Molecular properties of endogenous RFamide-related peptide-3 and its interaction with receptors

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Abstract

Based on database searches of DNA sequences, we previously reported a gene encoding peptides possessing Arg-Phe-NH$_2$ (RFamide) at their C termini. This gene, RFamide-related peptide (RFRP), was expected to encode several different peptides (i.e., RFRP-1, -2, and -3). In the present study, we purified endogenous RFRP-3 from bovine hypothalamus, and demonstrated that it consisted of 28 amino acid residues. After constructing a sandwich enzyme immunoassay for RFRP-3, we analyzed the tissue distribution of endogenous RFRP-3 in rats and found its concentration to be highest in the hypothalamus. In binding assays, [^{125}I]-labeled RFRP-3 bound to OT7T022 with high affinity, but its binding affinity to HLWAR77 was low. On the other hand, [^{125}I]-labeled neuropeptide FF (NPFF) bound to both OT7T022 and HLWAR77 with high affinity. By serial deletion in the N-terminal portions of RFRP-3 and NPFF, we found that four C-terminal amino acid residues (i.e., PQRFamide), which were common between the two peptides, comprised a core sequence responsible for binding with the receptors, whereas three amino acid residues (i.e., PNL in RFRP-3 and LFQ in NPFF) added to the N terminus of PQRFamide played crucial roles in the agonistic activities of RFRP-3 and NPFF for OT7T022 and HLWAR77, respectively.
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Keywords: RFamide-related peptide-3; OT7T022; HLWAR77; Neuropeptide FF; Receptor

1. Introduction

Biologically active peptides having RFamide structures at their C termini are found widely in the animal kingdom from invertebrates to mammals, and are generally referred to as 'RFamide peptides'. We have previously reported the identification of a novel mammalian RFamide peptide gene which we named RFamide-related peptide (RFRP) [1]. Employing bioinformatics, we isolated it originally from databases of human genomic and cDNA sequences. RFRP preproproteins encoded by the human or bovine gene have been expected to

generate three kinds of mature peptides through processing, that is, RFRP-1, -2, and -3. However, in the case of rats, only RFRP-1 and RFRP-3 are expected to be generated. Synthetic RFRP-1 and RFRP-3 have been demonstrated to bind to the G protein-coupled receptor (GPCR), OT7T022, and to suppress forskolin-induced cAMP production in Chinese hamster ovary (CHO) cells expressing OT7T022. As RFRP is mainly distributed in the rat's central nervous system (i.e., hypothalamus), it is thought to play a role in the regulation of the endocrine system [1]. In another previous paper, we purified endogenous RFRP-1 from bovine hypothalamic tissue extracts, and demonstrated that endogenous bovine RFRP-1 consisted of 35 amino acid residues [2].

In the present study, we prepared an antibody for the C-terminal portion of RFRP-3, and purified endogenous RFRP-3 from bovine hypothalamic tissue extracts on the basis of immunoreactivity to this antibody. We then determined the molecular structure of bovine endogenous RFRP-3. We also analyzed the tissue distribution of endogenous RFRP-3 in rats by enzyme immunoassay (EIA). OT7T022 and HLWAR77 receptors are highly homologous, and both receptors have been reported to interact with neuropeptide

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FF (NPFF) [3–5]. We therefore examined the differences between RFRP and NPFF in their interaction with these two receptors. Finally, we analyzed the amino acid residues of these peptides to determine those critical for receptor interaction.

2. Materials and methods

2.1. Peptides

Human RFRP-1 (SLNFEELKDWGPKNVIKMSTPAV-NKMPHSFANLPLRFamide: hRFRP-1-37), human RFRP-3 (ATANLPLRSGRNMEVSLVRRVPNLPQRFamide: hRFRP-3-28), human RFRP-3(21–28)amide (VPNLPQR-Famide: hRFRP-3-8), human RFRP-1(26–37)amide (MPH-SFANLPLRFamide: hRFRP-1-12), rat Cys-RFRP-3(13–20) amide (CFPSLPQRFamide: rRFRP-3C), rat RFRP-3(4–20)-Cys (NMEAGTMSHFPSLPQRFC: rRFRP-3N), and rat SPR-RFRP-3 (SPRARANMEAGTMSHFPSLPQRFamide: rRFRP-3-23) were synthesized using an automatic peptide synthesizer (Model 430, PE Biosystems) as described previously [1,6]. Bovine RFRP-3 (AMAHLPLRLGKN-REDSLSRWVPNLPQRFamide: bRFRP-3-28), bovine RFRP-1 (SLTFEEVKDWAPKIKMNKPVVNKMPPSAA-NLPLRFamide: bRFRP-1-35), NPFF (FLFQPQRFamide), bovine neuropeptide AF (AGEGLSSPFWSLAAPQR-Famide: NPAF), bovine SPA-NPFF (SPAFLFQPQRFamide: bNPSF), human SQA-NPFF (SQAFLFQPQRFamide: hNPSF), bovine prolactin-releasing peptide (PrRP) with 20-amino acid length (TPDINPAWYAGRGIRPVGRF-amide: bPrRP20), PQRFamide, and LPLRFamide were purchased from commercial suppliers (Peptide Institute or Bachem).

N-terminal truncated peptides, that is, human RFRP-3(22–28)amide (PNLPQRFamide: hRFRP-3-7), human RFRP-3(23–28)amide (NLPQRFamide: RFRP-3-6), human RFRP-3(22–28)amide (LPQRFamide: hRFRP-3-5), bNPSF(2–11)amide (PAFLFQPQRFamide: bNPSF-10), bNPSF(3–11)amide (AFLFQPQRFamide: bNPSF-9), NPFF(2–8)amide (LFQPQRFamide: NPFF-7), NPFF(3–8)amide (FQPQRFamide: NPFF-6), and NPFF(4–8)amide (QPQRFamide: NPFF-5) were prepared from hRFRP-3-8 or bNPSF by Edman degradation as described elsewhere [7].

2.2. Immunological procedures

A monoclonal antibody for the C-terminal portion of RFRP-3 was prepared according to our previously described method [2,8]. Briefly, rRFRP-3C was conjugated with bovine serum albumin (BSA) and then used as an antigen to immunize mice. By fusing myeloma and spleen cells from the immunized mice, we obtained a hybridoma cell clone (7F6) that produced an antibody for rRFRP-3C. A competitive EIA to detect RFRP-3 was constructed by utilizing the monoclonal antibody and horseradish peroxidase (HRP)-

conjugated rRFRP-3C as a tracer. In this competitive EIA, we used hRFRP-3-8 as a reference to quantify RFRP-3.

To quantify rat endogenous RFRP-3, we constructed a sandwich EIA by a procedure similar to that which we previously used to construct EIA for rat endogenous RFRP-1 [2]. Briefly, we prepared a rabbit polyclonal antibody for the N-terminal portion of rat RFRP-3 by immunizing rRFRP-3N conjugated with BSA. The Fab fragment of the polyclonal anti-rRFRP-3N antibody was labeled with HRP, and then used for constructing the sandwich EIA with monoclonal 7F6 antibody. Rat tissue extracts subjected to the sandwich EIA for RFRP-3 were prepared by the same method used to quantify RFRP-1 [2]. The monoclonal antibody was coated onto 96-well microplates, following which the tissue extracts were added to the wells and the plates were incubated at 4 °C for 24 h. After the HRP-conjugated anti-rat RFRP-3N Fab was added to the wells, the plates were incubated again at 4 °C for 16 h. HRP activity trapped in each well was then measured. To quantify rat endogenous RFRP-3, we used rRFRP-3-23 as a reference.

2.3. Chromatography

Bovine hypothalamic tissues (2 kg) were boiled, homogenized in 1 M acetic acid, and extracted overnight. The resultant supernatant was collected by centrifugation. After adding 0.05% trifluoroacetic acid (TFA), the solution was applied to a Preparative C18 column (Waters) and elution was performed using stepwise increments of 10%, 30%, and 50% CH_3CN with 0.1% TFA. The immunoreactive RFRP-3 (ir-RFRP-3) content was determined by the competitive EIA described above. Ir-RFRP-3-positive fractions were eluted at 30% CH_3CN. They were then further fractionated by a HiPrep CM-Sepharose FF (Amersham Pharmacia Biotech) column with stepwise increments of 0.1, 0.2, 0.5, and 1.0 M NaCl in 20 mM CH_3COONH_4 (pH 4.7) containing 10% CH_3CN. After precipitation with acetone, the fraction eluted at 0.2 M NaCl was serially fractionated through high-performance liquid chromatography (HPLC) using a RESOURCE RPC column (Amersham Pharmacia Biotech) with a linear gradient from 10% to 30% CH_3CN with 0.1% TFA, and a TSK-gel CM-2SW column (Toso) with a linear gradient from 0.2 to 0.6 M NaCl in 20 mM CH_3COONH_4 at pH 4.7. The resultant fractions containing ir-RFRP-3 were finally applied to HPLC using a μRPC C2/C18 SC 2.1/10 column (Amersham Pharmacia Biotech) in a SMART system (Amersham Pharmacia Biotech).

2.4. Sequencing and mass spectrometry

Sequencing of the purified peptides was performed on a 491cLC Protein Sequencer (PE Applied Biosystems). Electrospray ionization mass spectrometry (ESI-MS) and ESI-MS/MS were preformed on an LCQ ion-trap mass spectrometer (Thermoquest) equipped with a nanospray ion source (Protana).

2.5. Receptor binding assays

For receptor binding assays, CHO cells expressing either human OT7T22 (hOT7T022) or HLWAR77, and [125I] hRFRP-3-8 were prepared according to our method described previously [1,6]. [125I]NPFF was purchased from Amersham Pharmacia Biotech (IM345).

2.6. cAMP production assays

The inhibitory activities of the peptides on forskolin-induced cAMP production in CHO cells expressing hOT7-T022 or HLWAR77 were determined by our method described previously [1,9].

3. Results

3.1. Purification of endogenous RFRP-3 from bovine hypothalamus

To detect endogenous bovine RFRP-3, we prepared a mouse monoclonal antibody, (7F6 with IgG1 heavy and κ light chains) that was capable of recognizing the C-terminal portion of RFRP-3, and then used this antibody to construct a competitive EIA. The EIA detected hRFRP-3-8, PQRF-amide, NPFF, and NPAF, but failed to detect hRFRP-1-12, LPLRFamide, bPrRP20, and PQRF-OH (data not shown). These results indicate that the EIA detected the epitope, PQRFamide, which is the consensus C-terminal sequence shared among RFRP-3, NPFF, and NPAF. We therefore employed this EIA to pursue bovine endogenous RFRP-3 in every purification step.
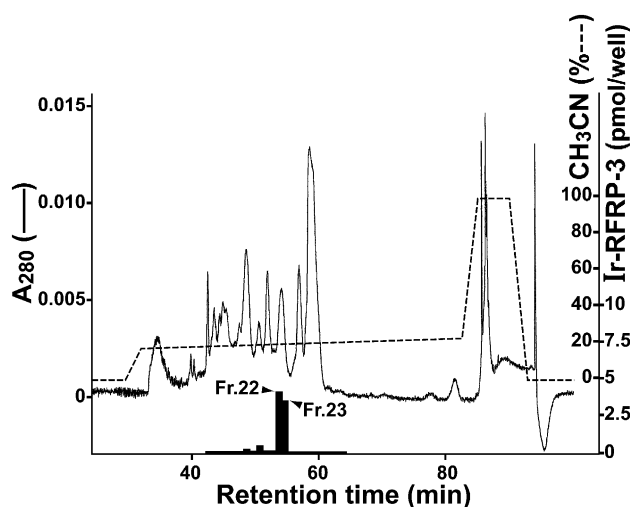

Fig. 1. Profile of μRPC C2/C18 SC 2.1/10-column chromatography in purification of endogenous RFRP-3. The amount of ir-RFRP-3 in each fraction detected with the competitive EIA is shown as a solid column. The dotted and jagged lines indicate the percentage of CH_3CN and absorbance, respectively. Arrowheads indicate the fraction numbers.
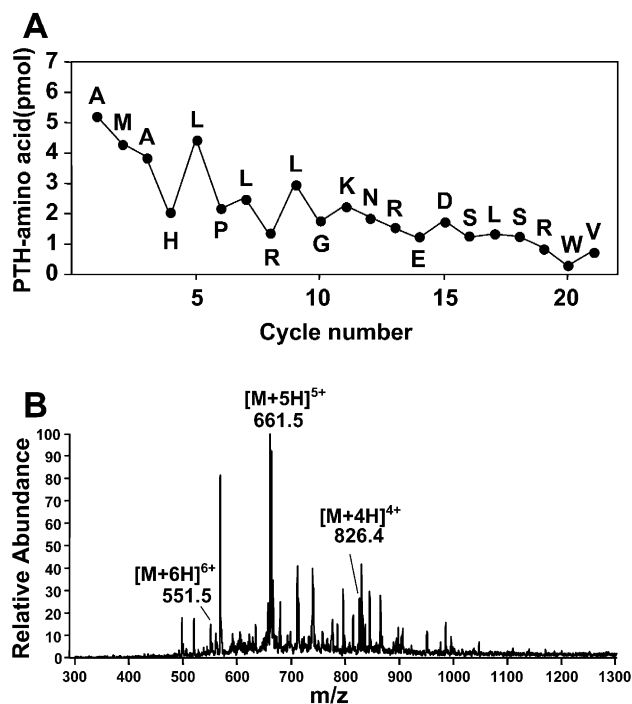

Fig. 2. Structural analyses for purified endogenous bRFRP-3. (A) Yield of phenylthiohydantoin (PTH)-amino acid at each cycle in Edman degradation. (B) ESI mass spectrum showing the multiply charged ions. Purified bRFRP-3 (11 pmol) was dissolved in 3 μl of 50% CH_3CN with 1% CH_3COOH, loaded in a gold-coated capillary, and analyzed with a LCQ mass spectrometer. Based on each m/z value of the multiply charged ion signals, the molecular weight of bRFRP-3 was calculated using Bioworks software.

Having previously succeeded in purifying endogenous RFRP-1 from bovine hypothalamic tissue extracts [2], we employed the same tissue as a starting material to purify endogenous RFRP-3. Purification was performed with a combination of various chromatographies through monitoring ir-RFRP-3 with the competitive EIA. In CM-Sepharose FF column chromatography, ir-RFRP-3 was eluted mainly at 0.1 and 0.2 M NaCl (data not shown). As the amounts of ir-RFRP-3 were greater in the fractions eluted at 0.2 M than at 0.1 M NaCl, we first performed purification on the former. Fig. 1 shows the profile of the final chromatography with HPLC using a μRPC C2/C18 SC 2.1/10 column. Ir-RFRP-3 was mainly eluted in fraction numbers 22 and 23. As fraction number 23 appeared to contain fewer impurities than number 22, we subjected it to microsequencing and mass spectrometry. As shown in Fig. 2A, microsequencing revealed the N-terminal sequence, AMAHLPLRLGKNREDSLSRWV, which corresponded to the sequence from Ala104 to Val124 in the bovine RFRP preproprotein [2]. Considering this N-terminal sequence together with the C-terminal structure deduced on the basis of immunoreactivity, the purified RFRP-3 was expected to consist of 28 amino acid residues, that is, AMAHLPLRLGKNREDSLSRWVPNLPQRF-amide. Using ESI-MS, we detected the signals of the multiple charged ions (i.e., $M+6H^{6+}$, $M+5M^{5+}$ and $M+4H^{4+}$) which would be expected from this structure (Fig. 2B). From

these signals, the molecular weight of the purified RFRP-3 was determined to be 3302.3 ± 0.4, which agreed well with the calculated molecular weight of 3301.8. By conducting ESI-MS/MS analysis for $M + 5M^{5+}$ (m/z 661) as a precursor ion, we confirmed the above 28-amino acid length RFRP-3 sequence with the b- and y-ions which would be expected (data not shown).

We subsequently purified the fractions eluted at 0.1 M NaCl in the CM-Sepharose FF column chromatography. By ESI-MS and microsequencing, the main immunoreactive peak of the final chromatography was revealed to be SPAFLFQPQRFamide, the three-amino acid prolonged form of NPFF (data not shown). As RFRP-3 and NPFF both possess the same C-terminal structure detected by our EIA, we concluded that NPFF was obtained by the purification.

3.2. Analyses for the distribution of RFRP-3 in rat tissues

Comparing the sequences of bovine and rat prepropro-teins [1,2], endogenous bovine RFRP-3 with 28-amino acid length seemed to correspond to rat RFRP-3 with 23-amino acid length (i.e., rRFRP-3-23). To quantify rat endogenous RFRP-3, we prepared an antibody for its N-terminal portion by immunizing rRFRP-3N. The antibody recognized rRFRP-3-23 and rRFRP-3-17, but neither rRFRP-1-37, NPFF, nor NPAF (data not shown). By utilizing both this antibody labeled with HRP and the monoclonal antibody 7F6, we constructed a sandwich EIA to quantify rat endogenous RFRP-3. This sandwich EIA detected at least 5 fmol/well of rRFRP-3-23. However, it did not detect other peptides with the RFamide structure, including rRFRP-1-37, NPFF, or NPAF (data not shown). These results indicate that it specifically detected rat endogenous RFRP-3.

Using the sandwich EIA, we measured rat endogenous RFRP-3 in various tissues. As shown in Fig. 3, significant levels of ir-RFRP-3 were detected in the central nervous system, with its highest concentration found in the hypo-
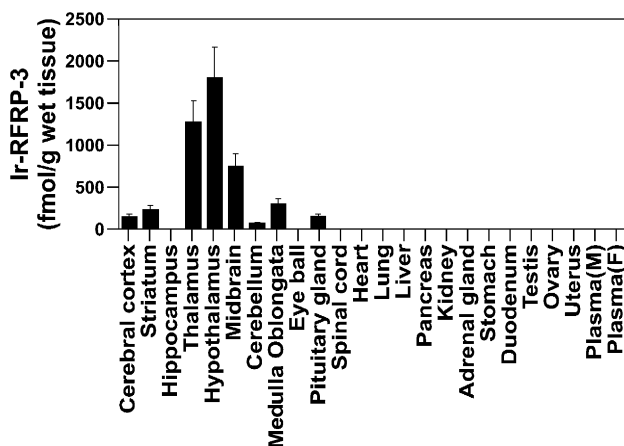

Fig. 3. Quantification of endogenous RFRP-3 in rat tissues by EIA. The ir-RFRP-3 contents were determined with the sandwich EIA. A calibration curve for quantification was obtained by using synthetic rat RFRP-3-23. Data are expressed as the mean $\pm$ S.E. in multiple assays ($n = 4$ or 5).
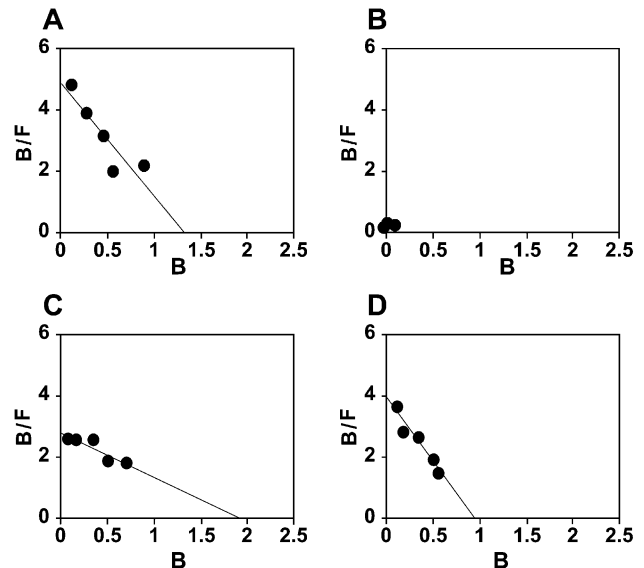

Fig. 4. Scatchard analyses for the binding of radiolabeled human RFRP-3 and NPFF to hOT7T022 and HLWAR77. The membrane fractions prepared from CHO-hOT7T022 (A, C) and CHO-HLWAR77 (B, D) cells were incubated with increasing concentrations of ^{125}I-labeled hRFRP-3-8 (A, B) or NPFF (C, D), and the bound and free ligands were separated when binding reached equilibrium. Data are plotted as bound (B, pmol mg^{-1} protein) versus bound/free (B/F, pmol mg^{-1} nM) radiolabeled ligands. Each symbol represents the mean value with standard error in triplicate determinations.

thalamus (1800 fmol/g wet tissue). High concentrations of ir-RFRP-3 were detected in the thalamus (1300 fmol/g wet tissue) and midbrain (750 fmol/g wet tissue). Moderate levels of ir-RFRP-3 were detected in the cerebral cortex (150 fmol/g wet tissue), striatum (240 fmol/g wet tissue), medulla oblongata (310 fmol/g wet tissue), and pituitary grand (150 fmol/g wet tissue), and a low level in the cerebellum (20 fmol/g wet tissue). It was, however, unde-tectable (< 20 fmol/g wet tissue) in the hippocampus, eye-ball, peripheral tissues, and plasma.

3.3. Scatchard analyses for binding of $[^{125}I]RFRP$-3 and $[^{125}I]NPFF$ with OT7T022 and HLWAR77

HLWAR77 has been reported as a receptor for NPFF [3] and shows high homology with hOT7T022 (56% according to a Gapped Blast program). We therefore examined the interactions of both RFRP-3 and NPFF with hOT7T022 and HLWAR77. In Scatchard analyses, $[^{125}I]hRFRP$-3-8 bound with hOT7T022 (Fig. 4A) at the K_d value of 270 pM, but significant binding was not detected with HLWAR77 (Fig. 4B). On the other hand, $[^{125}I]NPFF$ significantly bound with both hOT7T022 (Fig. 4C) and HLWAR77 (Fig. 4D) at the K_d values of 680 and 240 pM, respectively.

3.4. Properties of RFRP-3 and NPFF in interaction with receptors

We prepared various lengths of C-terminal peptides of RFRP-3 and NPFF, and analyzed their interactions with

hOT7T022 and HLWAR77 (Table 1). To assess the interaction, we performed competitive binding and cAMP-production-inhibitory assays. Competitive binding assays were performed to determine binding affinity in combinations of $[^{125}I]$hRFRP-3-8 with hOT7T022 and $[^{125}I]$NPFF with HLWAR77, respectively. cAMP-production-inhibitory assays with CHO cells expressing hOT7T022 or HLWAR77 were performed to determine each peptide's agonistic activity. Bovine endogenous RFRP-3 (bRFRP-3-28), hRFRP-3-31, and hRFRP-3-28 showed similar profiles in their interaction with the receptors. These peptides efficiently inhibited the binding of $[^{125}I]$hRFRP-3-8 to hOT7T022 at median inhibitory concentrations (IC_{50}) of 1–2 nM. They showed potent cAMP-production-inhibitory activity on hOT7T022 at median effective concentrations (EC_{50}) of around 5 nM. The IC_{50} values of these peptides in the binding of $[^{125}I]$hRFRP-3-8 to HLWAR77 ranged from 7 to 16 nM. On the other hand, their cAMP-production-inhibitory activities on HLWAR77 were from 40 to 100 times weaker than those on hOT7T022. A similar pattern was observed for the interaction of hRFRP-1-37 and bovine endogenous RFRP-1 (bRFRP-1-35) with hOT7T022 and HLWAR77, although the binding activity of RFRP-1 on hOT7T022 appeared to be slightly weaker than that of RFRP-3. Human and bovine peptides were almost equipotent in these assays. Our results suggest that RFRPs are principally directed to hOT7T022 rather than HLWAR77, though they cross-react with HLWAR77 to some extent.

By serially deleting the N-terminal portion of RFRP-3, we isolated the sequence critical for interaction with the receptors. In the assays of binding affinity and cAMP-production-inhibitory activity on hOT7T022, hRFRP-3-8 and hRFRP-3-7 were almost equivalent to hRFRP-3-17, hRFRP-3-31, and hRFRP-3-28, suggesting that the seven-residue C-terminal peptide, PNLPQRFamide, was sufficient for full interaction with hOT7T022. However, in the assays of binding affinity and cAMP-production-inhibitory activity on HLWAR77, the interactions of hRFRP-3-8 and hRFRP-3-7 appeared to be attenuated by the deletion of the N-terminal sequence, suggesting that RFRP-3 interacted with HLWAR77 in a different manner than with hOT7T022. The potency of hRFRP-3-6 and hRFRP-3-5 reduced to about one-tenth that of hRFRP-3-7 in cAMP-production-inhibitory activity on CHO cells expressing hOT7T022, suggesting that Pro at the N terminus of hRFRP-3-7 was important for exhibiting full cAMP-production-inhibitory activity on hOT7T022. Although the four-residue C-terminal peptide of RFRP-3 (hRFRP-3-4: PQRFamide) — identical to that of NPFF (NPFF-4) — still retained a weaker but evident binding activity to hOT7T022 ($IC_{50} = 15$ nM), its cAMP-production-inhibitory activity on hOT7T022 was almost completely eliminated, suggesting that Leu at the N terminus of RFRP-3-5 is crucial for cAMP-production-inhibitory activity on hOT7T022. It is notable that the five-amino acid residue C-terminal peptide of RFRP-1 (hRFRP-1-5: LPLRFamide) showed a profile similar to hRFRP-3-5 in

Table 1
Comparison between RFRP-3 and NPFF in their interactions with hOT7T022 and HLWAR77 by competitive binding and cAMP-production-inhibitory assays

Peptide	Sequence	hOT7T022		HLWAR77	
		Binding (IC_{50}, nM)	cAMP (EC_{50}, nM)	Binding (IC_{50}, nM)	cAMP (EC_{50}, nM)
hRFRP-3-31	SAGATANLPLRSGRNMEVSLVRRVPNLPQRFamide	1.3	4.8	16	780
hRFRP-3-28	ATANLPLRSGRNMEVSLVRRVPNLPQRFamide	1.1	5.1	14	520
bRFRP-3-28	AMAHLPLRLGKNREDSLSRWVPNLPQRFamide	1.6	6.4	7.0	290
hRFRP-3-17	NMEVSLVRRVPNLPQRFamide	3.4	6.8	18	730
hRFRP-3-8	VPNLPQRFamide	1.2	4.1	150	>1000
hRFRP-3-7	PNLPQRFamide	0.62	3.0	110	>1000
hRFRP-3-6	NLPQRFamide	2.6	28	230	>1000
hRFRP-3-5	LPQRFamide	2.1	51	76	>1000
hRFRP-3-4/NPFF-4	PQRFamide	15	>1000	26	>1000
hRFRP-1-37	SLNFEELKDWGPKNVIKMSTPAVNKMPHSFANLPLRFamide	15	12	17	470
bRFRP-1-35	SLTFEEVKDWAPKIKMNKPVVNKMPPSAANLPLRFamide	21	14	47	>1000
hRFRP-1-20	MSTPAVNKMPHSFANLPLRFamide	5.0	2.8	3.1	410
hRFRP-1-12	MPHSFANLPLRFamide	2.7	4.8	3.8	330
hRFRP-1-5	LPLRFamide	2.5	23	16	790
hNPSF	SQAFLFQPQRFamide	4.2	47	0.34	0.9
bNPSF	SPAFLFQPQRFamide	4.0	81	0.36	1.2
bNPSF-10	PAFLFQLQRFamide	4.1	110	0.38	1.4
bNPSF-9	AFLFQPQRFamide	4.5	110	0.58	2.0
NPFF	FLFQPQRFamide	2.4	120	1.0	6.9
NPFF-7	LFQPQRFamide	4.6	140	3.0	8.5
NPFF-6	FQPQRFamide	13	360	28	130
NPFF-5	QPQRFamide	21	430	69	>1000

its binding affinity and cAMP-production-inhibitory activity on the receptors, further supporting the view that N-terminal Leu is crucial for cAMP-production-inhibitory activity on hOT7T022. In the binding assays for HLWAR77, hRFRP-3-4 and hRFRP-3-5 appeared to be more potent than hRFRP-3-6, hRFRP-3-7, and hRFRP-8, suggesting that Asn at the N terminus of RFRP-3 might slightly hinder the binding of RFRP-3 to HLWAR77, although this residue did not appear to hinder binding to hOT7T022.

In the competitive binding assays, NPFF efficiently bound with both hOT7T022 ($IC_{50} = 2.4$ nM) and HLWAR77 ($IC_{50} = 1.0$ nM). In addition, it showed potent cAMP-production-inhibitory activity on HLWAR77 ($EC_{50} = 6.9$ nM), but weak activity on hOT7T022 ($EC_{50} = 120$ nM), suggesting that NPFF is principally directed to HLWAR77 rather than OT7T022 although it binds with both receptors. Identifying a novel form of bovine NPFF having three additional amino acids at the N terminus (i.e., bNPSF), which corresponded to hNPSF [10], we compared these peptides to NPFF in their interaction with the receptors. In binding and cAMP-production-inhibitory assays for hOT7T022, they showed potency almost equivalent to NPFF. However, in binding to HLWAR77, they were more potent than the authentic form, suggesting that the three additional amino acid residues enhanced interaction with HLWAR77.

NPFF-7 was almost equivalent to NPFF in its interaction with the two receptors when examined in the binding and cAMP-production-inhibitory assays, suggesting that the seven-residue C-terminal (i.e., LFQPQRFamide) was enough for complete interaction with the receptors. However, NPFF-6 showed lower binding affinity and cAMP-production-inhibitory activity for the two receptors than NPFF-7, suggesting that the N-terminal Leu of NPFF-7 played an important role for full interaction. NPFF-5 almost completely lost cAMP-production-inhibitory activity for HLWAR77, although it still retained binding activity for OT7T022 and HLWAR77 and a very weak cAMP-production-inhibitory activity for hOT7T022, suggesting that Phe at the N terminus of NPFF-6 partly contributed to cAMP-production-inhibitory activity on HLWAR77. As NPFF-5 was almost equivalent to NPFF-4 in its binding and cAMP-production-inhibitory activity for the two receptors, Glu at the N terminus of NPFF-5 did not appear to bear much influence on the interaction.

4. Discussion

To demonstrate the existence of RFRP-3, we purified it from bovine hypothalamus. The purified endogenous bovine RFRP-3 was found to consist of 28 amino acid residues, suggesting that its N terminus is produced by cleavage between Arg103 and Ala104 in the RFRP preproprotein. This was unexpected for the following reasons. (1) As basic amino acid residues are known to be potential sites for cleavage by proteases, the cleavage between Arg103 and

Ala104 in bovine preproRFRP appears to be rational. However, in the endogenous bovine RFRP-3 sequence, there are four other basic amino acid residues, that is, Arg111, 116, and 122 and Lys114. This unexpected processing of endogenous bovine RFRP-3 is similar to that seen in endogenous RFRP-1 which also has a long N-terminal portion [2]. Thus, not only the primary sequences, but also other factors such as three-dimensional conformation might determine the selective cleavage of the N-terminal portions. (2) The N-terminal portion of the endogenous RFRP-3 sequence included the motif to generate the putative RFRP-2 sequence, that is, AMALLPLRLGR. We have previously demonstrated that synthetic human RFRP-2 did not bind to OT7T022 receptor [1]. In consideration of this, RFRP-2 should not be produced from bovine and human RFRP preproproteins. Nevertheless, they appeared to be able to generate RFRP-2 in the primary sequence. Another group has recently reported the identification of gonadotropin inhibitory hormone (GnIH) in quail [11], which is thought to be the orthologue of RFRP. Interestingly, GnIH preproprotein is also reportedly able to produce three different peptides, corresponding to RFRP-1, -2, and -3. Two of these peptides have been verified to exist in the quail brain, that is, GnIH and GnIH-related peptide II [12]. These are encoded in the preproprotein at positions corresponding to RFRP-2 and RFRP-3, respectively. However, we have previously demonstrated that rat and mouse RFRP preproproteins completely lack the sequence corresponding to RFRP-2, suggesting that RFRP-2 retrogressed in the evolution of mammals. As for endogenous RFRP-3, rat hypothalamic RFRP-3 was recently determined to be an 18-amino acid peptide [13], differing in length from that of bovine endogenous RFRP-3. However, both peptides appear to be correspondingly produced by cleavage at the C-terminal side of Arg in the preproRFRPs, suggesting that the same processing mechanism produces different lengths of peptides between rat and cow.

We analyzed the tissue distribution of RFRP-3 in rats by sandwich EIA, and found that RFRP-3 agreed well with RFRP-1 in tissue distribution [2]. Both RFRP-1 and -3 were localized principally in the central nervous system. The highest concentrations of both peptides were detected in the hypothalamus at comparable levels. These results suggest that the two peptides are produced almost equally from the RFRP preproprotein in tissues.

RFRP-3 and NPFF are closely related because they possess the identical C-terminal sequence, PQRFamide. HLWAR77 has recently been reported to be a specific receptor for NPFF and NPAF [3]. NPGPR is nearly identical to HLWAR77, and thought to be its counterpart in rat [14]. In addition, another group has reported two receptors, that is, NPFF1 and NPFF2, for NPFF [5]. OT7T022 and HLWAR77/NPGPR are identical to NPFF1 and NPFF2, respectively. In this study, we prepared CHO cells expressing hOT7T022 or HLWAR77 and analyzed the interactions of RFRP-3 and NPFF with these receptors. We assessed their interactions by competitive binding and cAMP-pro-

duction-inhibitory assays. In our experiments with RFRP-3 and NPFF C-terminal peptides, we found that PQRFamide, which was common between the C-terminal portions of RFRP-3 and NPFF, showed evident binding activity to both receptors, but almost lost its cAMP-production-inhibitory activity. This suggests that PQRFamide is a core structure essential for binding to the receptors, but is not sufficient to exhibit cAMP-production-inhibitory activity. We showed that the seven-amino acid length C-terminal peptide of RFRP-3 (hRFRP-3-7: PNLPQRFamide) and that of NPFF with the same length (NPFF-7: LFQPQRFamide) showed full interactions with hOT7T022 and HLWAR77, respectively. These results suggest that the three amino acid residues (i.e., PNL in RFRP-3 and LFQ in NPFF) added to the N terminus of PQRFamide played important roles not only in determining receptor specificities, but also in exhibiting full cAMP-production-inhibitory activities. Among these three amino acid residues, Pro and Leu were found to be particularly critical in the interaction of RFRP-3 with hOT7T022, and Leu and Phe in that of NPFF with HLWAR77.

Our results indicate that at least in vitro, shorter RFRP-3 peptides — minimally, PNLPQRFamide — exhibit interaction with the receptors as fully as the long endogenous RFRP-3. Important residues in the sequence, that is, Pro, Leu and PQRFamide, are conserved between human, bovine, rat and mouse [1,2], whereas the N-terminal portion shows diversity in both length and sequence. Endogenous bovine RFRP-3, composed of 28 amino acids, is 10 amino acids longer than endogenous rat RFRP-3 [13]. Although the reason why natural RFRP-3 has such long, varying N-terminal portions remains to be elucidated by future studies, they may possess various functions in vivo.

Acknowledgements

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