Identification of a Neuropeptide Modified with Bromine as an Endogenous Ligand for GPR7*

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Ryo Fujii†‡, Hiromi Yoshida†§, Shoji Fukusumi†, Yugo Habata‡, Masahiko Hosoya‡, Yuji Kawamata†, Takahiko Yano†, Shuji Hinuma†, Chieko Kitada†, Taiji Asami‡, Masaaki Morit*, Yukio Fujisawa††, and Masahiko Fujino‡‡

From the Discovery Research Laboratories 1, Pharmaceutical Research Laboratories 1, Pharmaceutical Research Division, Takeda Chemical Industries Ltd., 10 Wada, Tsukuba, Ibaraki 300-4293, Japan

We isolated a novel gene in a search of the Celera data base and found that it encoded a peptide ligand for a G protein-coupled receptor, GPR7 (O’Dowd, B. F., Scheideler, M. A., Nguyen, T., Cheng, R., Rasmussen, J. S., Marchese, A., Zastawny, R., Heng, H. H., Tsui, L. C., Shi, X., Asa, S., Puy, L., and George, S. R. (1995) Genomics 28, 84–91; Lee, D. K., Nguyen, T., Porter, C. A., Cheng, R., George, S. R., and O’Dowd, B. F. (1999) Mol. Brain Res. 71, 96–103). The expression of this gene was detected in various tissues in rats, including the lymphoid organs, central nervous system, mammary glands, and uterus. In situ hybridization showed that the gene encoding the GPR7 ligand was expressed in the hypothalamus and hippocampus of rats. To determine the molecular structure of the endogenous GPR7 ligand, we purified it from bovine hypothalamic tissue extracts on the basis of cAMP production-inhibitory activity to cells expressing GPR7. Through structural analyses, we found that the purified endogenous ligand was a peptide with 29 amino acid residues and that it was uniquely modified with bromine. We subsequently determined that the C-6 position of the indole moiety in the N-terminal Trp was brominated. We believe this is the first report on a neuropeptide modified with bromine and have hence named it neuropeptide B. In vitro assays, bromination did not influence the binding of neuropeptide B to the receptor.

A large number of new genes have been discovered in the progress of analyses for the human genome. How to determine the functions of these genes is an important issue. G protein-coupled receptors (GPCRs) play important roles in the regulation of physiological phenomena including sense, growth, reproduction, metabolism, and homeostasis. In the human genome, numerous genes have been found encoding GPCRs with as yet unknown ligands. The identification of ligands for these “orphan” GPCRs is a key to revealing their functions. In addition, since GPCRs have been historically important as drug targets, it is hoped that the identification of ligands for orphan GPCRs will bring new drug targets. We have developed our own unique methods to determine ligands for such orphan GPCRs (3). First, we sought for ligands in tissue extracts and, subsequently, in a library with synthetic compounds. Recently, we have developed a method to search for genes encoding ligands in databases providing genomic and cDNA sequences. By utilizing these methods, we have already succeeded in the identification of several ligands for orphan GPCRs (4–8).

GPR7 has been cloned as an orphan GPCR resembling opioid or somatostatin receptors (1). Another GPCR, GPR8, sharing 59% amino acid identity with GPR7, has been also reported, but ligands for GPR7 and GPR8 have not been identified (1). By utilizing the Celera data base, we searched for candidate genes encoding ligands for orphan GPCRs. In this paper, we report on the identification of a novel gene encoding a ligand for GPR7. In addition, we show here that this endogenous GPR7 ligand purified from tissue extracts is a peptide modified with bromine.

EXPERIMENTAL PROCEDURES

Cloning of Neuropeptide B (NPB) cDNAs—Celera Discovery Systems- and Celera Genomics-associated databases were used to search for genes encoding proteins with the motif of the secretory signal sequence. In our search, we found a gene, NPB, encoding a novel secretory protein. Based on the sequence information provided by the data base, we isolated a cDNA from human brain cDNAs by PCR using a primer set (5’-GTCGACATGGCCCGGTCGCGGACACTGGCGGC-3’ and 5’-GCTAGACCGGTCGCGGACACTGGCGGC-3’). We subsequently designed several primers on the basis of this human NPB cDNA and isolated rat, mouse, and bovine NPB cDNAs from brain cDNAs by the rapid amplification of cDNA ends method using a Marathon cDNA amplification kit (CLONTECH, Palo Alto, CA).

Cloning of Bovine GPR7 and GPR8 cDNAs—Utilizing several primers designed from the published sequences of human GPR7 and GPR8 cDNAs (1), we isolated bovine GPR7 and GPR8 cDNAs by rapid amplification of cDNA ends. The complete coding regions of bovine GPR7 and GPR8 were amplified from bovine hypothalamus cDNAs by PCR with primer sets (5’-GTCGACATGGCCCGGTCGCGGACACTGGCGGC-3’ and 5’-GCTAGACCGGTCGCGGACACTGGCGGC-3’) for GPR7 and 5’-GTCGACCATATGAGCCGACACTGGCGGC-3’ for GPR8.

Preparation of Chinese Hamster Ovary (CHO) Cells Expressing NPB—2
GP7, and GPR8 cDNAs—The entire coding regions of NPB, GP7, and GPR8 cDNAs were cloned, respectively, into the downstream region of an SV40 promoter in the expression vector pAKKO-111H (9). The resultant expression vector plasmids were transfected into dhfr-CHO cells, following which dhfr-CHO cells were selected, respectively, as previously described (9).

Assays for cAMP Production—The inhibition of forskolin-induced cAMP production in CHO cells was determined as previously described (10).

Quantitative RT-PCR Analyses of NPB and GPR7 mRNAs—Poly(A)+ RNA fractions were prepared from tissues of 8–12-week-old Wistar rats, and cDNAs were synthesized from these (11). Poly(A)+ RNA was prepared from placenta, mammary gland, and whole tissue of female rats 17 days pregnant. Rat NPB and GPR7 mRNA expressions were determined with a Prism 7700 sequence detector (Applied Biosystems) (6) with primers and fluorescence-labeled probes (5).

Assays for Receptor-binding Assays—Receptor-binding assays were conducted with 125I-labeled human desBr-NPB-23 (100 pM) and incubated at room temperature for 90 min. To determine the amount of nonspecific binding, 1 μM unlabeled human desBr-NPB-23 was added to the mixture. The amounts of 125I-labeled human desBr-NPB-23 bound to the membrane fractions were measured after rapid filtration.

RESULTS

Cloning of NPB cDNAs—In order to find novel secretory protein genes, we searched for candidate proteins with possible secretory signal peptides in hypothetical proteins deduced from human genomic sequences in the Celera data base. Since one of these candidate proteins appeared to be derived from a novel secretory protein gene, NPB, we cloned a cDNA on the basis of the sequence information. We subsequently cloned NPB cDNAs in other species also. As shown in Fig. 1, these cDNAs encoded proteins with secretory signals. Homology among the proteins ranged from 53 to 92%, and they seemed to possess preproprotein structures. For example, human NPB contained an N-terminal secretory signal peptide with 24-amino acid length and potential sites (i.e. Arg3–Arg4 and Arg10–Arg15) for cleavage with proteases.

Demonstration of a Peptidic Ligand for GPR7—We anticipated that the preproprotein we found might produce a ligand for orphan GPCRs. We therefore expressed the human NPB cDNA in CHO cells and examined whether ligands were secreted in the culture supernatants. The culture supernatants were screened by adding them to CHO cells expressing orphan GPCRs. We detected specific cAMP production-inhibitory activities to CHO cells expressing GPR7 in the culture supernatant. Based on this, we purified a ligand for GPR7 from the culture supernatant. Open column chromatography was applied wherein the culture supernatant (2 liters) was boiled and then eluted through a C18 column (Prep C18125A; Waters) with stepwise increments of 10, 40, and 60% CH3CN in 0.05% trifluoroacetic acid. Activity was detected in the fraction eluted with 40% CH3CN. Therefore, this fraction was purified serially.
through a HiPrep CM-Sepharose FF column (Amersham Biosciences) with 0–0.5 M NaCl in 20 mM CH₃COONH₄ at pH 4.7, a RESOURCE RPC column (HPLC) with 15–30% CH₃CN, a TSK-gel CM-2SW column (HPLC; Tosoh, Tokyo, Japan) with 0.2–0.5 M NaCl in 20 mM CH₃COONH₄ at pH 4.7, and a HiPrep CM-Sepharose FF column with 0–0.5 M NaCl in 20 mM CH₃COONH₄ at pH 4.7. The purified ligand for GPR7 that we thus obtained was then analyzed by N-terminal sequencing and mass spectrometry (data not shown). Our results revealed that this purified ligand was a peptide consisting of the 24 amino acid residues WYKPAAGHSSYSVGRAAGLLGLR, indicating that this peptide was generated from the preproprotein through processing. We could not detect any modification in the peptide purified from the culture supernatant.

Shimomura et al. (13) have recently identified a peptide which acts as a natural ligand for both GPR7 and GPR8, and named it neuropeptide W (NPW). We found that our purified peptide and NPW shared 61% identity (Fig. 2), indicating that the two peptides are closely related, although they are derived from different genes.

**Tissue Distribution of NPB and GPR7 mRNAs in Rats**—We analyzed the tissue distribution of NPB and GPR7 mRNAs in rats. Although the expression of NPB mRNA was detected in a variety of tissues, high levels were found in the lymphoid organs, central nervous system, mammary glands, and uterus (Fig. 3, upper panel). Human NPB mRNA was highly expressed in the central nervous system (data not shown). GPR7 mRNA expression was mainly detected in the rats’ central nervous system and uterus (Fig. 3, lower panel). We subsequently conducted in situ hybridization to detect NPB mRNA in the rat brain. NPB mRNA was found to be widely distributed here. Dense signals from antisense NPB riboprobes were detected in the hypothalamus and hippocampus. In the hypothalamus, specific signals were detected by the antisense probe in the medial preoptic area (Fig. 4A), ventromedial hypothalamic nucleus (Fig. 4B), and lateral hypothalamic area (Fig. 4C). In the hippocampus, moderate expression was detected in the CA fields of the Ammon’s horn but not in the dentate gyrus (Fig. 4G). No hybridization signals were detected by a sense probe in the same areas (Fig. 4, D–F and H).

**Structural Analyses of Endogenous NPB**—To determine the molecular structure of endogenous NPB, we purified it from bovine hypothalamic tissue extracts by a combination of various chromatographies on the basis of cAMP production-inhibitory activity to CHO cells expressing GPR7. After boiling, bovine hypothalami (2 kg) were homogenized in 1 M acetic acid. The resultant supernatant was then fractionated by open column chromatography through a C18 column with stepwise increments of 10, 40, and 60% CH₃CN in 0.05% trifluoroacetic acid. We then fractionated the 40% CH₃CN fraction through a HiPrep CM-Sepharose FF column with 0–0.5 M NaCl in 20 mM CH₃COONH₄ at pH 4.7. The resultant fractions showing the activities were precipitated with 66% acetone and again fractionated through a RESOURCE RPC column (HPLC) with 20–30% CH₃CN. After precipitation with 75% acetone to remove proteins, these were fractionated serially through a Vydac C18 218TP5415 column (HPLC) with 20–30% CH₃CN, a TSK-gel CM-25S column (HPLC) with 0.3–0.5 M NaCl in 20 mM CH₃COONH₄ at pH 4.7, and a μRPC C2/C18 SC 2.1/10 column (HPLC) with 16–24% CH₃CN. In the final chromatography, the endogenous NPB was eluted as a single peak at 21% CH₃CN (Fig. 5). As the N-terminal residue of the endogenous bovine NPB was expected to start from Trp25 in the preproprotein (Fig. 1), we analyzed its structure by nanoelectrospray...
ionization ion-trap mass spectrometry and MS/MS (data not shown). However, unexpectedly, the observed molecular weight (i.e. 3241.4 mass units) did not correspond to any lengths of peptides derived from the preproprotein and was 80 mass units higher than the calculated molecular weight (i.e. 3161.5 mass units) of 29-amino acid length bovine NPB. Consideration of the MS/MS spectrum and the isotope distribution of the signals suggested that the endogenous bovine NPB was modified with bromine at the first or second amino acid residue of the N-terminal. To confirm whether the endogenous NPB was indeed modified with bromine, we subjected it to electrospray ionization Fourier transform mass spectrometry. The mass values and isotope distribution of (M+H)^+ ions (Fig. 6A, upper panel) agreed well with the theoretical profile of brominated 29-amino acid bovine NPB (Fig. 6A, lower panel). N-terminal sequencing revealed XYPAGGQGYYSGAAAGLLSGFHR (X not identified), which corresponded to the sequence from Tyr^{26} to Arg^{49} in the preproprotein (Fig. 1). Only the PTH-derivative at cycle 1, which was expected to be Trp from the cDNA sequence, was eluted at a different retention time from that of a standard PTH-derivative. Therefore, Trp1 was determined to be the brominated residue. Bromination was originally reported in peptides derived from marine invertebrates, where Trp residues were found to be brominated at the C-6 position of the indole moiety (14, 15). After preparing PTH-5BrW and PHT-6BrW as standards, we subjected the purified NPB to FIG. 5. Profile of chromatography with a µRPC C2/C18 SC 2.1/10 column in purification of endogenous NPB. Specific responses of CHO cells expressing GPR7 to the ligand were detected through the inhibition of forskolin-induced cAMP production (black areas). The dotted and solid lines indicate the percentage of CH_{3}CN and absorbance at 215 nm, respectively. Evident responses were detected in fractions in accordance with the absorbance peak.

FIG. 6. Structural analyses for endogenous NPB. A, (M+5H)^5+ ions in the electrospray ionization Fourier transform mass spectrum of the purified peptide (upper panel) and the theoretical isotope distribution of (M+5H)^5+ ions for NPB-29 (lower panel). The mass values are indicated in the figure. B, elution profiles in a standard sample mixed with PTH-6BrW, PTH-5BrW, and 20-amino acid PTH standards (Applied Biosystems) (upper panel) and in cycle 1 of the purified NPB (lower panel). The purified peptide was analyzed with an ABI 491 cLC protein sequencer according to a slightly modified elution program in which elution was conducted for 6 min in isocratic mode following the usual elution for 22 min in gradient mode. PTH-derivative standards and reagent peaks are marked with asterisks. The first residue of the purified NPB was eluted at the same position as PTH-6BrW. The chemical structure of 6BrW is indicated below the elution profiles.
N-terminal sequencing by a protocol modified to fit these standards. The PTH-derivative peak at position 1 from the endogenous NPB coincided exactly with PTH-6BrW (Fig. 6B). Our results indicate that the endogenous bovine NPB has the following structure: 6BrW-YKPTAGQGYYSVGRAAGLLSGFHRSPYA. We could not detect NPB without bromination as a major component in the fractions obtained from bovine hypothalamic tissue extracts.

Figure 7. Amino acid sequences of human and bovine GPR7 and GPR8. Amino acid residues that are identical in at least two sequences are boxed. The predicted seven transmembrane domains (TM1 to -7) are indicated by bars above the sequences. Bovine GPR7 shared 85 and 57% amino acid identity with human GPR7 and bovine GPR8, respectively.

Figure 8. Scatchard plot for binding of [125I-Tyr11]desBr-NPB-23 to GPR7. Membrane fractions prepared from CHO cells expressing human GPR7 were incubated with increased concentrations of [125I-Tyr11]desBr-NPB-23, and bound and free ligands were separated when the binding reached equilibrium. Data are plotted as bound (B; pmol mg⁻¹ H₁₁₀²⁻¹) versus bound/free (B/F; pmol mg⁻¹ nM⁻¹). Each symbol represents the mean value with S.E. in triplicate determinations.

Table 1. Interaction of NPB with GPR7 and GPR8

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Inhibition of cAMP production&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Binding&lt;sup&gt;b&lt;/sup&gt; (IC₅₀) for human GPR7</th>
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<tr>
<td></td>
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<td>Human</td>
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<tr>
<td>Bovine</td>
<td></td>
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<tr>
<td>NPB-29</td>
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<tr>
<td>desBr-NPB-29</td>
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<tr>
<td>NPW-23</td>
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<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>desBr-NPB-29</td>
<td>1.2</td>
<td>14</td>
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</table>

<sup>a</sup> desBr-NPB, NPB without bromination. The numbers after NPB indicate amino acid lengths.

<sup>b</sup> Median inhibitory concentration (IC₅₀) values were determined from dose-response curves.
Interaction of NPB with Receptors—We cloned bovine GPR7 and GPR8 cDNAs (Fig. 7) and then examined the interaction of NPB with these. Synthetic NPB specifically inhibited cAMP production in CHO cells expressing GPR7 or GPR8 in various species (Table I). NPB inhibited cAMP production in cells expressing GPR7 more efficiently than those expressing GPR8. Both bovine NPB-29 and desBr-NPB-29 efficiently suppressed the forskolin-induced production of cAMP. We also measured the effects of NPBs on forskolin-induced cAMP production in CHO cells expressing human GPR8 and found that these peptides were less effective upon GPR8. We could not detect any apparent difference in the cAMP production-inhibitory activities between authentic NPB and desBr-NPB or between human and bovine NPB.

To examine the binding of NPB to GPR7, we prepared human desBr-NPB-23 labeled with 125I at either of two tyrosine residues (Tyr2 and Tyr11). After iodination, we separated the two labeled peptides by HPLC using a TSK gel ODS-80TMCTR column (TOSO) with 48–58% CH₃CN. The positions of the iodination were confirmed by mass spectrometry (data not shown). Since the agonistic activity of NPB was obviously reduced by iodination at Tyr2 but not at Tyr11 (data not shown), we used [125I-Tyr11]desBr-NPB-23 for binding assays. Scatchard plot analysis showed that the membrane fractions of CHO cells expressing human GPR7 had a single class of high affinity binding sites for [125I-Tyr11]desBr-NPB-23 at the dissociation constant (K_D) of 3.6 × 10⁻¹¹ M and maximal binding sites (B_max) of 1.3 pmol mg⁻¹ protein, indicating that NPB binds to GPR7 as a specific ligand with high affinity (Fig. 8). Even in the competitive binding assays, authentic NPB and desBr-NPB showed no difference in their inhibitory potency. Bovine NPB-29 and desBr-NPB-29 both efficiently inhibited the binding of [125I-Tyr11]desBr-NPB-23 (Table I), suggesting that bromination in NPB does not directly alter interaction with the receptor.

DISCUSSION

In this paper, we have identified a novel brominated peptide, NPB, as an endogenous ligand for GPR7. Synthetic NPB showed very potent inhibitory activity to cAMP production in CHO cells expressing GPR7 and specifically bound to their membrane fractions with high affinity. However, we could not detect Ca²⁺ influx in the NPB-treated CHO cells (data not shown), suggesting that GPR7 coupled to Gαi. NPB cross-reacted with GPR7 and specifically bound to their membrane fractions of CHO cells expressing human GPR7 had a single class of high affinity binding sites for [125I-Tyr11]desBr-NPB-23 at the dissociation constant (K_D) of 3.6 × 10⁻¹¹ M and maximal binding sites (B_max) of 1.3 pmol mg⁻¹ protein, indicating that NPB binds to GPR7 as a specific ligand with high affinity (Fig. 8). Even in the competitive binding assays, authentic NPB and desBr-NPB showed no difference in their inhibitory potency. Bovine NPB-29 and desBr-NPB-29 both efficiently inhibited the binding of [125I-Tyr11]desBr-NPB-23 (Table I), suggesting that bromination in NPB does not directly alter interaction with the receptor.

**A Brominated Peptide Ligand for GPR7**

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