Identification and Characterization of Two Neuromedin U Receptors Differentially Expressed in Peripheral Tissues and the Central Nervous System

Two structurally related, G-protein-coupled receptors were identified as receptors for the neuropeptide, neuromedin U. This peptide is found in highest levels in the gut and genitourinary system where it potently contracts smooth muscle but is also expressed in the spinal cord and discrete regions of the brain. Binding sites for neuromedin U have been characterized in rat uterus, however, little is known about the activity of this peptide in the regions of the central nervous system where it is expressed. The receptors characterized in this report are activated by neuromedin U at nanomolar potency in heterologous expression systems and bind radiolabeled neuromedin U with high affinity. Localization of the receptor RNA by quantitative reverse transcription-polymerase chain reaction in a variety of human tissues shows distinct expression patterns for the two receptors. NMU1 is expressed predominantly in peripheral tissues, whereas NMU2 is more highly expressed in the central nervous system. Identification of neuromedin U receptor subtypes will greatly aid in the determination of the physiological roles of this peptide.

A number of neuropeptides are known to produce distinct effects within the nervous system as well as on peripheral targets. More recently, the identification and cloning of neuropeptide receptors has accelerated progress toward determining their physiological roles. Along with several other biologically active peptides, neuromedin U (NMU) was first isolated from porcine spinal cord (1, 2) and was named for its uterine contractile activity. Indeed, receptors for this peptide and related analogues were first suggested by binding of 125I-labeled rat NMU in rat uterus membranes (3). Furthermore, the GTP analogue, GTPγS, inhibited binding of 125I-labeled rat NMU in this tissue suggesting that the binding site is a G-protein-coupled receptor (GPCR). Although NMU is present in various regions of the brain and spinal cord, binding sites for the peptide in the CNS have not been well characterized.

Two forms of the peptide (porcine NMU and porcine NMU18–25)2 were identified and their amino acid sequences were shown to be distinct from other known peptide families (Fig. 1). NMU peptides from various species share the greatest homology in their C-terminal regions (Fig. 1). Similarly, the region of rat NMU critical for smooth muscle contractile activity was found to reside in the C terminus of the peptide (between residues 17 and 22), supporting the importance of this region for contractile activity (4, 5).

Along with many other neuropeptides, NMU is present in nerves throughout the gastrointestinal tract (6), and it stimulates contraction of isolated muscle tissue such as longitudinal muscle of human ileum (7) and rat stomach circular muscle (8). High doses of this peptide also produce a hypertensive effect when given intravenously to rats (9). NMU has been shown to affect hormonal systems, including a profound effect on the in vivo release of stress-related modulators from the anterior pituitary and adrenal glands (12). Corticotrophs within the anterior lobe of rat and human pituitary gland contain high levels of NMU-like immunoreactivity (13), suggesting a hormonal role for NMU. NMU is present in the same secretory granules as ACTH and galanin (14). A small population of NMU-positive parafollicular C-cells is also found in rat thyroid gland (15, 16). High doses of this peptide also produce a hypertensive effect when given intravenously to rats (17).

In addition to its roles in smooth muscle contraction and gut/genitourinary system function, NMU is also present in various brain regions, suggesting as yet unidentified central functions for this peptide (11, 18, 19). Several structures associated with sensory processing are rich in NMU-containing fibers, including spinal cord (dorsal horn > ventral horn), trigeminal sensory nuclei, vestibular nuclei, and other nuclei associated with descending spinal pathways (18). Neuronal cell bodies containing NMU-like immunoreactivity have been identified in the arcuate nucleus of the hypothalamus, an area

2 Porcine NMU18–25 has historically been referred to as NMU-8.
identified as important for the regulation of food intake and neuroendocrine control. Relatively high levels of NMU-like immunoreactivity were also detected in the nucleus accumbens and substantia nigra (19), suggesting a role for NMU in the modulation of dopaminergic actions. NMU-like immunoreactivity is found in the hippocampus, amygdala, and other portions of the limbic system, suggesting a role for NMU and NMU receptors in neuropsychiatric disorders. The identification and characterization of NMU receptors expressed in these CNS regions will greatly aid in understanding the role of this peptide in brain functions.

This report describes the identification of two structurally related, G-protein-coupled receptors for NMU with distinct tissue expression patterns. Identification and characterization of these receptors will provide the first evidence for NMU receptor subtypes.

**EXPERIMENTAL PROCEDURES**

**Materials—**Rat NMU, porcine NMU, and porcine NMU18–25 were purchased from Bachem (Torrance, CA). Human NMU was synthesized by Synaptic Pharmaceutical Corp. (Paramus, NJ) using solid phase peptide synthesis with the Fmoc (N-(9-fluorenylmethoxycarbonyl)) protection protocol, with Rink/amide methylenebenzhydryl amine resin. 125I-labeled rat NMU and 125I-labeled porcine NMU18–25 were custom-labeled by NEN Life Science Products Inc. (Boston, MA).

**SNORF62 (NMU1) Cloning—**The GENEMBL data base SWPLUS was searched for G-protein-coupled receptor sequences (GPCRs) using a select set of known GPCRs and the Wisconsin Package (CCG, Genetics Computer Group, Madison, WI). One sequence, O43664, was selected for use in ligand identification screens. Before cloning the full-length receptor, the 5' and 3'-ends of the coding sequence were verified by 5'/3' rapid amplification of cDNA ends (RACE), using human hypothalamic Marathon-Ready cDNA (CLONTECH, Palo Alto, CA), the Marathon adaptor primers AP1 and AP2 (CLONTECH), and the following primers: for 5' RACE, 5'-CCACGAAATGACGATCATGTTG-3' and 5'-GCCATGACATGTCGTGCTGG-3'; for 3' RACE, 5'-CCGCCAGGAGACACGCTATTG-3' and 5'-GCTGCTGATGCCTCACTG-3'. A band of approximately 550 bp from the 5' RACE reaction was isolated using the Qiaquick gel extraction kit (Qiagen, Valencia, CA) and sequenced with the primer: 5'-GCCCGAGTACTTGTGCTCAGTGGTAAGAGGACATTCTACCTC-3'). The sequence of this band revealed an open reading frame 69 bp longer than the sequence represented in the public data base with an additional potential initiating methionine 23 amino acids upstream from the 5' untranslated sequence. The new (longer) coding sequence was named SNORF62.

**Radiolabeled Ligand Binding Assays—**Radioligand binding assays were performed by diluting membranes prepared from cells expressing the receptor in 50 mM Tris buffer (pH = 7.4 at 0 °C) containing 0.1% bovine serum albumin (Sigma), aprotinin (0.005 mg/ml, Roche Molecular Biochemicals), and bestatin (0.1 mM, Sigma) as protease inhibitors. The final protein concentration in the assay was 12–40 μg/ml. Membranes were then incubated with either 125I-labeled rat NMU or [125I]NMU18–25 (specific activity 2200 Ci/mmol) in the presence or absence of competing ligands on ice for 60 min in a total volume of 250 μl in 96-well microtiter plates. The bound ligand was separated from free ligand by filtration through GF/B filters presoaked in 0.5% polyethyleneimine, using a Tomtec (Wallace) vacuum filtration device. After addition of Ready Safe (Beckman) scintillation fluid, bound radioactivity was quantitated using a Trilux (Wallace) scintillation counter (approximately 40% counting efficiency of bound counts). Data was fit to nonlinear curves using GraphPad Prism.

**Inositol Phosphate Assay—**COS-7 cells expressing NMU1 were plated at a density of 70,000 cells per well, allowed to incubate for 24 h, and exposed to [3H]inositol (10 μCi/mg, specific activity 2000 Ci/mmol) in presence or absence of competing ligands on ice for 60 min in a total volume of 100 μl in 96-well microtiter plates. The bound ligand was separated from free ligand by aspiration of the media, followed by acidification with 1.2 M ammonium formate/0.1 M formic acid solution, and radioassay of the supernatant using a Micromass isotope separator. The compounds of interest were diluted in wash buffer (Hanks' balanced salt solution without phenol red), 20 μM HEPES, 2.5 μM probenecid, and added to the cell plates. Responses were measured using the FLIPR instrument and data were analyzed using the FLIPR software and GraphPad Prism.
**NMU Receptors**

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**Fig. 2.** Sequence alignment of deduced amino acid sequences for NMU1 (SNORF62) and NMU2 (SNORF72) receptors. Pairwise GAP comparison (Wisconsin Package, Genetics Computer Group, Madison, WI) of the amino acid sequences of NMU1 (upper sequences) and NMU2 (lower sequences). The periods (.) indicate gaps in the sequences to demonstrate optimum alignment. Symbols above the alignment correspond to NMU1, whereas symbols below the alignment correspond to NMU2; asterisks indicate potential N-linked glycosylation sites, large dots indicate potential PKC phosphorylation sites, and the solid diamonds indicate potential casein kinase II phosphorylation sites.

**Preparation of cDNA for Quantitative PCR—**Total RNA was purchased from CLONTECH (Palo Alto, CA) or isolated from human tissues using TRIzol reagent (Life Technologies, Gaithersburg, MD) and used according to the vendor’s protocol. After isopropanol precipitation, RNA was resuspended in RNase free water and treated with DNase to remove residual genomic DNA. RNA was quantified by measuring absorbance (A_{260/280}) and by using a dye intercalation assay (RiboGreen, Molecular Probes, Eugene, OR). cDNA was synthesized by reverse transcription from total RNA using SuperScriptII RNase H (-) and random hexamers, extracted with phenol/chloroform, and precipitated. Control reactions were carried out with

**RESULTS AND DISCUSSION**

**Cloning of SNORF62 (NMU1) and SNORF72 (NMU2)**

**Isolation of a Full-length Human SNORF62 Receptor—**A search of the SWPLUS data base with a search set of known GPCRs yielded several orphan GPCR sequences. One sequence, O43664, was found to be most similar to the neurotransnin receptor 1 (31% identity) as well as the recently identified motilin receptor, GPR36 (33% identity). O43664 was then chosen to be cloned for use in ligand identification screens. In the process of verifying the 5'- and 3'-ends of the coding sequence for O43664 by RACE, an additional methionine was found upstream from the initiating methionine of O43664, which was in-frame with the rest of the sequence. This new receptor sequence was 69 bp longer, coding for a protein 23 amino acids longer than O43664. The deduced amino acid sequence of this novel protein (SNORF62) is represented in Fig. 2. There are three potential N-linked glycosylation sites in the extracellular N-terminal domain at amino acid positions 7, 27, and 41, and one potential N-linked glycosylation site in the second extracellular loop at position 196. The C-terminal tail contains two potential casein kinase II phosphorylation sites at threonines 366 and 387, and one potential protein kinase C phosphorylation site at serine 360. As expected, a Kyte-Doolittle hydrophobicity plot of the amino acid sequence of SNORF62 (NMU1) indicated that there are seven transmembrane domains, as shown in Fig. 2.

**Isolation of a Full-length Human SNORF72 Receptor—**A receptor sequence that was 46% identical to SNORF62 was found in the public domain data bases. The full-length receptor by the Stanford Human Genome Center (SHGC) and distributed by Research Genetics, Inc. The Stanford G3 panel of 83 radiation hybrids was analyzed by PCR using the same primers, probes, and thermal cyclers as profiles used for localization (above). 20 ng of DNA was used in each PCR reaction. Data was submitted to the RH server (SHGC), which linked the NMU1 and NMU2 gene sequences to specific markers. NCBI LocusLink and NCBI GeneMap '99 were used for further analysis of gene localization.
SNORF62 and SNORF72 were cloned from human whole brain cDNA and was named SNORF72. After comparing several independent PCR products, the actual sequence of SNORF72 was found to be slightly different from the published sequence. There were seven nucleotide differences, six of which changed the amino acid sequence of SNORF72. The predicted amino acid sequence and putative transmembrane domains are shown in Fig. 2. There is a 47% amino acid identity between the two receptors, suggesting that they are likely to be members of the same receptor subfamily. SNORF72 also shares 31% amino acid identity to the neurotensin receptors NT1 and NT2, 32% identity to the growth hormone secretagogue receptor 1, and 33% identity to the neurotensin receptors NT1 and NT2, 32% identity to the growth hormone secretagogue receptor 1, and 33% identity to the neurotensin receptors NT1 and NT2, respectively. For comparison, in isolated rat uterus preparations the EC50 concentration for these receptors as NMU receptors. For comparison, in isolated rat uterus preparations the EC50 concentration for NMU1, NMU2, and mock-transfected COS-7 cells transiently transfected with NMU1 (open squares, A), NMU2 (open circles, B), or vector (mock-transfected, closed squares) were stimulated with increasing concentrations of human NMU, and intracellular Ca2+ was measured by FLIPR. The data presented are representative of six to seven experiments performed in duplicate.

Identification of SNORF62 and SNORF72 as NMU Receptors

Increase in Intracellular Ca2+—Ligand screening experiments were conducted to identify the agonist for SNORF62 in Ca2+ release assays (data not shown). Subsequent experiments were then performed to further characterize this receptor along with the structurally related SNORF72. Application of human NMU to COS-7 cells transiently expressing SNORF62 or SNORF72 (but not in vector-transfected cells) resulted in concentration-dependent release of intracellular Ca2+ (Fig. 3, A and B). The EC50 values obtained for the stimulation of SNORF62 (NMU1) and SNORF72 (NMU2) by NMU-related peptides are listed in Table I. The peptides that activated these receptors produced similar maximum responses and were therefore considered full agonists (data not shown).

High potency of NMU-induced stimulation of SNORF62 (NMU1) and SNORF72 (NMU2) provides support for classifying these receptors as NMU receptors. For comparison, in isolated rat uterus preparations the EC50 concentration for contraction by rat NMU is 0.2 nM (22). The slightly lower potency of rat NMU observed in the NMU1- and NMU2-transfected COS-7 cells (average EC50 = 2.1 ± 0.5 and 5.0 ± 1.7 nM, respectively) may be due to species differences in the receptors as well as the heterogeneous expression system.

Radioligand Binding—Receptor binding was performed on NMU1-, NMU2-, and mock-transfected COS-7 membranes using 125I-labeled rat NMU and 125I-labeled porcine NMU18–25 as radioligands. Binding of 125I-labeled rat NMU and 125I-labeled porcine NMU18–25 to the NMU1 and NMU2 membranes was time-dependent (reaching equilibrium by 30 min, data not shown) and saturable (Fig. 4, A and B). No saturable, specific binding sites for either radioligand were present in the mock-transfected COS-7 cell membranes (data not shown).

In membranes from NMU1-transfected cells 125I-labeled rat NMU and 125I-labeled porcine NMU18–25 bound with high affinity (Kd = 0.61, 0.72 nM and Kd = 1.2, 2.8 nM, respectively; n = 2). 125I-Labeled porcine NMU18–25 bound with high affinity (Kd = 0.61, 0.72 nM and Kd = 1.2, 2.8 nM, respectively; n = 2). 125I-Labeled porcine NMU18–25 bound with high affinity (Kd = 0.61, 0.72 nM and Kd = 1.2, 2.8 nM, respectively; n = 2). 125I-Labeled porcine NMU18–25 bound with high affinity (Kd = 0.61, 0.72 nM and Kd = 1.2, 2.8 nM, respectively; n = 2). 125I-Labeled porcine NMU18–25 bound with high affinity (Kd = 0.61, 0.72 nM and Kd = 1.2, 2.8 nM, respectively; n = 2). 125I-Labeled porcine NMU18–25 bound with high affinity (Kd = 0.61, 0.72 nM and Kd = 1.2, 2.8 nM, respectively; n = 2).

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The high affinity binding of $^{125}$I-labeled rat NMU at NMU1 and NMU2 in COS-7 cell membranes is similar to the $K_d$ determined for this radioligand in isolated rat uterus ($K_d = 0.35\, \text{nM}$ (3)). The binding site in this tissue demonstrated lower affinity for NMU18–25 than for rat NMU with average IC$_{50}$ values of 60 and 1 nM, respectively (3). Careful study of both the pharmacology and distribution of the rat homologues would be required to establish a link between the cloned receptor subtypes and the uterine NMU receptor.

Because NMU was demonstrated to bind with high affinity and be a potent agonist for both NMU1 and NMU2, these receptors likely mediate responses to NMU in vivo. We propose the renaming of SNORF62 as NMU1 and SNORF72 as NMU2. The potential activities of these NMU receptors were further explored by performing a series of experiments to identify the signaling pathways stimulated by receptor activation.

**Signaling Pathways Activated by NMU1**

**Inositol Phosphate Release**—Exposure of NMU1-transfected COS-7 cells (but not mock-transfected cells) to human NMU caused the release of IP second messengers with an EC$_{50}$ of $0.25 \pm 0.09$ nM (Fig. 6). Rat NMU and porcine NMU18–25 were also full agonists in this assay with EC$_{50}$ of $0.23 \pm 0.10$ nM and $0.23 \pm 0.06$ nM, respectively ($n = 3$). The EC$_{50}$ values measured for IP release are lower than the EC$_{50}$ values measured for increases in intracellular Ca$^{2+}$ (see Table I). This may be due to differences in experimental conditions between the two types of assays, including the nonequilibrium nature of Ca$^{2+}$ measurements. In addition, the Ca$^{2+}$ release response to human NMU was present in NMU1-transfected cells following pretreatment with pertussis toxin (100 ng/ml for 18–20 h, $n = 2$, data not shown) indicating that the Ca$^{2+}$ signal is not predominantly generated by G-proteins of the G$i/Go$ family. Taken together these results indicate that NMU1 couples to phospholipase C stimulation via a $G_q$-type G-protein in COS-7 cells.

**Activation of Calcium-activated Cl$^-$ Currents in Human NMU1-expressing Xenopus Oocytes**—Heterologous expression of GPCRs in Xenopus oocytes has been widely used to determine the identity of signaling pathways activated by agonist stimulation (23, 24). Human NMU elicited Cl$^-$ currents with
average amplitude of 1065 ± 211 nA (n = 3) in oocytes injected with NMU1 (SNORF62) mRNA, whereas mock-injected oocytes typically showed no response to human NMU (n = 5) (Fig. 7). A detailed concentration-response relation was not obtained, but nearly maximal responses were observed at a human NMU concentration of 1 μM. Porcine NMU also elicited a strong response (2150 ± 330 nA (n = 3)) from oocytes injected with NMU1 mRNA, but not in control oocytes (data not shown). The agonist-induced oscillatory Cl current seen in oocytes injected with NMU1 mRNA are indicative of activation of phospholipase C and the resulting Ca2 second messenger system. Consistent with the experiments described in the COS-7 cells, such activation in oocytes is exhibited by GPCRs that couple to Gq or Gi1.

Tissue Expression Pattern of NMU1 and NMU2 RNA

Detection of RNA Coding for Human NMU1—To further characterize the NMU receptors, quantitative reverse transcription-PCR using fluorogenic probes specific for NMU1 or NMU2 was used to determine the tissue distribution of RNA encoding each receptor (Fig. 8). Human NMU1 RNA was localized in greatest abundance in peripheral organs, particularly in elements of the gastrointestinal and urogenital systems with highest levels in testes. The uterus and prostate express NMU1 RNA, consistent with functional studies and localization of NMU in these organs. Although NMU1 mRNA was measured in kidney (both cortex and medulla), the peptide has not been identified in this tissue and its role in kidney function is not known. However, the presence of the receptor in the kidney may be associated with NMU’s effects on arterial blood pressure.

The gastrointestinal system also has considerable amounts of NMU1 RNA. The stomach, small intestine, and the duodenum express NMU1 RNA. This is consistent with the high levels of NMU in the GI tract found by radioimmunoassay (19) in both myenteric and submucosal plexuses of the gut (11) and the postulated role of NMU as a potent constrictor of smooth muscle. NMU1 RNA is also present in the pancreas at levels equivalent to that seen in other regions of the GI tract, but it is not known if the NMU1 receptors are found on the pancreatic islets, acinar cells, ducts, or are present on vasculature within the gland. Sumi et al. (10) demonstrated an increase in blood flow in the pancreas after administration of NMU suggesting a vascular localization or function.

Other tissues expressing NMU1 RNA include the lung, trachea, adrenal gland, and mammary gland, with lower levels in skeletal muscle and heart. This widespread distribution implies a broad regulatory or modulatory activity, perhaps at the level of smooth muscle contraction or secretagogue actions within these tissues.

Central nervous system structures express NMU1 RNA at levels much lower than those seen in peripheral organs. Within the CNS, NMU1 RNA has been detected in highest abundance in the cerebellum, dorsal root ganglia, hippocampus, and spinal cord. NMU-like immunoreactivity was identified in each of these regions (19). Within the CNS, it is found in levels that are 5- to 25-fold less than that found in peripheral organs. The role of NMU1 RNA in the CNS is not clear, however, its widespread distribution is consistent with the distribution of NMU found in the brain (19). The presence of NMU as well as NMU1 RNA in the spinal cord, dorsal root ganglia, and medulla oblongata implies a role in sensory transmission or modulation.

Detection of RNA Coding for Human NMU2—NMU2 RNA was found in highest abundance in the CNS, particularly the
dominantly in the periphery, and NMU2, predominantly in the CNS. Differential expression of NMU1, predominantly in peripheral organs. The exception to this CNS/peripheral organ pattern are the testes, which express high levels of both NMU1 and NMU2 RNA.

The hippocampus, hypothalamus, and cerebral cortex all express moderate to high levels of NMU2 RNA. Other CNS structures expressing NMU2 RNA include the amygdala and cerebellum. Dorsal root ganglia also express NMU2 RNA albeit at substantially lower levels than those found in the spinal cord.

The expression pattern of NMU2 RNA in the CNS is consistent with the hypothesis that its ligand, NMU, is a sensory transmitter/modulator. NMU is found in the spinal cord, dorsal root ganglia, and medulla oblongata using radioimmunoassay (19) and immunohistochemistry (11). Its presence in other regions, including hippocampus, hypothalamus, and cerebral cortex, implies a modulatory role in multiple systems within the CNS.

Peripheral organs expressing NMU2 RNA include the kidney (medulla), lung, and trachea. NMU1 RNA is found in equivalent amounts in both the cortex and medulla of the kidney, whereas NMU2 RNA is found primarily in the medulla. It is not known at this time which cells in the kidney express NMU2 and/or NMU2 RNA. It is interesting to note that NMU2 RNA is expressed in low levels in the gastrointestinal tract. In contrast, both NMU1 RNA and the NMU peptide are expressed in high levels in the gastrointestinal tissues.

Chromosomal Localization—The human NMU1 gene maps to SHGC-33253, which is localized to chromosome 2q34-q37. NMU2 maps to SHGC-8848, which is localized to chromosome 5q31.1-q31.3.

In conclusion, a variety of experimental results support the identification of NMU1 and NMU2 as functional receptors for the neuropeptide NMU. Differential expression of NMU1, predominantly in the periphery, and NMU2, predominantly in the CNS, suggests different roles for these receptors in vivo. Identification of these two distinct NMU receptors also opens the possibility of developing chemical tools useful to distinguish CNS and peripheral effects of NMU.

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Addendum—During the preparation of this manuscript two reports were published, which relate to a portion of this work (27, 28).

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Identification and Characterization of Two Neuromedin U Receptors Differentially Expressed in Peripheral Tissues and the Central Nervous System
Rita Raddatz, Amy E. Wilson, Roman Artymyshyn, James A. Bonini, Beth Borowsky, Lakmal W. Boteju, Siqun Zhou, Evguenia V. Kouranova, Raisa Nagorny, Maricel S. Guevarra, Meng Dai, Gabriel S. Lerman, Pierre J. Vaysse, Theresa A. Branchek, Christophe Gerald, Carlos Forray and Nika Adham

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