Identification of a Novel Neuromedin U Receptor Subtype Expressed in the Central Nervous System*

Received for publication, August 3, 2000, and in revised form, September 18, 2000
Published, JBC Papers in Press, September 28, 2000, DOI 10.1074/jbc.C000522200

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Neuromedin U is a neuromedinic peptide prominently expressed in the upper gastrointestinal tract and central nervous system. Recently, GPR66/FM-3 (NmU-R1) was identified as a specific receptor for neuromedin U. A BLAST search of the GenBank™ genomic database using the NmU-R1 cDNA sequence revealed a human genomic fragment encoding a G protein-coupled receptor that we designated NmU-R2 based on its homology to NmU-R1. The full-length NmU-R2 cDNA was subsequently cloned, stably expressed in 293 cells, and shown to mobilize intracellular calcium in response to neuromedin U. This response was dose-dependent (EC₅₀ = 5 nM) and specific in that other neuromedins did not induce a calcium flux in receptor-transfected cells. Expression analysis of human NmU-R2 demonstrated its mRNA to be most highly expressed in central nervous system tissues. Based on these data, we conclude that NmU-R2 is a novel neuromedin U receptor subtype that is likely to mediate central nervous system-specific neuromedin U effects.

The neuropeptides (Nm)³ are a group of smooth muscle-stimulating peptides commonly divided into four groups: bombesin-like (NmB, NmC), kassinin-like (NmL and -K or neurokinins A and B, respectively), neurotensin-like (NmN), and neuromedin U (NmU). Among this group of peptides, neuromedin U has been the least well understood, in large part due to the lack of a known receptor. Neuromedin U was first reported in 1985 by Minamino et al. (1, 2) as a peptide isolated from porcine spinal cord. These investigators isolated two active peptides, NmU-25 and an additional cleavage product, NmU-8, and characterized them as having smooth muscle contractile activity. Neuromedin U was subsequently isolated from a variety of species including rat (3, 4), guinea pig (5), dog (6), rabbit (7), chicken (7, 8), and frog (9).

The cDNAs for rat and human NmU have been cloned, and analysis of the nucleic acid sequence suggests that NmU is produced as a 174-amino acid precursor (10, 11). The precursor contains a signal peptide and several dibasic cleavage sites that give rise to a number of possible secreted peptides, including NmU, which is present near the carboxyl terminus. Neuromedin U shows remarkable conservation throughout evolution, and a core active peptide (Phe-Leu-Phe-Arg-Pro-Arg-Asn-NH₂) is absolutely conserved among mammalian species.

A variety of biological activities have been reported for NmU although its role in normal physiology is unclear. The first biological activity ascribed to NmU was smooth muscle contraction (1, 2). These experiments have not been consistent among different species, however, in regard to the specific tissues that respond to NmU (1, 12–15). Neuromedin U has also been reported to increase arterial blood pressure (16, 17) and modify ion transport in the intestinal tract (15). Finally, NmU injected subcutaneously into rats has been reported to result in a short term increase in circulating ACTH levels and a long term increase in serum corticosterone levels (18, 19), suggesting a role in regulation of the hypothalamo-pituitary-adrenal axis.

Recently, several groups, including our own, have reported that GPR66/FM-3 is a specific receptor for NmU (20–23). Originally identified as a partial mouse expressed sequence tag residing in GenBank™, full-length mouse GPR66/FM-3 was cloned from a T cell library and subsequently used as a probe to identify a human clone (24). Comparison of GPR66/FM-3 to other known G protein-coupled receptors (GPCRs) shows it is most similar to the human growth hormone secretagogue and neurotensin receptors (33 and 29% amino acid identity, respectively) as well as the recently described motilin receptor (25). This receptor (NmU-R1) is expressed in peripheral tissues, particularly in the upper gastrointestinal tract and lymphoid tissues (20–24), but is essentially absent from central nervous system tissues. In the present study we report the identification and characterization of a second specific NmU receptor that is predominantly expressed in the central nervous system.

**Experimental Procedures**

Cloning and Expression of NmU-R2—A genomic fragment encoding NmU-R2 (GenBank accession number AC008571) was identified using the deduced amino acid sequence of NmU-R1 as “bait” for a TBLASTN search of the GenBank™ high throughput genomic database. A putative deduced amino acid sequence of NmU-R1 as “bait” for a TBLASTN search of the GenBank™ high throughput genomic database. A putative deduced amino acid sequence of NmU-R1 as “bait” for a TBLASTN search of the GenBank™ high throughput genomic database. A putative deduced amino acid sequence of NmU-R1 as “bait” for a TBLASTN search of the GenBank™ high throughput genomic database. A putative deduced amino acid sequence of NmU-R1 as “bait” for a TBLASTN search of the GenBank™ high throughput genomic database. A putative deduced amino acid sequence of NmU-R1 as “bait” for a TBLASTN search of the GenBank™ high throughput genomic database. A putative deduced amino acid sequence of NmU-R1 as “bait” for a TBLASTN search of the GenBank™ high throughput genomic database. A putative deduced amino acid sequence of NmU-R1 as “bait” for a TBLASTN search of the GenBank™ high throughput genomic database. A putative deduced amino acid sequence of NmU-R1 as “bait” for a TBLASTN search of the GenBank™ high throughput genomic database. A putative deduced amino acid sequence of NmU-R1 as “bait” for a TBLASTN search of the GenBank™ high throughput genomic database. A putative deduced amino acid sequence of NmU-R1 as “bait” for a TBLASTN search of the GenBank™ high throughput genomic database. A putative deduced amino acid sequence of NmU-R1 as “bait” for a TBLASTN search of the GenBank™ high throughput genomic database. A putative deduced amino acid sequence of NmU-R1 as “bait” for a TBLASTN search of the GenBank™ high throughput genomic database. A putative deduced amino acid sequence of NmU-R1 as “bait” for a TBLASTN search of the GenBank™ high throughput genomic database. A putative deduced amino acid sequence of NmU-R1 as “bait” for a TBLASTN search of the GenBank™ high throughput genomic database. A putative deduced amino acid sequence of NmU-R1 as “bait” for a TBLASTN search of the GenBank™ high throughput genomic database. A putative deduced amino acid sequence of NmU-R1 as “bait” for a TBLASTN search of the GenBank™ high throughput genomic database.
Identification of a Novel Neuromedin U Receptor Subtype

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**RESULTS AND DISCUSSION**

**Single Cell Ca
2
+
 Imaging**—293 cells stably expressing NmU-R2 plated onto poly-D-lysine-coated coverslips that subsequently formed the base of a perfusion chamber. Cultures were loaded with fura-2 acetoxymethyl ester (5 μM, 45 min, 37 °C, Molecular Probes, Eugene, OR) in a buffered salt solution (149 mM NaCl, 3.25 mM KCl, 2.0 mM CaCl₂, 2.0 mM MgCl₂, 10 mM HEPES, 11 mM glucose). The perfusion chamber was continuously perfused with the buffered salt solution (3 ml/min), and drug additions were made using a pinch valve arrangement with minimal dead space. Intracellular Ca
2
+ concentration was estimated using an Attofluor RatioVision digital fluorescence imaging system (Atto Instruments, Rockville, MD). Fura-2 was excited, alternately, at 334 and 380 nm, and the emission was collected at 510 nm and 1.25-s intervals. Calibration of the 334/380 nm fura-2 signal was performed in vitro with fura-2 pentapotassium salt in the presence of 1 mM Ca
2
+ or 1 mM EGTA, and the 334/380 nm excitation ratio was converted to Ca
2
+ concentration values using the procedure of Grynkiewicz et al. (28).

**Messenger RNA Expression Analysis**—Expression of NmU-R2 was examined using dot blots and Northern blots obtained from a commercial source (CLONTECH). Hybridization to blots was carried out using PCR-generated DNA fragments encompassing 1200 base pairs of the coding region of NmU-R2 beginning at the 3’-end and including most of the coding region. The DNA fragments were random-prime labeled with [32P]dCTP, and the blots were hybridized for 14 h in ExpressHyb (CLONTECH) containing 2 × 10⁶ cpm/ml of radiolabeled probe. The following day the blots were washed and exposed to Kodak Biomax MS film for 3 days at −70 °C. The dot-blot films were analyzed for NmU-R2 expression levels using the MCID M4 image analysis system (Imaging Research, Ontario, Canada), and the data were displayed as absolute optical density.

**Cellular Calcium Imaging**—Fluo-3AM (Sigma) according to the FLIPR manufacturer's protocol. Cells stably expressing NmU-R2 were selected using LipofectAMINE 2000 (Life Technologies, Inc.) according to the manufacturer's protocol. Cells expressing NmU-R2 were transfected with poly-D-lysine-coated coverslips that subsequently formed the base of a perfusion chamber. Cultures were loaded with fura-2 acetoxymethyl ester (5 μM, 45 min, 37 °C, Molecular Probes, Eugene, OR) in a buffered salt solution (149 mM NaCl, 3.25 mM KCl, 2.0 mM CaCl₂, 2.0 mM MgCl₂, 10 mM HEPES, 11 mM glucose). The perfusion chamber was continuously perfused with the buffered salt solution (3 ml/min), and drug additions were made using a pinch valve arrangement with minimal dead space. Intracellular Ca
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**RESULTS AND DISCUSSION**

A peripheral NmU receptor (NmU-R1) has been recently identified that is expressed in peripheral tissues (20–22). To identify potential subtypes of this receptor we performed a TBLASTN search of the high throughput genomic database

**FIG. 1.** Deduced amino acid sequence and genomic structure of NmU-R2. **A**, the predicted amino acid sequence of NmU-R2 is shown aligned with that of NmU-R1. Amino acid identities and regions of high overall homology are boxed while similar amino acids are indicated with shading. Potential N-linked glycosylation sites are noted (*) as are the predicted transmembrane domains (overscore). The boundaries of exons for NmU-R2 are also indicated (solid arrowheads) with the predicted splice junction nucleotide acid sequences written as exon/intron/exon. The position of the single exon boundary in NmU-R1 is indicated for comparison (open arrowhead). **B**, genomic structure and alternate splicing of NmU-R2. The open reading frame of NmU-R2 and an assembly EST (GenBank™ accession numbers R13353, R13890, H11359) representing an alternatively spliced NmU-R2 transcript were exon mapped onto an unordered genomic fragment (GenBank™ accession number AC008571) using Sequencher. Exons unique to the alternative (sterile) transcript are indicated as filled boxes, exons shared by both the NmU-R2 transcript and the alternative transcript are filled in gray, and exons unique to the NmU-R2 transcript are unfilled. Solid lines indicate the splicing pattern of NmU-R2 whereas splicing of the alternative transcript is indicated with dashed lines. Distances between exons are drawn to scale although exon sizes are not. Numbers at either end of the schematic are distances (kilobases) not including a gap of unknown length (indicated by a break).
subset of GenBank™ using the deduced amino acid sequence of NmU-R1 as bait. One such search identified a genomic fragment from chromosome 5 (GenBank™ AC008571) containing a region of relatively high homology (~57%) to NmU-R1. Although the genomic sequence was unordered, a predicted open reading frame was assembled based on sequence homology to NmU-R1. PCR primers were designed based upon this predicted open reading frame, and a cDNA was subsequently obtained that was designated NmU-R2 based on its homology to NmU-R1 (Fig. 1A). The genomic structure of NmU-R2 differs significantly from that of NmU-R1 in that the predicted open reading frame is encoded on four exons instead of the two found in the NmU-R1 gene (Fig. 1). Interestingly, the intron 2-exon 3 boundary of NmU-R2 coincides with the intron 1-exon 2 boundary of NmU-R1, and the overall homology between the open reading frames is far higher upstream of this point (TM1–TM6) than downstream. The conservation of one of the intron/exon boundaries and the relatively high homology of the two genes suggests that these two receptors arose from a duplicative event sometime in the past. The position of exon 2 of NmU-R2 is such that it encompasses only the third intracellular loop and the very beginning of transmembrane domain six. The length of this loop is also shortened in NmU-R2 as compared with NmU-R1 (Fig. 1A). Finally, both the amino- and carboxy-terminal sequences show considerable divergence with NmU-R2 having extended termini.

In addition to the NmU-R2 genomic fragment, we also identified several expressed sequence tag (EST) sequences in the GenBank™ database that proved to have identity with NmU-R2. Four of five of these ESTs originated from brain-related cDNA libraries with the fifth coming from a heart library. Three of these ESTs appear to represent an alternative NmU-R2 transcript. This transcript includes exons 2 and 3 of the NmU-R2 gene but do not splice upstream to exon 1. Instead, two novel exons are spliced in, creating a presumably sterile transcript with multiple stops in all reading frames (Fig. 1B). Although the “aberrant” transcripts are all from fetal brain libraries, it is possible that such transcripts might exist elsewhere and suggests caution in interpreting the results of expression studies. The downstream splicing of exon 3 to exon 4 in these alternate transcripts could not be assessed from the sequence data present in GenBank™. The expression data presented in this study were confirmed with probes that do not hybridize to the alternate NmU-R2 transcript (data not shown).

To gain some insight into the possible physiological role of a second NmU receptor, the expression of this receptor was comprehensively assessed using dot blots and Northern blots of human tissues. The expression of NmU-R2 on dot blots was highest in testis and central nervous system tissues, particularly spinal cord (Fig. 2A). This is in contrast to NmU-R1, which showed relatively little expression in central nervous system tissues (20–22). Low levels of NmU-R2 expression were detected by dot blot in stomach and duodenum (Fig. 2A), but unlike NmU-R1, NmU-R2 expression was absent or very low in other gastrointestinal tract tissues. Similarly, expression of NmU-R2 in lymphoid tissues was either very low or undetectable (Fig. 2A). Aside from the expression in central nervous system tissues and gastrointestinal tract, expression of NmU-R2 mRNA was also observed in kidney, lung, and thyroid (Fig. 2A). No expression of NmU-R2 was detected in uterus, despite the fact that NmU binding has been reported in rat uterus (29). This may reflect a species-specific difference as discussed in the introduction. Alternatively, the expression of NmU receptor was reported to be estrogen-dependent (30) and may vary depending on when tissue was obtained. In any case, this is clearly an area where further investigation is warranted.

The expression of NmU-R2 in the central nervous system and gastrointestinal tract was examined in more detail by Northern blot analysis. Spinal cord and corpus callosum demonstrated the highest expression with a NmU-R2 message of 2.4 kilobases (Fig. 2B). In contrast, expression of NmU-R2 in the gastrointestinal tract as detected by Northern blot was very low and could not be detected even upon very long exposure (1 week, data not shown). Despite the inability to detect NmU-R2 in the Northern blot, the dot-blot results suggest that NmU-R2 may have some overlapping expression with NmU-R1 in the gastrointestinal tract. Thus, interpretation of the physiological effects of NmU in this tissue will need to be carefully considered in regard to which receptor is mediating a given event.

The identification of NmU-R2 as a neuromedin receptor was accomplished in human embryonic kidney cells (29) that were transiently transfected with NmU-R2 cDNA. The NmU-R2 transfected cells were assessed for their ability to mobilize intracellular calcium when stimulated with each of more than 1000 known or suspected GPCR ligands including small molecules and various peptides, among them neuromedins B, C, K, L, N, and U (human NmU-25, rat NmU-23, and pig NmU-8). From among this library of potential ligands, only the NmUs
generated a specific, dose-dependent calcium flux in the transfected cells (Fig. 3A). This response was dose-dependent with an EC50 of 5 nM and a maximal response observed between 80 and 800 nM (Fig. 3B). Maximal intracellular calcium concentration reached 600–800 nM when cells were stimulated with 100 nM NmU-25 (Fig. 3C). We observed no significant difference in the ability of NmU-25/23 to stimulate NmU-R2 when compared with NmU-8 (data not shown). We did find amidation of NmU-8 to be necessary for activity as a nonamidated form did not activate the receptor even at concentrations in excess of 10 μM (data not shown). In addition, we found that pertussis toxin (100 ng/ml, overnight incubation) did not significantly alter NmU-R2 receptor signaling, suggesting that in 293 cells this receptor couples to calcium through the Gq subset of G proteins (Fig. 3A, inset).

Recently, a number of other investigators have also reported characterization of NmU-R2 (23, 31, 32). All of these manuscripts report an NmU EC50 of 1–5 nM in functional assays, which is consistent with our findings. These reports agree also that coupling appears to be primarily through Gi although Hosoya et al. (31) reported some Gq coupling. The reports vary, however, in their findings regarding distribution of NmU-R2 mRNA. Hosoya et al. (31) reported only rat expression and provided no data regarding the expression pattern of human NmU-R2. Furthermore, these investigators performed their analysis using only quantitative PCR and provided no other confirmation of their results. Similarly, Raddatz et al. (32), while providing human expression data, relied solely on quantitative PCR for expression analysis. Howard et al. (23) discussed expression of human NmU-R2 but presented no data, reporting only that NmU-R2 expression was weakly observed in some tissues. Hosoya et al. (31) found rat NmU-R2 most prominently expressed in uterus (31); however, neither Raddatz et al. (32) nor our group found any significant expression of human NmU-R2 in uterus (Fig. 2). In contrast, expression of NmU-R2 was very high in human testis (Fig. 2) (32) but low in rat testis (31).

The recent reports on NmU-R2 do agree that this receptor is most prominently expressed in the brain of both human and rat; however, the exact nature of the message detected and
whether it is translated remains unknown. This question becomes more important in light of our finding of alternative transcripts for NmuU-R2 (Fig. 1B).

In particular, Raddatz et al. (32) reported expression of neuromedin U receptor in dorsal root ganglion; however, we have been unable to demonstrate NmuU responsiveness in either mouse or rat dorsal root ganglion, even at doses as high as 1 μm, although this could be species-related (data not shown).² Howard et al. (23) have shown intracerebral injection of NmuU altered feeding behavior in rats but did not demonstrate dose responsiveness of this effect, only that 1 μg of NmuU was not sufficient to alter feeding behaviors, while 3 or 10 μg produced similar effects. These studies also failed to demonstrate that the effect observed was mediated by central nervous system receptors because no similar studies were reported using peripheral administration of NmuU. Given that NmuU-R1 is highly expressed in gastrointestinal tract tissue and that nothing is known about the ability of NmuU to cross the blood-brain barrier, it is not unreasonable to assume that some of the observed effects upon feeding behavior might be mediated directly or indirectly via peripheral receptors. Finally, given the history of species-specific effects of NmuU on muscle contraction (see introduction) and differences in the species-specific expression of the NmuU receptors, it will be important to develop additional animal models for NmuU function and to confirm any findings in human cells or tissues whenever possible.

In summary, we have demonstrated the existence of a second specific neuromedin U receptor that we have designated NmuU-R2. Cells transfected with NmuU-R2 show a dose-dependent intracellular Ca²⁺ mobilization in response to NmuU stimulation. We also show that NmuU-R2 calcium signaling in 293 cells is pertussis toxin insensitive, suggesting that NmuU-R2, like NmuU-R1, is coupled to the Gq family of G proteins in these cells. Unlike NmuU-R1, NmuU-R2 mRNA is highly expressed in the central nervous system and suggests that NmuU-R2 mediates the effects of NmuU in these tissues. However, given that NmuU-R1 and NmuU-R2 expression overlap in some peripheral tissues and that NmuU itself is broadly expressed it will be necessary to conduct further studies to understand the precise role each receptor plays in mediating the peripheral and central effects of neuromedin U. Targeted gene knockouts of each of the NmuU receptors, as well as of NmuU itself, would undoubtedly be invaluable in such studies.

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² J. Crona, personal communication.