Receptor for the Pain Modulatory Neuropeptides FF and AF Is an Orphan G Protein-coupled Receptor*

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Opiate tolerance and dependence are major clinical and social problems. The anti-opioid neuropeptides FF and AF (NPAF and NPAF) have been implicated in pain modulation as well as in opioid tolerance and may play a critical role in this process, although their mechanism of action has remained unknown. Here we describe a cDNA encoding a novel neuropeptide Y-like human orphan G protein-coupled receptor (GPCR), referred to as HLWAR77 for which NPAF and NPFF have high affinity. Cells transiently or stably expressing HLWAR77 bind and respond in a concentration-dependent manner to NPAF and NPFF and are also weakly activated by FMRF-amide (Phe-Met-Arg-Phe-amide) and a variety of related peptides. The high affinity and potency of human NPAF and human NPFF for HLWAR77 strongly suggest that these are the cognate ligands for this receptor. Expression of HLWAR77 was demonstrated in brain regions associated with opioid activity, consistent with the pain-modulating activity of these peptides, whereas the expression in adipose tissue suggests other physiological and pathophysiological activities for FMRF-amide neuropeptides. The discovery that the anti-opioid neuropeptides are the endogenous ligands for HLWAR77 will aid in defining the physiological role(s) of these ligands and facilitate the identification of receptor agonists and antagonists.

Two highly studied mammalian neuropeptides, NPAF (A-18-F-amide) and NPFF (F-8-F-amide), also known as mor-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF2572109.

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† The abbreviations used are: NPAF, neuropeptide AF (A-18-F-amide); NPFF, neuropeptide F (F-8-F-amide); hu, human; HEK, human embryonic kidney; GRK2, G protein-coupled receptor kinase 2; GFP, green fluorescent protein; FLIPR, fluorescence imaging plate reader; CRE, cAMP response element; MRE, multiple response element; PLC, phospholipase C; FMRF-amide, Phe-Met-Arg-Phe-amide.

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EXPERIMENTAL PROCEDURES

Receptor Cloning—Expressed sequences tag analysis (16) of cDNA clones derived from a human placenta cDNA library (oligo(dT)3-primed and constructed in the ZAPII vector (Stratagene)) identified a 900-base...
pair clone demonstrating low homology to the neuropeptide Y-like receptors. This cDNA clone encoded an incomplete GPCR. Nested primers (outside 5′-AAGACTGAAGCTGCGACAGATATTC-3′ and inside 5′-CCTGGAACCAATCCACTGATCTTGC-3′) were designed at the 5′ end of the gene and used to extend the 5′ sequence using Marathon human brain, placenta, and fetal brain cDNA templates (CLONTECH). Primers were synthesized corresponding to the amino and the carboxyl termini of the predicted open reading frame and were used to obtain the full-length cDNA clone from human brain cDNA by PCR. The full-length cloning process was confirmed twice using the human brain and fetal brain cDNA as templates. The cDNAs were completely sequenced on both strands using an ABI sequencer. The HLWAR77 cDNA was subcloned into the mammalian expression vector pCDN, and transfections were performed as described (17).

Expression in Mammalian Cells—RNA purification, Northern blot, and TaqMan mRNA analysis were performed as described previously (18). HLWAR77-specific reagents, forward primer 5′-TTGCACACTG-GCTGGCATT-3′ and reverse primer 5′-AACCACGGCGGAAAT-TCTC-3′ and TaqMan probe 5′-CAGTGTCAATCCCATCATTTATG-GTTTCTTCAA-3′, were used.

Ca\textsuperscript{2+} Mobilization—Ca\textsuperscript{2+} mobilization was performed as described previously (18). HLWAR77-specific reagents, forward primer 5′-TTGCACACTG-GCTGGCATT-3′ and reverse primer 5′-AACCACGGCGGAAAT-TCTC-3′ and TaqMan probe 5′-CAGTGTCAATCCCATCATTTATG-GTTTCTTCAA-3′, were used.

\textit{CaMP Reporter Assay—MRE/CRE luciferase reporter assays were performed as described previously (19).}

\textit{Radioligand Binding—Binding studies, using \textsuperscript{125}I-labeled Y-8-F (YLFQPQRF-amide, Amersham Pharmacia Biotech), were performed on membranes prepared from HEK 293 cells stably expressing HLWAR77 and G\textsubscript{qi5} (14). Membranes were incubated in a final volume of 200 μl containing 50 mM HEPES (pH 7.4), 10 mM CaCl\textsubscript{2}, 10 mM MgCl\textsubscript{2}, 0.1% bovine serum albumin (pH 7.4), and 0.2 nM \textsuperscript{125}I-Y-8-F (specific activity, 2000 Ci/mmol) for 45 min at 25 °C. Nonspecific binding was determined in the presence of 1.0 μM cold NPFF. Membrane-bound ligand was captured on Whatman GF/C filters presoaked with 0.5% polyethyleneimine using vacuum filtration and then washed and the radioactivity was quantitated by scintillation spectrometry.}

\textit{Transient Transfection with GRK2 and β-Arrestin 2-GFP—HEK 293 cells stably expressing HLWAR77 receptor were grown to 40–80% confluency in T80 flasks and transiently transfected with 1.5 μg each of GRK2 and β-arrestin 2-GFP, using LipofectAMINE Plus as described by the manufacturer. Cells were seeded into chambered covered glass slides 24 h after transfection (Life Technologies, Inc., 1.8-cm\textsuperscript{2} well), at a density of 100–200,000 cells/well and incubated overnight at 37 °C before being analyzed by confocal microscopy.}

\textit{Confocal Microscopy—For assay, growth medium was removed from covered glass-based chamber slides and replaced with 200 μl of assay buffer (Hanks’ balanced salt solution with 10 mM HEPES, 10 mM CaCl\textsubscript{2}, 10 mM MgCl\textsubscript{2}, 0.1% bovine serum albumin (pH 7.4), and 0.2 nM \textsuperscript{125}I-Y-8-F (specific activity, 2000 Ci/mmol) for 45 min at 25 °C. Nonspecific binding was determined in the presence of 1.0 μM cold NPFF. Membrane-bound ligand was captured on Whatman GF/C filters presoaked with 0.5% polyethyleneimine using vacuum filtration and then washed and the radioactivity was quantitated by scintillation spectrometry.}

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RESULTS

As part of an ongoing effort to identify ligands for novel orphan GPCRs, we isolated an 1888-base pair cDNA from a human brain cDNA library that encoded a 420-amino acid protein that structurally resembled members of the GPCR superfamily. As shown in Fig. 1, this orphan receptor, HL-WAR77, encodes the typical GPCR signature motif of 7 distinct hydrophobic domains of 16–28 amino acids each linked by hydrophilic amino acid stretches of varying length. Two cysteines highly conserved in the GPCR family are present in the putative first and second extracellular loops. Among the GPCRs, HLWAR77 is most similar to the orexin-A receptor (15) (37% amino acid identity). Recently, an apparent splice variant of this cDNA (NPGR), with an additional 102 residues at the amino terminus, was described (20). Repeated attempts were made to clone the NPGR cDNA from different human cDNAs using primers designed at the 5’- and 3’-untranslated regions of the gene. The result was a gene product missing most of the 1st transmembrane domain; therefore, we concluded that this gene represents an aberrant variant.

Northern hybridization analysis of RNA isolated from various human tissues suggested that the HLWAR77 receptor is expressed at barely detectable levels, with a predominant 1.8-kilobase pair mRNA transcript present in placenta (data not shown). TaqMan quantitative reverse transcriptase-polymerase chain reaction (PCR) analysis confirmed the Northern blot data demonstrating that HLWAR77 was present in placenta at relatively low levels of expression (Fig. 2A). The sensitive TaqMan methodology showed that low levels of receptor RNA expression were detectable in adipose tissue and many brain regions, most notably cingulate gyrus (Fig. 2, A and B). Taq-Man was also used to test for HLWAR77 expression in 40 cell lines. Significant expression was observed in only 8 cell lines with the highest expression obtained in neuroblastoma and astrocytoma cell lines.

By using the “reverse pharmacological approach” (21), HEK 293 cells transiently co-transfected with HLWAR77 and a “promiscuous” G protein α subunit, G\(_{\alpha_{16}}\), which facilitates GPCR signaling through phospholipase C (22), were used in a microtiter plate-based (FLIPR) calcium mobilization assay to search for the ligand(s) activating this receptor (17). HLWAR77/G\(_{\alpha_{16}}\) cells were challenged, as described previously, with a collection of .1,000 different ligands (17). Unexpectedly, and in contrast to our experience with other orphan GPCRs, numerous peptide ligands functioned as selective agonists, albeit with very different potencies, generating transient calcium mobilization responses in HLWAR77/G\(_{\alpha_{16}}\)-expressing HEK 293 cells. The common feature of the diverse peptide ligands that functioned as HLWAR77/G\(_{\alpha_{16}}\) agonists was the occurrence at the carboxyl terminus of the dipeptide, Arg-Phe-amide (Table I). The response to the RF-amide peptides of HEK 293 cells expressing HLWAR77 was dependent on the co-transfection of both HLWAR77 and G\(_{\alpha_{16}}\) cDNAs. HEK 293 cells transfected with

![Fig. 2. TaqMan quantitative reverse transcriptase-PCR analysis of mRNA levels in human tissues.](image)

**TABLE I**

Peptides that stimulated calcium mobilization response in HLWAR77-expressing HEK 293 cells

| Peptide       | Sequence          | Species of origin | EC\(_{50}\) |
|---------------|-------------------|-------------------|------------|
| bNPAF         | AGEGLSSPFWSLAAARPQRF-amide | Bovine            | 1.56       |
| huNPAF        | AGEGLNSQFWSLAAPQRF-amide | Human             | 1.26       |
| bNPFF         | FLFQQPQRF-amide    | Bovine            | 2.42       |
| huNPFF        | SQAFLFQPQRF-amide  | Human             | 0.39       |
| PQRF-amide    | PQRF-amide         | Synthetic         | 925        |
| FMRF-amide    | FMRF-amide         | Clam              | 565        |
| Antho-RF-amide| pEGRF-amide       | Sea anemone       | 48,000     |
| Antho-RN-amide| L-3-phenyllactyl-LRN-amide | Sea anemone     | 53,500     |
| Antho-RW-amide I | pESLRW-amide      | Sea anemone       | 5,750      |
| Antho-RW-amide II | pELLGRW-amide   | Sea anemone       | 9,800      |
| Pol-RF-amide  | pELLGRF-amide     | Hydromedusa       | 52,000     |
| KSAYMRF-amide | KSAYMRF-amide     | Nematode          | 795        |
| Peptide F1    | TNRNFLRF-amide    | Lobster           | 535        |
| DF2           | DRNFLRF-amide     | Crayfish          | 875        |
| LPLRF amide   | LPLRF-amide       | Chicken           | 860        |
| Leu-enkephalin-RF-amide | YGGFGLRF-amide | Synthetic         | 125        |
| Met-enkephalin-RF-amide | YGGFMRF-amide | Synthetic         | 165        |
HLWAR77, or Ga16 alone, did not respond to the RF-amide peptides, nor did cells transfected with vector alone or vector encoding other orphan GPCRs (Fig. 3A). The 17 peptides identified in the initial screen that showed reproducible concentration-dependent calcium responses are presented in Table I. The most potent peptides were bovine NPFF (bNPFF) and bNPAF, exhibiting EC50 values of 1365 and 2266 nM, respectively. Our initial activities were dependent on HLWAR77 co-expression with Ga16. Subsequently, we co-transfected HLWAR77 with a variety of other reported promiscuous G proteins and the “chimeric” G protein subunits (22–24), composed of αq in which the five carboxyl-terminal residues are replaced with the carboxyl-terminal residues derived from αi2 or αo, referred to as Gqi5 or Gqo5, respectively. Co-transfection of HLWAR77 with these chimeras resulted in a 3–4-fold enhancement of the calcium mobilization signal compared with the amplitude of the response obtained when the receptor was co-expressed with Ga16 (Fig. 3B). However, the potency of the response was not altered significantly, since all responding cells exhibited EC50 values of 10–30 nM (Fig. 3B).

In light of the responses obtained in HEK 293 cells transiently transfected with HLWAR77 and Ga16, HEK 293 cell lines stably expressing both HLWAR77 and Gqi5 were generated. Several clones were identified in the FLIPR calcium assay where huNPFF induced concentration-dependent calcium response with varying potencies and magnitude of responses (Fig. 3C). The most responsive clonal cell line, AA18, responded to huNPFF with an EC50 of 0.39 ± 0.08 nM, and the least potent cell line, AB5, produced a maximal response 25% that of AA18, and the EC50 was 10-fold weaker at 5.2 nM (Fig. 3C). These data may reflect the critical importance of the ratio of receptor to G protein to obtain efficient coupling of the PLC pathway (25). Clone AA18 was used to assess the relative potency of RF-amide-like peptides. As shown in Fig. 3D, bNPFF, huNPFF, bNPAF, and huNPAF were the most potent with EC50 values of 2.4, 0.4, 1.56, and 1.26 nM, respectively. The next most potent agonists identified were synthetic peptides, Leu-enkephalin-RF-amide and Met-enkephalin-RF-amide (MERF), and FMRFamide-like peptides in Table I had EC50 values of 0.5–60 μM.

The calcium response induced by huNPFF or huNPAF is rapid and sustained (Fig. 4A). Preliminary studies characterizing the calcium response in the AA18 cell line indicated that the large sustained calcium response was completely inhibited by removing extracellular calcium, resulting in a rapid transient response that returns to base line in less than 2 min (Fig. 4A). The huNPFF-induced calcium response was blocked by the PLC inhibitor, U 73122, with an IC50 of 400 nM, and was not significantly affected by the inactive analog U 73343, at concentrations as high as 10 μM. Additionally, nifedipine, the
calcium channel blocker, did not significantly inhibit the response at concentrations up to 100 μM (data not shown).

Since the calcium response induced by huNPAF and huNPFF in HLWAR77 expressing cells was dependent on co-transfection with promiscuous Ga protein subunits, we performed experiments to elucidate a natural second messenger pathway of HLWAR77 in HEK 293 cells. We hypothesized that HLWAR77 would normally activate Gi/o subunits of G proteins because Gqi5 or Gqo5 was able to elicit potent robust calcium mobilization responses from HLWAR77 in response to huNPAF and huNPFF peptides. Activation of the Gi/o pathway results in inhibition of forskolin-stimulated cAMP accumulation and subsequent decreases in transcription from promoters containing the cAMP response element (CRE). Therefore, a CRE/MRE-directed luciferase reporter assay (19) was used to assess the activation of this second messenger pathway. In cells co-transfected with HLWAR77 and reporter vector, bNPAF, bNPFF huNPFF, and huNPFF inhibited forskolin-stimulated luciferase activity in a concentration-dependent manner, exhibiting IC50 values of 1.3 ± 0.4, 0.41 ± 0.10, 0.25 ± 0.08, and 0.18 ± 0.04 nM, respectively (Fig. 4B). Cells co-transfected with reporter vector alone showed no response to these ligands.

The amino-terminal residue of NPFF was replaced with tyrosine (Y-8-F-amide) to facilitate the radioiodination of the peptide for use in binding studies (14). A high affinity saturable binding site was obtained with membranes of HEK 293 cells

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**FIG. 4. Characterization of NPFF and NPAF activity in calcium, reporter gene, and binding assays.** A, cells stably expressing HLWAR77 were stimulated with 10 nM bNPFF in the presence (solid line) and in the absence (dashed line) of extracellular Ca2+. B, bNPAF (○), bNPFF (▲), huNPFF (●), and huNPAF (■) inhibit forskolin-stimulated luciferase activity in reporter gene assay. HEK293 cells transiently transfected with an MRE/CRE-directed luciferase reporter, and HLWAR77 expression vectors responded in a concentration-dependent manner. Data are from three experiments with each point determined in duplicate. C, 125I-Y-8-F binds with high affinity to membranes of HEK 293 cells stably co-transfected with HLWAR77 and Gqo5. Saturable and specific binding of 125I-Y-8-F to membranes and Scatchard analysis of the saturation data are shown. Data presented are typical of two saturation studies with each point the mean of triplicate samples. D, competition binding studies with 125I-Y-8-F and cold bNPFF (○), bNPAF (▲), huNPFF (●), and huNPAF (■). Data presented are representative of three studies run, with each point the mean of duplicate samples.

**FIG. 5. Translocation of β-arrestin 2-GFP in response to agonist binding to the NPFF receptor.** HEK 293 cells stably expressing the NPFF receptor were transiently co-transfected with cDNA plasmids encoding β-arrestin 2-GFP and GRK2. The distribution of β-arrestin 2-GFP was visualized by confocal microscopy before (A) and 7–8 min after (B) stimulation with 0.5 μM NPFF and C is a different cell 60 min after NPFF activation.
(AA18) stably expressing HLWAR77, with a $K_d$ of $0.5 \pm 0.2$ nm and a $B_{max}$ of $34 \pm 6$ fmol/mg protein (Fig. 4C). In competition binding studies bNPFF, bNPFF, huNPFF, and huNPAF were evaluated for their ability to displace $^{125}$I-Y-8-F-amide peptide from these membranes. The peptides competed with IC$_{50}$ values of $1.6 \pm 0.2$, $2.5 \pm 0.8$, $0.15 \pm 0.02$, and $0.90 \pm 0.23$ nm, respectively (Fig. 4D). There was no specific binding observed for HEK 293 cells stably expressing the pCDN vector or to the parental HEK 293 cells.

An alternate functional assay for GPCRs is to study receptor-mediated translocation of $\beta$-arrestin-GFP from the cytoplasm to the plasma membrane following receptor activation (26). Following transient expression of both GRK2 and $\beta$-arrestin 2-GFP in cells stably expressing HLWAR77, the cells were visualized by confocal microscopy. In the absence of receptor activation, fluorescence corresponding to $\beta$-arrestin 2-GFP was evenly distributed throughout the cytoplasm of the cells (Fig. 5A). Upon activation with 0.5 $\mu$M NPFF, cytosolic $\beta$-arrestin 2-GFP translocated to the cell periphery within 4–5 min, visualized as punctate foci at the plasma membrane (Fig. 5B), suggesting that it was interacting with the agonist-occupied receptor. Subsequent images showed that distribution of $\beta$-arrestin 2-GFP was not further altered in the presence of ligand for up to 60 min (Fig. 5C), and there was no evidence for internalization of $\beta$-arrestin 2-GFP from the plasma membrane over this time period.

**DISCUSSION**

NPFF and NPAF were originally isolated from bovine brain (27), and recently cDNAs encoding both human and bovine peptides were reported (28, 29). Both peptides are encoded by the same cDNA in man and bovine and are flanked by consensus sequences for peptide processing (27–29). Human and bovine NPAF are both 18 amino acid peptides that differ in sequence by only 2 amino acid residues (Table I). However, the sequence of NPFF predicted from the conserved processing site in human cDNA (huNPFF predicted sequence, SQAFLQPQPQRF-NH$_2$) is 3 residues longer than the sequence of bNPFF isolated from bovine brain or found in human serum (FLQFQRQR-NH$_2$) (29, 30). There are conflicting reports on the relative potency of the short and long forms of huNPFF. It was originally reported that the extended, 11-residue human NPFF sequence, SQAFLFQPQRF-NH$_2$) is 3 residues longer than the sequence of NPFF predicted from human NPAF expressed in other tissues, e.g. adipose, further investigation is required to determine other potential pathophysiological roles associated with this receptor.

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Neuropeptide FF Is the Cognate Ligand for HLWAR77

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