-TOF mass spectrometry of the purified RFa-A (A) and -B (B). The synthetic RFa-A and -B are 2752.37 and 2327.30 m/z, respectively.

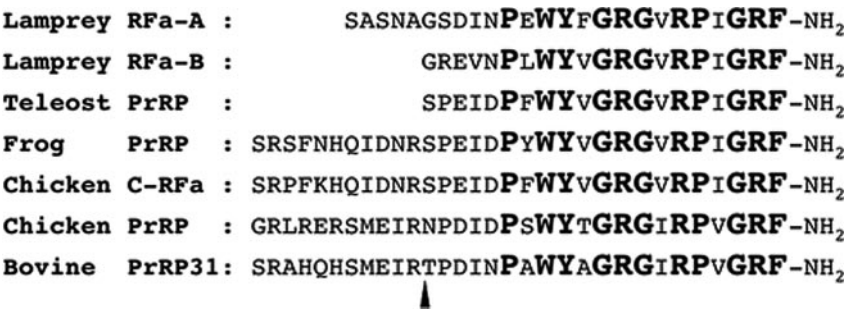

FIG. 3. Sequence comparison of lamprey RFa-A and -B with teleost PrRP (20, 21), putative frog PrRP (36), putative chicken PrRP and C-RFa (35), and bovine PrRP31 (4). *Bold and big characters* represent identical residues. *Arrowhead* indicates processing site of bovine PrRP20.

RFa-A and -B (Figs. 9B and 10B). A significant decrease in POM mRNA levels was also observed at concentrations of 100 and 1000 pM of RFa-A and -B (Figs. 9D and 10D), but no change was observed in POC mRNA levels (Figs. 9C and 10C).

Discussion

In the present study, we identified novel RFamide peptides in sea lamprey, isolation, and cDNA cloning, and compared the sequences with those of newly identified PrRPs in teleost such as Japanese crucian carp (19), chum salmon (20), and tilapia (21), as well as bovine PrRP (4). We further demonstrated by immunocytochemistry that cells immunoreactive with anti-salmon PrRP serum were located not only in the hypothalamus but also in the PI of the pituitary. RFamide peptides inhibited the expression of GH and POM mRNAs in the pituitary organ cultures of sea lamprey pituitary. These results are the first to demonstrate that a homolog of teleost PrRP is not only a hypothalamic peptide but is also an adenohypophysic peptide regulating GH and MSH release in basal vertebrates, the agnathans.

Identification of lamprey RFamide peptides

We first isolated two RFamide peptides possessing PrRP immunoreactivity with anti-salmon PrRP serum from acid extracts of sea lamprey brain/hypothalamus. On the basis of the results of structure determinations, such as aa sequence, molecular weight presumption, and comparison of elution position on a reverse-phase HPLC, the isolated RFamide peptides were considered to be 25 and 20 aa peptides with the structures: SASNAGSDINPEWYFGRGVRPIGRF-NH_2 and GREVNPLWYVGRGVRPIGRF-NH_2. These identified RFamide peptides are novel peptides having a similar C-terminal structure with those of teleost and mammalian PrRPs (4, 19–21). Thus, we named the 25 aa peptide RFa-A and the 20 aa peptide RFa-B, respectively. In nonmammalian, recently, PrRP homologs were identified in chicken (35) and frog (36). C-terminal regions of RFa-A (11–25 aa position) and RFa-B (6–20 aa positions) are highly conserved in relation to known PrRPs and its homologs, including teleosts, frog, chicken, and mammals (Fig. 3). RFa-A shows similar sequence identity to putative chicken PrRP and C-RFa, while RFa-B shows higher sequence identity to putative chicken

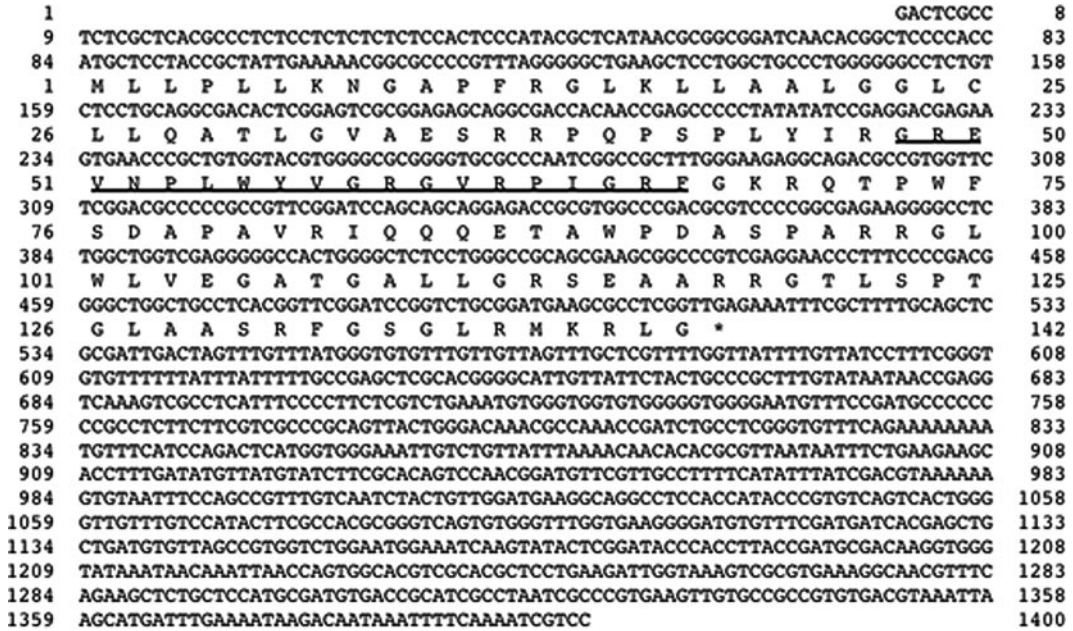

FIG. 4. Nucleotide and deduced aa sequence of RFa-B cDNA without the poly(A) tail. The number of nucleotides and aa residues are indicated on either side of the sequence. *Underline* shows RFa-B. The stop codon is marked with an *asterisk*.

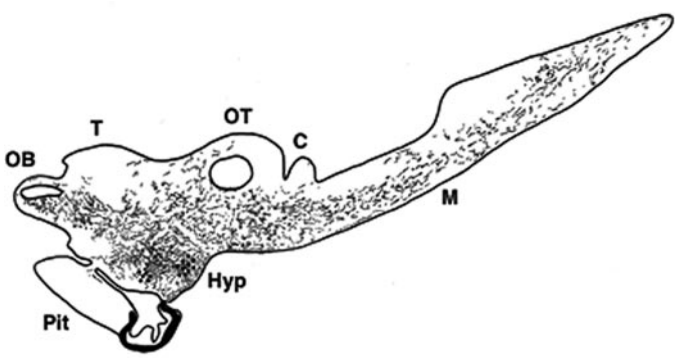

FIG. 5. Schematic distribution of RFamide peptide-immunoreactive cell bodies (*dots*) and fibers (*line*) with anti-synthetic salmon PrRP serum in a sagittal section of the lamprey brain, hypothalamus (Hyp), and pituitary (Pit). C, Cerebellum; M, medulla oblongata; OB, olfactory bulb; OT, optic tectum; T, telencephalon.

C-RFa than to putative chicken PrRP. We previously demonstrated that salmon and tilapia PrRPs, which are identical to crucian carp C-RFa, stimulate PRL release from the pituitary (20, 21). Together, this evidence suggests that lamprey RFa-A and -B may be PrRP homologs.

In the present study, we cloned the cDNA encoding the RFa-B from lamprey brain, including hypothalamus, by a combination of 5′ and 3′ RACE. RFa-B cDNA encodes a preprohormone with 142 aa, the same as in teleost PrRPs (20, 21, 38). The lamprey prepro-RFa-B sequence at positions 48–67 was identical with the sequence of the isolated RFa-B. Analysis of the deduced aa sequence of the preprohormone indicates that lamprey RFa-B consists of 20 aa with C-terminal amidation motif as in the case of teleost PrRPs (20, 21, 38). This also indicates that RFa-B consists of 47 aa of signal peptide, which is longer than teleost PrRP. The typical proteolytic cleavage sequence, Lys and Arg, is located at positions 69 and 70. Thus, after cleavage between Gly at position 68 and Lys at position 69, Phe at the C terminus of the mature peptide would be amidated by reacting with Gly, similar to teleost and mammalian PrRPs (4, 20, 21, 38).

On the other hand, RFa-A cDNA was not cloned by PCR from the lamprey brain, including hypothalamus, in the present study, even though various primers designed using the conserved nucleotide sequences of teleost PrRPs, including lamprey RFa-B, were used. The RFa-B cDNA did not contain the RFa-A sequence. These data suggest that RFa-A and -B might be transcribed from different mRNAs. These results, together with sequence comparison, indicate that PrRP homologs structurally related to teleost PrRP exist in the lamprey brain/hypothalamus, and these peptides may possess hypophysiotropic functions.

Tissue distribution of lamprey RFamide peptides

In the present study, cell bodies immunoreactive with anti-salmon PrRP serum were observed in the NAPv of the hypothalamus of lamprey, and immunoreactive fibers were widely distributed from the hypothalamus to the brain. In rainbow trout (20) and guppy (39), PrRP somata were located only in the posterior part of the hypothalamus, and the fibers were projected widely from the hypothalamus to the brains. In goldfish, RFamide peptide cell bodies were observed in the telencephalon, medulla oblongata, diencephalons, midbrain tegmentum, and olfactory bulb in the brain, and immunoreactive fibers were widely distributed in the hypothalamus and brain (40). In rat, neuronal perikarya with PrRP immunoreactivity and PrRP mRNA signals were distributed in the ventromedial and dorsomedial nuclei of the hypothalamus and nucleus of solitary tract, and ventral and lateral reticular nuclei in the medulla oblongata (12, 13), and PrRP fibers projected into a wide range of areas in the brain. It has also been reported that PrRP axon terminals appeared to contact tyrosine-hydroxylase immunoreactive neurons in the arcuate nucleus, as well as oxytocin, CRH, and somatostatin-immunoreactive neuronal elements in the rat (15, 16, 18). Together, these results suggest that lamprey RFamide peptides may play a role as neurotransmitters or neuromodulators, as in mammals.

In rainbow trout, a few PrRP axon terminals were projected in the RPD and PI, close to PRL- and SL-producing

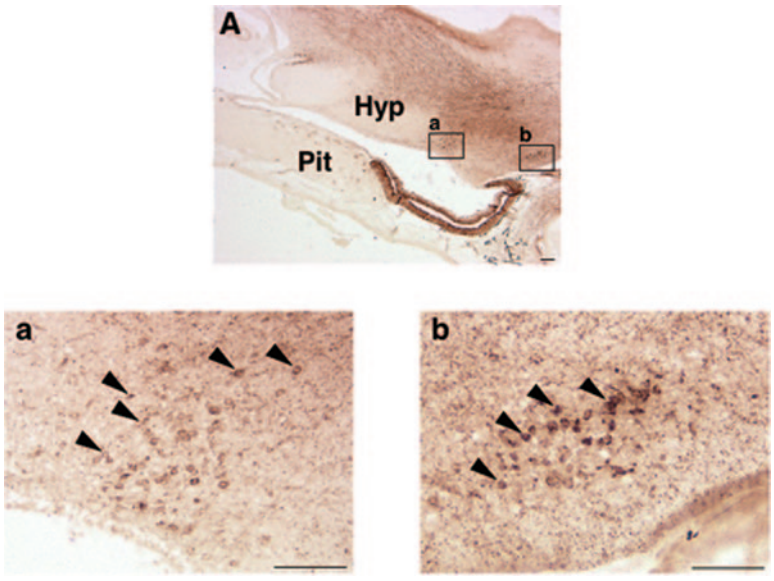

FIG. 6. A, RFamide peptide-immunoreactive cell bodies and fibers in the brain, hypothalamus (Hyp), and pituitary (Pit). *Boxed areas* in A are enlarged in a and b. RFamide peptide immunoreactive cell bodies in the NAPv of the hypothalamus (a, b). *Arrowheads* indicate RFamide peptide cell bodies. *Bar*, 100 μm.

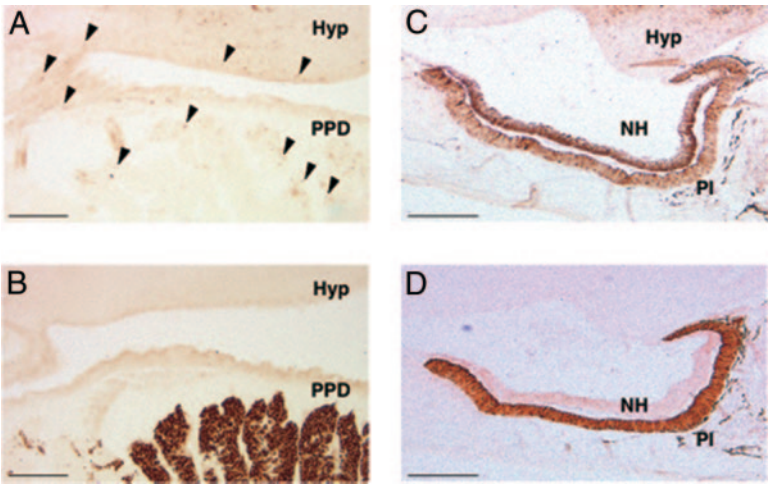

FIG. 7. A, RFamide peptide-immunoreactive fibers in the dorsal of the PPD. *Arrowheads* indicate RFamide peptide-immunoreactive fibers in the hypothalamus (Hyp) and the PPD. B, GH-immunoreactive cells in the dorsal part of the PPD. C, RFamide peptide-immunoreactive cells in the PI. D, MSH-B-immunoreactive cells in the PI. *Bar*, 100 μm.

cells (20). In goldfish and guppy, RFamide peptide-immunoreactive fibers were also projected in the pituitary (39, 40). Therefore, PrRP seems to be a candidate for a physiological regulator of the pituitary function in teleosts. Indeed, salmon and tilapia PrRPs regulate the secretion of PRL, GH, and SL from the pituitary gland (20–22). The adenohypophysis of the lamprey pituitary gland is divided into three regions, the RPD, PPD, and PI, as in gnathostome fish. In our previous studies, we have demonstrated that the ACTH- and MSH-producing cells are localized in the RPD and PI, and the GH- and GTH-producing cells are localized in the dorsal and ventral half of PPD, respectively (30, 32, 41). In the present study, some RFamide peptide-immunoreactive axon terminals were projected in the dorsal half of the PPD, close to GH-producing cells. These immunoreactive fibers disappeared when tissue were incubated in preabsorbed with excess amounts of the synthetic salmon PrRP. Thus, the immunocytochemical staining was considered to be specific for the peptide, suggesting that lamprey RFamide peptides may regulate GH secretion in sea lamprey, as in teleost.

Interestingly, strong immunoreactions with anti-salmon PrRP serum were observed in the PI and NH of the lamprey pituitary. Although PrRP-immunoreactive fibers were observed in the pituitary of teleost, no immunoreactive cells in the pituitary were observed in the pituitary gland (20, 39, 40). Immunoreactivity with anti-salmon PrRP serum in the PI was observed in MSH-producing cells. The immunocytochemical staining was considered to be specific for the peptide because preabsorption of the antiserum with the synthetic salmon PrRP resulted in a complete disappearance of the reaction product. In addition to the results of immunocytochemistry, we also demonstrated that RFa-B mRNA signal was detected in the brain, including hypothalamus, and PI, but not in the RPD and PPD. These results indicate that at least RFa-B is produced not only in the hypothalamus but also in PI of the lamprey pituitary. It is also suggested that lamprey RFamide peptides may regulate MSH secretions.

Hypophysiotropic activities of lamprey RFamide peptides

PRL has not yet been identified in lamprey. Therefore, in the present study, we examined the effects of RFamide peptides on the expression of GH, GTHβ, POC, and POM mRNAs using a pituitary organ culture system because no specific radioimmunoassays are available to measure these pituitary hormones in sea lamprey.

Both of the synthetic RFa-A and -B inhibited GH mRNA expression in a concentration dependent fashion *in vitro*. In teleosts, salmon PrRP inhibited GH secretion from the pituitary *in vivo*, but no change in GH release was observed *in vitro* (20, 22). In rat, PrRP inhibited GH release from the pituitary (42, 43). The inhibitory effects of PrRP on GH were diminished by depletion or neutralization of somatostatin (42). Together, these results suggest that, in lamprey, RFamide peptides may directly inhibit GH secretion from the pituitary. However, further investigation is necessary to elucidate the possible effects of RFamide peptides on GH secretion.

A significant inhibition of POM mRNA expression was also seen in pituitaries incubated with 100 and 1000 pM of RFa-A *in vitro*, but no changes in POC mRNA levels were observed. It has been reported that, in rat, PrRP regulates food intake and body weight (44–46). The effects on food intake seem to be mediated by stimulatory effects of PrRP on the release of α-MSH, which is important in the inhibition of food intake (44). After an intracerebroventricular injection of PrRP, plasma α-MSH levels were increased, while food intake decreased in rats. On the other hand, central administration of PrRP stimulates plasma ACTH levels, an effect that is dependent on CRH receptor activation in rat (47, 48). In gnathostomes, proopiomelanocortin is the common precursor of ACTH, MSHs, and β-endorphin. In sea lamprey, MSH and ACTH were found to be encoded by two distinct genes,

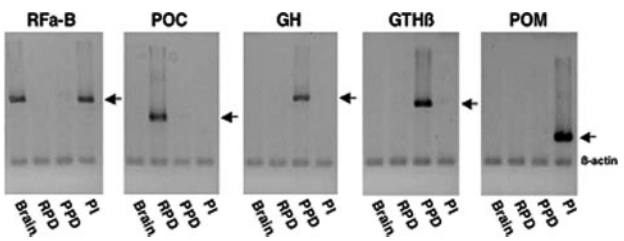

FIG. 8. Localized expression of lamprey RFa-B, POC, GH, GTHβ, and POM mRNAs in the brain, RPD, the PPD, and PI. *Arrows* indicate target mRNAs.

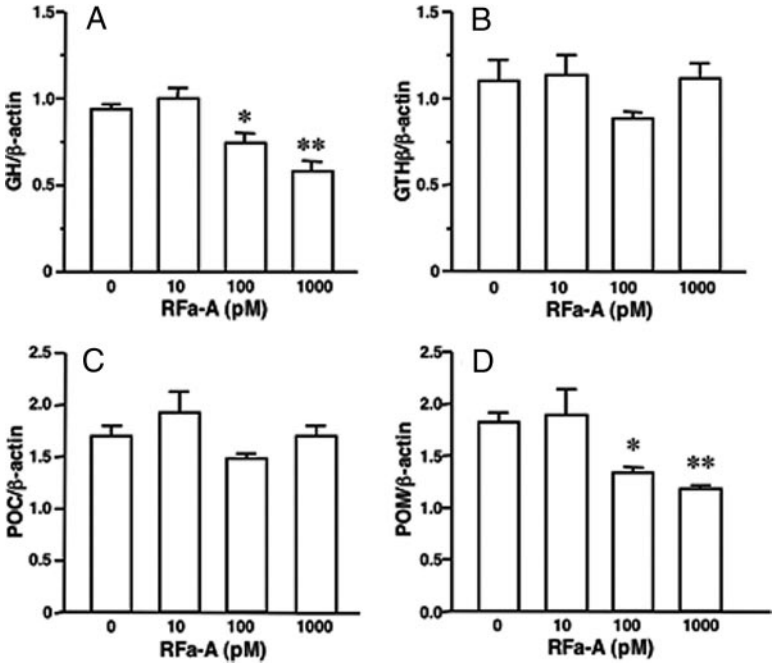

FIG. 9. Effects of RFa-A (0, 10, 100, and 1000 pM) on expression of GH (A), GTHβ (B), POC (C), and POM (D) in the pituitary of adult lamprey after 24-h preincubation in HBSS with 25-mM HEPES (pH 7.0), followed by incubation with or without (control) RFa-A for 24 h. Data are shown as means ± SEM (n = 6). Significant differences from the control are indicated by * ($P < 0.05$) and ** ($P < 0.01$), respectively.

POM and POC, respectively. POM is expressed in the PI, while POC is expressed in the RPD (27–29). Thus, in the present study, it is suggested that RFamide peptides may regulate MSH production, but not ACTH production, in lamprey because RFamide peptides inhibited POM mRNA. However, the present findings are in contrast to the situation in mammals.

Rat hypothalamus contains a factor that inhibits the release of MSH (49). The tripeptide, Pro-Lue-Gly-NH_2 [melanotropins-release-inhibiting factor (MIF)], which is derived from the C terminal of oxytocin, inhibits MSH release from the pituitary both *in vivo* and *in vitro*. In contrast, Thody *et al.* (50) reported that MIF did not affect the release of MSH from the rat pituitary both *in vivo* and *in vitro*. Therefore, the function of MIF is still unclear. Although the structure of lamprey RFamide peptide shows no homology to MIF, RFamide peptides inhibited the POM mRNA levels. It is suggested that one of the functions of lamprey RFa-A and -B may be inhibition of MSH production. Thus, further investigation is necessary to elucidate the possible effects of RFamide peptides on MSH secretion.

In the present study, no changes in the GTHβ mRNA

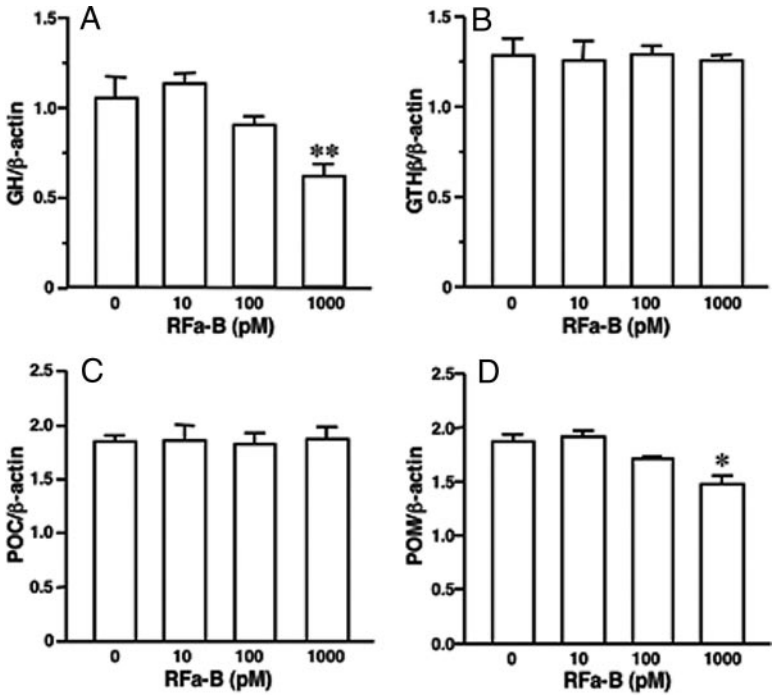

FIG. 10. Effects of RFa-B (0, 10, 100, and 1000 pM) on expression of GH (A), GTHβ (B), POC (C), and POM (D) in the pituitary of adult lamprey after 24-h preincubation in HBSS with 25-mM HEPES (pH 7.0), followed by incubation with or without (control) RFa-A for 24 h. Data are shown as means ± SEM (n = 6). Significant differences from the control are indicated by * ($P < 0.05$) and ** ($P < 0.01$), respectively.

levels were observed in cultured pituitaries of lamprey incubated with RFamide peptides. In rat, PrRP stimulates LH and FSH secretion after intracerebroventricular injection (51–53). However, no changes in LH and FSH levels were observed *in vitro* (44). Thus, it is considered that the effect of PrRP on gonadotropin release is mediated by stimulatory effects of PrRP on GnRH release (44, 52). Watanobe (53) reported that, before GnRH secretion and LH surge, PrRP is released in the medial preoptic area. These results suggest that, like in mammals, lamprey RFamide peptides may not directly affect GTH secretion. Further investigation is necessary to elucidate the possible secondary effects of RFamide peptides on GTH secretion.

In conclusion, we identified two RFamide peptides, which are structurally related to teleost PrRP, by peptide isolation and cDNA cloning from lamprey brain/hypothalamus. Immunocytochemical localization of RFamide peptide-immunoreactive cells and fibers suggests a role for the peptides as neurotransmitters or neuromodulators, and as hypophysiotropic factors for GH and MSH. In the present study, we report that at least RFa-B is produced in the pituitary. These results provide evidence that RFamide peptides are major hypothalamic and/or pituitary peptides that may be involved in inhibition of GH and MSH release in lamprey.

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