Neuromedin U Is a Potent Agonist at the Orphan G Protein-Coupled Receptor FM3*

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Neuromedins are a family of peptides best known for their contractile activity on smooth muscle preparations. The biological mechanism of action of neuromedin U remains unknown, despite the fact that the peptide was first isolated in 1985. Here we show that neuromedin U potently activates the orphan G protein-coupled receptor FM3, with subnanomolar potency, when FM3 is transiently expressed in human HEK-293 cells. Neuromedins B, C, K, and N are all inactive at this receptor. Quantitative reverse transcriptase-polymerase chain reaction analysis of neuromedin U expression in a range of human tissues showed that the peptide is highly expressed in the intestine, pituitary, and bone marrow, with lower levels of expression seen in stomach, adipose tissue, lymphocytes, spleen, and the cortex. Similar analysis of FM3 expression showed that the receptor is widely expressed in human tissue with highest levels seen in adipose tissue, intestine, spleen, and lymphocytes, suggesting that neuromedin U may have a wide range of presently undetermined physiological effects. The discovery that neuromedin U is an endogenous agonist for FM3 will significantly aid the study of the full physiological role of this peptide.

G protein-coupled receptors (GPCRs)1 represent one of the largest gene superfamilies identified to date, with more than 1000 members cloned from a wide range of species. The current explosion in the availability of human genomic sequence data is allowing many more members of this family to be identified in man. Most if not all of these newly identified GPCRs fall into the category of orphan receptors, for which the endogenous ligand(s) remain to be identified. Typically these orphan receptors show only low levels of similarity (less than 35% identity) with known GPCRs, too low to classify them with any confidence into a specific receptor subfamily, although one can often predict the likely class of ligand for these receptors, e.g., peptide, nucleotide, lipid, etc., by using phylogenetic analysis.

Recently, naturally occurring ligands have been identified for a number of these orphan GPCRs using a "reverse-pharmacological" approach (1), that is, using the recombinant orphan receptor as a specific sensor component of a bioassay. Tissue extracts have often been the source of these natural ligands (2, 3), although more recently the ligands for several orphans have been identified as a result of screening large libraries of known or putative GPCR ligands (4–6). Here, we describe how this latter approach has been used to identify neuromedin U (NmU) as a naturally occurring ligand for the orphan receptor FM3.

Neuromedin U was first isolated over 15 years ago from extracts of porcine spinal cord, using a uterine smooth muscle contraction bioassay to monitor purification (7). Two molecular forms were isolated; neuromedin U-8 (NmU-8) and neuromedin U-25 (NmU-25). NmU-like immunoreactivity has since been detected in neurones in the mammalian brain and gastrointestinal tracts of various species (8–10) and in the thyroid and endocrine cells of the pituitary gland (8, 11). The smooth muscle-contracting effects of NmU are now well documented: NmU causes hypertension (7), can regulate local blood flow in the intestine (12–14), and exhibits contractile activity in various smooth muscle preparations in vitro, such as human ileum and urinary bladder (15), chicken crop (16), and rat stomach (17). In addition, complex effects on steroid secretion from the adrenal cortex have also been reported (18, 19). Despite this large body of literature strongly suggesting an extracellular transmitter role for NmU, a receptor for this neuropeptide has not been discovered, until now.

The human orphan GPCR FM3 was originally identified (20) by similarity to the mouse orthologue. The mouse gene was identified from a mouse T-cell expressed sequence tag library by similarity screening of GenBankTM using the human GHS-R sequence as a query sequence (GHS-R was an orphan subsequently characterized as a receptor for the naturally occurring peptide ghrelin (21)). The FM3 sequence has many of the characteristics of Family 1 GPCRs, including the ERY variant of the DRY motif at the boundary of transmembrane domain 3 and the second intracellular loop, and consensus sites for asparagine-linked glycosylation and phosphorylation on amino- and carboxyl-terminal sequences, respectively. Phylogenetic comparison with other GPCR sequences (data not shown) suggests that the natural ligand for FM3 is likely to be a peptide, as the closest relatives to FM3 are receptors for neuropeptides.

To investigate putative physiological roles of this newly discovered ligand/receptor pairing, we also describe the first quantitative comparison of both NmU precursor and receptor mRNA expression in a wide variety of human tissues using the technique of RT-PCR.
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**Experimental Procedures**

**Receptor Cloning and Transient Expression in Mammalian Cells**—We cloned FM3 from a human placenta library using gene-specific primers. A 403-amino acid protein was obtained that was 100% identical to the originally published sequence for this receptor (20) (GenBankTM accession number NP_006047). Receptors were subcloned into the mammalian expression vector pcDNA (22) and transiently transfected into HEK-293 cells using LipofectAMINE plus (Life Technologies, Inc.), according to the manufacturer’s instructions.

**Calcium Mobilization Assays**—Intracellular calcium assays were carried out as follows. HEK-293 cells transiently expressing FM3 were seeded (50,000 cells/well) into poly-L-lysine coated 96-well black-wall, clear-bottom microtiter plates (Becton Dickinson) 24 h prior to assay. Cells were loaded for 1 h with 1 μM Fluoro-4-AM fluorescent indicator dye (Molecular Probes) in assay buffer (Hanks’ balanced salts solution, 10 mM HEPES, 200 μM Ca²⁺, 0.1% bovine serum albumin, 2.5 mM probenecid), washed three times with assay buffer, then returned to the incubator for 10 min before assay on a fluorometric imaging plate reader (FLIPR, Molecular Devices). Maximum change in fluorescence over baseline line was used to determine agonist response. Cells were screened against a large library of over 1500 known and putative GPCR agonists, including all known mammalian neuropeptides (tachykinins, neureomeds, etc.), bioactive lipids (leukotrienes, prostaglandins, etc.), steroids (aldosterone, testosterone, etc.), amines (catecholamines, etc.), cannabinoids (anandamide, etc.), nucleotides (ATP, ADP, UTP, etc.), and sugar nucleotides (UDP-glucose, UDP-galactose, etc.). Peptides were tested at a final concentration of 100 nM, and other ligands at >1 μM. Concentration response curve data were fitted to a four parameter logistic equation using GraFit (Erlithacus Software Limited).

**cAMP Assays**—Assays for intracellular cAMP were carried out as described previously (4).

**Total Inositol Phosphate Accumulation Assays**—Inositol phosphate accumulation assays were performed as described previously (23) with minor modifications. Briefly, HEK-293 cells transiently transfected with FM3 were loaded overnight with myo[3H]inositol. Cells were treated for 30 min at 37 °C with NmU-25. The reaction was stopped with trichloroacetic acid. Samples were purified using columns containing AG 1 × 8 anion exchange resin. Total inositol phosphates were eluted using 1 M ammonium formate containing 0.1 M formic acid.

**Tissue Localization Studies Using Taqman RT-PCR**—Quantitative RT-PCR analysis was carried out as described previously (5) using the following forward, probe, and reverse (respectively) FM3-specific primers: 5′-GGCTCCAGCAAGCAGATC-3′, 5′-GCCGGAGACAAATGGAAGC-3′, and the following forward, probe, and reverse (respectively) NmU precursor-specific primers: 5′-CGAAGACAGAACAGCTGGTTCG-3′, and 5′-CTTGTGGTCTGCATGC-3′ and the following forward, probe, and reverse (respectively) NmU precursor-specific primers: 5′-CGAAGACAGAACAGCTGGTTCG-3′, and 5′-CTTGTGGTCTGCATGC-3′.

**Peptides**—Porcine neuromedin U-8 (NmU-8) and human neuromedin K were obtained from Sigma. Porcine neuromedin U-25 (NmU-25), rat neuromedin U-23 (NmU-23), and human neurotensin, motilin, and neuromedins B, C, and N were obtained from Bachem. Ghrelin was obtained from Phoenix Pharmaceuticals.

**Results**

**Functional Screening and Characterization of FM3 in Mammalian Cells**—As part of a large program to identify natural ligands for orphan GPCRs, we transiently expressed FM3 in human embryonic kidney (HEK-293) cells and functionally screened these cells on a FLIPR to measure mobilization of intracellular calcium in response to a large library of over 1500 putative GPCR ligands in potential peptide ligands for FM3. NmU-8, NmU-25, and NmU-23 were the only peptides that produced orphan-mediated calcium mobilization in HEK-293 cells transiently expressing FM3. Responses to NmU in these cells were large, transient, and robust (Fig. 1A). Mock-transfected HEK-293 cells did not respond to any injection of NmU (Fig. 1A), nor did cells that had been transiently transfected with a number of other orphan GPCRs (data not shown).

We investigated the concentration dependence of FM3 activation by porcine and rat NmU isoforms (Fig. 1B). In a FLIPR assay, all 3 NmU isoforms tested caused the same maximal activation of FM3 with EC₅₀ values of 0.21 ± 0.02 nM (n = 6), 0.38 ± 0.03 nM (n = 6), and 0.17 ± 0.02 nM (n = 6) for NmU-8, NmU-25, and NmU-23, respectively. Thus all three peptides cause potent activation of FM3, suggesting that NmU is the natural agonist for this receptor. Neuromedins B, C, K, and N were all inactive at FM3 (Fig. 1B) in this assay, as were neurotensin, ghrelin, motilin, vasoactive intestinal peptide, and pancreatic polypeptide, when tested at concentrations up to 1 μM (data not shown).

The calcium mobilization response seen following activation of FM3 by NmU suggests that this receptor is coupled to G proteins of the Gi/o subfamily. In agreement with this hypothesis, NmU-8 (10 nM; added at t = 10 s) induces a robust, transient rise in intracellular Ca²⁺ in FM3-transfected cells (continuous line), but not in mock-transfected cells (dashed line). B, noradrenaline (NE), and neuropeptide Y (NPY) were all inactive at FM3 (Fig. 1B). In contrast, neuropeptide Y (100 nM) induced calcium mobilization in HEK-293 cells transiently expressing FM3 transduced cells.

**Fig. 1.** Neuromedin U causes concentration-dependent activation of FM3 transiently expressed in HEK-293 cells. HEK-293 cells were transiently transfected with FM3 and intracellular calcium responsive HEK-293 cells transiently expressing FM3 increased intracellular calcium in response to a large library of over 1500 putative GPCR agonists, including all known mammalian neuropeptides (tachykinins, neureomeds, etc.), bioactive lipids (leukotrienes, prostaglandins, etc.), steroids (aldosterone, testosterone, etc.), amines (catecholamines, etc.), cannabinoids (anandamide, etc.), nucleotides (ATP, ADP, UTP, etc.), and sugar nucleotides (UDP-glucose, UDP-galactose, etc.). Peptides were tested at a final concentration of 100 nM, and other ligands at >1 μM. Concentration response curve data were fitted to a four parameter logistic equation using GraFit (Erlithacus Software Limited).

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![Image of calcium mobilization response](image-url)
from the reverse transcription of 1 ng of poly(A)

romedin U, as demonstrated by quantitative RT-PCR. The cDNA

another individual's large intestine.

except the intestine, which is an equal pool of one individual's small and

as the mean ± S.E. of four individual's mRNA level for each tissue, except the intestine, which is an equal pool of one individual's small and another individual's large intestine.

does not contribute to the functional response observed. In addition, neither basal nor forskolin-elevated levels of intracellular cAMP were modulated by any form of NmU (data not shown) in HEK-293 cells transiently expressing FM3, suggesting that this receptor does not couple strongly to G proteins of the Gs or Gi/o subfamilies.

To provide further proof that NmU is an agonist at FM3, we

studied changes in total inositol phosphates in HEK-293 cells

transiently expressing FM3 following challenge with NmU (Fig. 1C). NmU-25 produced a potent, dose-dependent increase in total inositol phosphates with an EC50 of 0.28 ± 0.02 nM (n = 3). No response was observed in mock-transfected cells.

This strongly supports the calcium mobilization data and provides further evidence that stimulation of FM3 causes activation of phospholipase C.

Human Tissue Localization of FM3—To further investigate the physiological role of FM3 we carried out studies to localise the expression of this receptor in human tissues. We performed quantitative RT-PCR analysis using FM3 specific primers to determine the relative levels of FM3 mRNA in a variety of tissues from four different nondiseased individuals was assessed by TaqMan PCR for FM3 and neuromedin U precursor mRNA and, as a control, mRNA for the housekeeper gene GAPDH. Data are presented as the mean ± S.E. of four individual's mRNA level for each tissue.

stomach, lymphocytes, placenta, and spleen. A more detailed study of the central nervous system distribution of human NmU (Fig. 3) showed that in addition to the pituitary, several cortical areas of the brain such as the cingulate gyrus and medial frontal gyrus also express moderate levels of NmU precursor. Low to moderate levels of expression were also observed in many other brain regions, including the hypothalamus, locus coeruleus, thalamus, medulla oblongata, and substantia nigra (Fig. 3).

DISCUSSION

For many years a receptor for NmU has remained elusive; this has hampered the full elucidation of its physiological roles. In this report we identify FM3 as a specific receptor for NmU and show that NmU causes potent activation of this receptor. This finding will stimulate research into the fundamental physiological effects of NmU.

NmU was first isolated in two molecular forms from extracts of porcine spinal cord and was so called because of its ability to stimulate contraction of uterine smooth muscle (7). The longer COOH-terminally amidated porcine peptide (NmU-25) contains 25 amino acids, while the shorter peptide (NmU-8) is a result of proteolytic processing of NmU-25 at the Arg16-Arg17 site and represents the amidated COOH-terminal 8 amino acids of the longer form. NmU was subsequently identified in many mammals, including humans (24), and the human gene encoding the NmU precursor peptide has been cloned (25). The sequence of the biologically active COOH-terminal region of the peptide is almost completely conserved across all species studied (26), indicating the strong degree of selection to retain this physiologically important region of sequence. Interestingly, the dibasic cleavage site is not conserved in many species, e.g. rat, rabbit, and humans, and only the long form of NmU has been isolated from these species.

Despite the fact that NmU has been known for more than 15 years, the broader physiological role of this peptide has not been extensively studied and is still not well understood. This is due in part to the fact that a receptor for NmU has, until now, been unknown. Nandha et al. (27) have shown that a high affinity, specific binding site for NmU exists in rat uterus. Furthermore, a nonhydrolyzable analogue of GTP, GTPγS, reduced NmU binding in a dose-dependent manner, suggesting
that this uterine receptor was indeed a member of the GPCR superfamily. The subnanomolar potencies of NmU, which we report here, are typical of many neuropeptides at their cognate receptors and are also consistent with the potent binding affinities reported with rat NmU binding to rat tissues (27).

To confirm the specificity of the response to NmU we re-tested, at higher concentrations, a number of peptides that were inactive in the original screen, but which might conceivably exhibit cross-reactivity with FM3 for various reasons. Thus all known neuremedins were re-tested over a range of concentrations up to 1 μM, in view of the similarity of reported functions of these peptides. Likewise, ghrelin, motilin, and neurotensin were similarly re-tested, since the corresponding receptors for these peptides show significant sequence similarity to FM3 (20). Last, pancreatic polypeptide and vasoactive intestinal peptide were also tested in this way in view of their reported sequence similarity to neuremedin U at the COOH terminus (12). The absence of detectable activity from any of these peptides confirms the specificity of the interaction of neuremedin U with FM3.

The physiological role of neuremedin U is not fully understood. The smooth muscle contracting activity of NmU has been well documented, but the complex effects on steroid secretion from the adrenal cortex suggest that there may be much to learn about the physiological role of NmU. Important clues that can suggest novel functions for NmU could be obtained most simply by measuring the expression levels of both peptide and cognate receptor throughout the body. Obviously, this has not been possible until now. In the present study we have described for the first time a quantitative, side-by-side comparison of both NmU precursor and receptor mRNA expression across a wide variety of human tissues. The low receptor expression levels seen in the brain are broadly consistent with similar Northern blot data available for mouse tissue (20), although the low levels previously observed in mouse spleen contrast with the relatively high levels of FM3 expression in human spleen observed in this study. Relatively high levels of FM3 expression were observed by us in both intestine and stomach, and these data are entirely consistent with NmUs well characterized role in smooth muscle contraction, may suggest novel functions for NmU, perhaps involving modulation of energy expenditure.

The distribution of NmU mRNA is widespread, including relatively high levels in intestine and pituitary. In addition, NmU message is also expressed in many other brain regions, especially the cortex (Figs. 2 and 3). This is concordant with early immunological studies that characterized NmU as a gut-brain peptide and is also in agreement with Northern blot studies showing mRNA expression throughout the rat (28) and human (25) intestine and in rat pituitary (29). NmU mRNA was also observed in several regions which had previously not been studied, such as adipose tissue, again supporting a putative role of this ligand/receptor pair in modulation of energy expenditure. Likewise, high levels of NmU mRNA expression in bone marrow suggests a potential role in hematopoietic function.

In conclusion, these data demonstrate the discovery of a specific receptor for NmU. The widespread tissue distribution of both FM3 and NmU suggest that this peptide, in addition to its well characterized role in smooth muscle contraction, may be involved in a number of additional, but as yet undefined, physiological roles.

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