A novel rat hypothalamic RFamide-related peptide identified by immunoaffinity chromatography and mass spectrometry

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Abstract Recently, cDNAs encoding novel RFamide-related peptides (RFRPs) have been reported in the mammalian brains by a gene database search and the deduced RFRPs have been suggested to participate in neuroendocrine and pain mechanisms in the rat. Two peptides have been predicted to be encoded in the cDNA of rodent RFRPs. To assess precise functions of rodent RFRPs in the brain, in the present study we identified a naturally occurring RFRP in the rat hypothalamus by immunoaffinity purification combined with mass spectrometry (MS). The affinity chromatography showed that the rat hypothalamus contained RFRP-like immunoreactivity. The immunoreactive material was analyzed by a nanoflow electrospray ionization time-of-flight MS followed by tandem MS analysis. The mass peak corresponding to octadecapeptide was detected at 1010.54 *m/z* ($[M+2H]^{2+}$) and its sequence, ANMEAGTMSHFPSLPQRF-NH_2, was revealed by the fragmentation, showing a mature form encoded in the cDNA sequence of RFRPs. The identified endogenous RFRP will aid not only in defining its physiological roles but also facilitate the development of its agonists and antagonists in the rodent brain.

Key words: RFamide-related peptide; Hypothalamus; Immunoaffinity chromatography; Mass spectrometry; Rat

1. Introduction

Since the cardioexcitatory Phe-Met-Arg-Phe-NH_2 (FMRFamide) was originally isolated from the ganglia of the venus clam [1], some neuropeptides containing carboxyl-terminal the RFamide motif have been found in several vertebrate brains and referred to as RFamide peptides. At the first time LPLRFamide, a chicken pentapeptide, has been purified in the vertebrate brain [2]. Two pain modulatory neuropeptides FF and AF [3], prolactin-releasing peptide [4] and its fish counterpart *Carassius* RFamide [5] are also RFamide peptides. To date, these RFamide peptides have been shown to play many important physiological roles, such as neuroendocrine, behavioral, sensory and autonomic functions (see reviews [6–8]). Recently, we have isolated and identified a novel avian hypothalamic RFamide peptide (SIKPSAYLPLRF-NH_2) inhibiting gonadotropin release from the quail brain and named gonadotropin-inhibitory hormone (GnIH) [9]. A cDNA encoding GnIH has also been cloned [10]. In addition, we have recently isolated the dodecapeptide (SLKPAANLPLRF-NH_2) closely related to GnIH from the bullfrog hypothalamus [11]. This frog peptide possessed growth hormone (GH)-releasing activity in both in vivo and in vitro studies and was designated frog GH-releasing peptide (fGRP) [11]. On the other hand, cDNAs of new RFamide peptides similar to GnIH have been reported in the mammalian brains by a gene database search and termed RFamide-related peptides (RFRPs) [12]. Two deduced RFRPs have been suggested to be present in the cDNA of mammals [12]. Hinuma et al. [12] have reported that the intracerebroventricular administration of the deduced human dodecapeptide (hRFRP-1; MPHSFANLPLRF-NH_2) increases prolactin release in the rat. On the other hand, Liu et al. [13] have supposed that the 37-residue RFamide peptide, which is an N-terminal extended form of the human dodecapeptide (hRFRP-1) [12], is one of the predicted mature RFRPs judging from the cleavage site and indicated that the rat 37-residue peptide modulates the action of morphine. However, the identification of endogenous mature RFRPs has not been determined in mammals. We have previously found that RFRP-like immunoreactive substances are highly concentrated in the rodent brainstem including hypothalamus [14]. To elucidate precise functions of RFRPs in mammals, in this study we identified a mature endogenous RFRP from the relative small amounts of rat hypothalami by the immunoaffinity chromatography and mass spectrometric (MS) analyses.

2. Materials and methods

2.1. Animals and tissue samples

Sixty adult male Donryu rats at the age of 5 months were employed in this study. The hypothalami were dissected out by fine scissors and forceps, frozen in liquid nitrogen, and kept at −80°C until used. The experimental protocol was approved in accordance with the Guide for the Care and Use of Laboratory Animals prepared by Hiroshima University, Japan.

2.2. Affinity purification and immunoassay

To identify an endogenous RFRP in the hypothalamus, we used the specific antibody against quail GnIH [9]. This antibody has previously been confirmed to recognize specifically deduced rat RFRPs [14].

The hypothalami were boiled and homogenized in 5% acetic acid as described previously [9,11,15,16]. The homogenate was centrifuged at 15 000 × *g* for 20 min at 4°C and the supernatant was collected. After the precipitation with 75% acetone, the supernatant was passed through a disposable C-18 cartridge column (Mega Bond-Elut; Varian, Harbor, USA) and the retained material was loaded onto an

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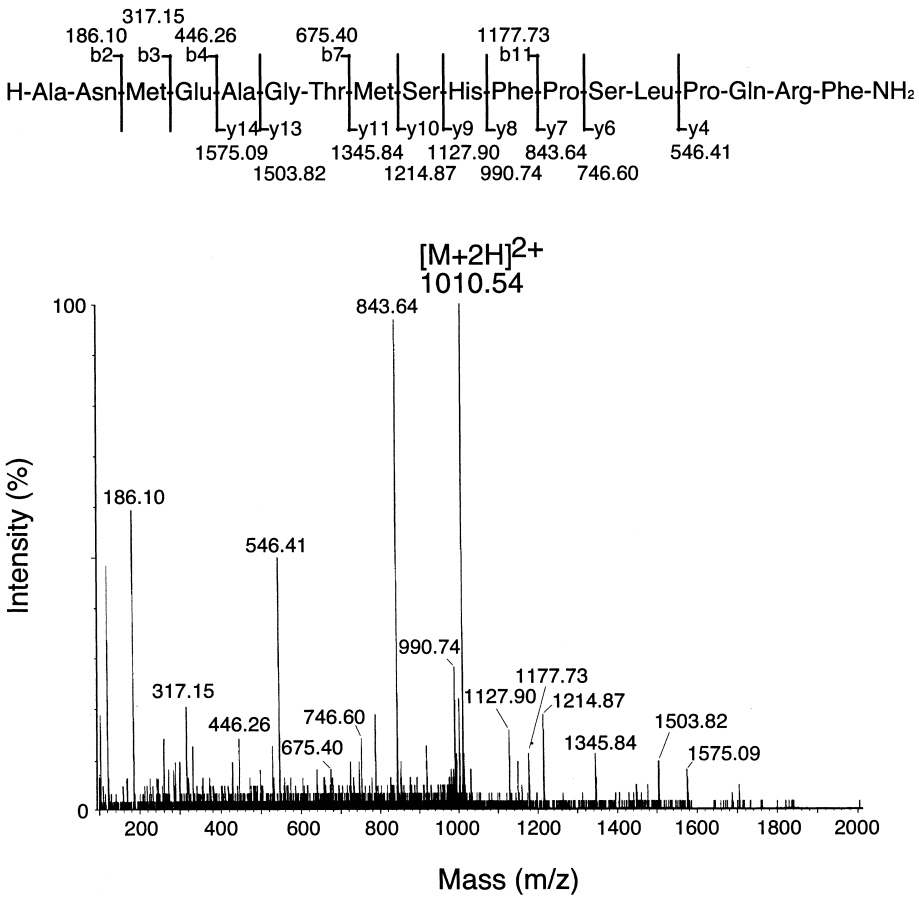

Fig. 1. Fragmentation patterns of the purified substance with the observed mass number of 1010.54 *m/z* ($[M+2H]^{2+}$) by a tandem MS analysis. The spectrum shows typical mass values of predicted octadecapeptide fragment ions. Observed N-terminal (b) and C-terminal (y) fragmentation ions are assigned in the sequence of the octadecapeptide.

immunoaffinity column chromatography. The affinity chromatography was carried out as described elsewhere [3]. The antibodies against GnIH were conjugated to CNBr-activated Sepharose 4B as an affinity ligand. The hypothalamic extract was applied to the column at 4°C and the adsorbed materials were eluted with 0.3 M acetic acid containing 0.1% 2-mercaptoethanol. An aliquot of each fraction (1 ml) was analyzed by a dot immunoblot assay with the antibody against GnIH according to our previous method [11]. The immunoreactive fractions were concentrated and subjected to a reversed-phase high-performance liquid chromatography (HPLC) column (ODS-80TM; Tosoh, Tokyo, Japan) with a linear gradient of 20–40% acetonitrile containing 0.1% trifluoroacetic acid for 100 min at a flow rate of 0.5 ml/min. The isolated immunoreactive substance was then subjected to MS analyses as described below.

2.3. MS and peptide synthesis

After evaporation of the immunoreactive material, the residue was dissolved in 50% methanol containing 0.1% formic acid and the molecular mass was analyzed by a nanoflow electrospray ionization time-of-flight MS (nano ESI-TOF-MS) (Q-TOF, Micromass UK, Wythenshawe, UK) as described previously [10,17]. The prospected mass value of each deduced rat RFRP was calculated using the ProteinProspector program (UCSF) and a corresponding peak was further examined in a tandem MS analysis. The needle voltage was optimized at 1000 V and the cone voltage was set at 50 V. Argon was used as the collision gas and the energy was set at 28 V.

The peptide having the suggested sequence was synthesized by a manual method followed by a hydrogen fluoride-anisole cleavage and purified by reversed-phase HPLC. The synthetic peptide was compared with the native one with regard to behavior on HPLC and MS.

3. Results and discussion

Acetic acid extracts of rat hypothalami were passed through a disposable C-18 reversed-phase cartridge column. The retained material eluted with 60% methanol was then subjected to an affinity chromatography with the antibody cross-reacted with deduced rat RFRPs. Immunoreactivity was measured in the eluted fractions by a dot immunoblot assay. Immunoreactive fractions were then subjected to the reversed-phase HPLC purification, and the eluate was fractionated every 2 min and assayed by immunoblotting. The fraction corresponding to the elution time of 36–38 min showed an intense immunoreactivity (Table 1). A purified substance was further

Table 1
Behavior of purified and synthetic peptide on HPLC and MS

	Retention time on HPLC (min)	Observed mass (*m/z* $[M+2H]^{2+}$)	Calculated mass (*m/z* $[M+2H]^{2+}$)
Purified peptide	36–38	1010.54	–
Synthetic peptide	37.2	1010.51	1010.48

Table 2
Sequences of characterized RFRP and its related peptides

Sequence	Animal	Name	Reference
ANMEAGTMSHFPSLPQRF-NH_2	Rat	RFRP	this study
SIKPSAYLPLRF-NH_2	Quail	GnIH	[9]
SSIQSLLNLPQRF-NH_2	Quail	GnIH-RP-2	[10]
SLKPAANLPLRF-NH_2	Frog	fGRP	[11]

examined in a MS. The mass values of predicted peptides were previously calculated using programming software on the basis of the sequence of rat preproprotein [12]. A molecular ion peak in the spectrum of the substance was observed at 1010.54 *m/z* ($[M+2H]^{2+}$) on the nano ESI-TOF-MS (Table 1). This value was close to the mass number 1010.48 *m/z* ($[M+2H]^{2+}$) calculated for octadecapeptide, ANMEAGTMSHFPSLPQRF-NH_2 (Table 1). Therefore, the sequence was determined by a tandem MS analysis (Fig. 1). Assignment of the observed typical fragment ions, i.e. N-terminal (b) and C-terminal (y) ions, indicated that the amino acid sequence of this peak was compatible with the sequence ANMEAGTMSHFPSLPQRF-NH_2 (Fig. 1). To confirm the data obtained by these structural analyses, the peptide having the suggested sequence was synthesized and compared with the purified peptide with regard to the retention time on HPLC and the mass number. Both purified and synthetic octadecapeptides showed a similar retention time on the reversed-phase HPLC and a similar molecular mass (Table 1). Furthermore, the fragmentation of synthetic peptide by the tandem MS analysis was completely coincident with that of the purified one (data not shown).

Hinuma et al. [12] have reported that the preproprotein of rat RFRPs contains two putative peptides (RFRP-1 and RFRP-2). Although deduced RFRPs may have prolactin-releasing and anti-opioid activities in the rat [12,13], the characterization of mature RFRPs has not been investigated in mammals. In the present study, we therefore demonstrated that the octadecapeptide, ANMEAGTMSHFPSLPQRF-NH_2, is a mature form of RFRP-2 in the rat hypothalamus. On the other hand, it has been speculated that RFRP-1 is a 37-amino acid residue peptide judging from the cleavage site [13]. However, endogenous RFRP-1 remains to be characterized in this study. In contrast to the mammal, we have identified the novel hypothalamic RFamide peptide, GnIH [9], and its related peptide, GnIH-RP-2 [10], in the quail brain. Subsequently, we have isolated fGRP closely related to GnIH from the frog hypothalamus [11]. As summarized in Table 2, the present and previous findings [9–11] strengthen the view that members of the novel RFamide family (RFRP and its related peptides) possess the LPXRF-NH_2 (X = L or Q) motif at the C-terminus in both mammalian and non-mammalian species.

We have previously found that RFRP-like immunoreactive cell bodies and fibers are distributed in the regions involved in feeding, pain and auditory systems in the mouse brain [14]. It is therefore possible that RFRPs may participate in several important physiological roles in rodents. On the other hand, the study of the receptor for RFRPs is now very confused. Hinuma et al. [12] have reported that a G protein-coupled receptor OT7T022 is a specific receptor for deduced RFRPs, while Bonini et al. [18] have proposed the same receptor as a receptor for another RFamide peptide, neuropeptide FF. The sequence of neuropeptide FF, FLFQPQRF-NH_2, is closely related to the present identified octadecapeptide and their C-terminal four amino acids are identical. To draw a firm conclusion for the RFRP receptor, further study is needed. We believe that the present findings can contribute to future studies concerning the physiological roles of an endogenous RFRP, the properties of its receptors and the development of its agonists and antagonists in the mammalian brain.

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