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A human gene encoding morphine modulating peptides related to NPFF and FMRFamide

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Abstract FMRFamide-related peptides have been isolated from both invertebrates and vertebrates and exhibit a wide range of biological effects in rats. We show here that in humans 2 FMRFamide-related peptides are encoded by a single gene expressed as a spliced mRNA. The larger predicted peptide (AGEGLNSQFWSLAAPQRFamide) differs from the peptide isolated from bovines (AGEGLSSPFWSLAAPQRFamide) by the substitutions of 2 amino acids. The shorter predicted peptide (NPSF, SQAFLFQPQRFamide) is 3 amino acids longer than the bovine 8 amino-acid NPFF (FLFQPQRFamide) or the human NPFF peptide isolated from serum [5], suggesting that the encoded protein is subject to cleavage by a tripeptidyl peptidase or by a novel processing mechanism. On rat spinal cord, the larger peptide is indistinguishable in activity from the equivalent bovine peptide whereas the smaller extended peptide is inactive.

Key words: NPFF; NPAF; FMRFamide; cDNA; Nociception; Human

1. Introduction

FMRFamide-related peptides were originally isolated from molluscs [1] and subsequently from many species, including *Drosophila* [2], *C. elegans* [3] and bovines [4].

Neuropeptide FF (NPFF, FLFQPQRF-NH_2) and the related neuropeptide AF (NPAF, AGEGLSSPFWSLAAPQRF-NH_2) were originally isolated from the bovine brain using an antibody raised against the molluscan peptide FMRF-NH_2 [4], and more recently NPFF has been detected in human serum [5]. These peptides have wide-ranging physiological effects, in particular the modulation of morphine-induced analgesia [6], elevation of arterial blood pressure [7] and increased somatostatin secretion from the pancreas [8].

By the use of immunohistochemistry and binding studies it has been shown that the rat spinal cord contains an intrinsic NPFF system. Cell bodies in the dorsal horn react to antibodies against NPFF and specific high-affinity binding sites for NPFF have been demonstrated [9,10]. A specific binding site for an iodinated analogue of NPFF, ^{125}I-YLFQPQRF-amide, has also been found in human spinal cord and medulla, the highest density being in the spinal trigeminal nucleus [11], suggesting that release of NPFF-related peptides from the immunoreactive neurons may bind to this highly specific receptor.

In bovine brain, 3 different forms of immunoreactive material were detected by HPLC separation and the use of an antibody raised against the molluscan peptide FMRF-NH_2. Two of these correspond to NPFF and NPAF; the structure of the third is unknown [12]. Similarly, in the rat hypothalamus, NPFFs have been shown to exist by HPLC and immunoreactivity but the predominant form of peptide is not NPFF [13].

The isolation of the human gene described here now allows a definitive description of the human FMRFamide peptides. We show that there is an additional 3 amino acids on the human NPFF precursor that must be cleaved to produced biologically active NPFF. If these amino acids are present on the precursor of the bovine and rat NPFF then this could account for the alternate form detected by HPLC. We also demonstrate that human NPAF is capable of modulating neural activity in the rat spinal cord in a manner indistinguishable from that of bovine NPAF while human N-terminally extended NPFF has no activity.

2. Materials and methods

2.1. Isolation of cDNA clones encoding human FMRFamide-like peptides

A full-length cDNA clone was isolated from a human testis cDNA λgt10 library (Clontech) using a random primed α-^{32}P-dCTP-radiolabelled cDNA probe encoding part of the human FMRFamide propeptide. Hybridization was performed on duplicate filters of 3×10^4 plaques (6 plates with 5000 pfu/plate) at 65°C overnight (hybridization buffer: 5×SSC, 0.1% w/v SDS, 8% w/v PEG_{8000}, 5×Denhardt's solution, 0.1 M Na_2HPO_4/NaH_2PO_4) followed by stringent washing (three 20-min washes at 65°C, with 0.1×SSC/0.1% SDS) [14]. Filters were placed on pre-flashed Kodak Xomat X-ray film overnight. Positively hybridizing plaques were purified through secondary and tertiary screens and the cDNA clones were isolated by PCR amplification from the λgt10 forward and reverse primer sites (PCR conditions: 50 mM KCl, 10 mM Tris-HCl pH 8.0, 2.5 mM $MgCl_2$, 200 μM each dNTP, 50 pmol each primer, 2 U *Taq* polymerase (Promega), ≈10^9 phage in phage suspension buffer; 25 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min). The products were cloned into the pTAG vector (R and D Systems, UK) and subjected to fluorescence-based cycle sequencing on an ABD 373A automated DNA sequencer.

2.2. PCR amplification of the human FMRFamide gene from genomic DNA

Oligonucleotide primers corresponding to positions within the cDNA sequence were used to amplify the equivalent regions from human genomic DNA (5′UTR: CATGAAGTCCTGGGGGCGC and 3′UTR: CAGAAGCCAGACAATTTTATTTGG; F1: GCAGGCTGCTGCACTGCT-GGT and F2rev: CTCAGCCATTCATTCCTCCAGGA; F2: TCACTGTTGCACTACC-TGCTCCA and F2rev). PCR reactions were performed in 50-μl vols. using 50 pmol of each

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primer, 200 ng of genomic DNA (isolated from human fibroblast tissue culture cells) or 10 pg of cloned cDNA (cycling conditions: 3 cycles at 94°C for 2 min and 63°C for 2 min, followed by 27 cycles at 94°C for 30 s, 63°C for 30 s, 72°C for 1 min). Products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. All PCR products generated from genomic DNA were cloned into the pTAG vector (R and D Systems, UK) and subjected to fluorescence-based cycle sequencing.

2.3. Dissection and preparation of rat spinal cords for electrophysiological recording

The method of dissection and preparation is previously described in [15]. Briefly, albino rats weighing 20–30 g were lightly anaesthetized with halothane prior to decapitation. A block of tissue containing the vertebral column was rapidly excised and chilled by immersion in artificial cerebrospinal fluid on ice (ACSF: 118 mM NaCl, 3 mM KCl, 24 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 12 mM glucose; gassed with 95% O_2/5% CO_2 pH 7.4). Under continuous flow of cold ACSF, the cord was exposed from the ventral surface, and the entire length of the cord, complete with intercostal nerves to segments T2, T6 and T12, and all the lumbar roots were dissected free from the vertebral column. The ventral roots were cut and the entire cord was mounted in the recording chamber. The dissection usually took 30–60 min to complete. The recording chamber was continually perfused with oxygenated ACSF at 18–20°C. Three or four lumbar dorsal roots were mounted on silver wire stimulating electrodes. Desiccation of the roots was prevented by covering all exposed tissue with a mixture of petroleum jelly and liquid paraffin.

2.4. Measurement of the effects of human and bovine RFamide peptides on an isolated rat spinal cord preparation

Stimulation with 0.5-ms 5–10-V pulses (5× threshold for each individual preparation) of lumbar dorsal roots through silver wire stimulating electrodes were made whilst perfusing the entire preparation with human or bovine NPAF, human NPSF or bovine NPFF dissolved in ACSF. Recordings were made from lumbar ventral roots using a suction electrode, and a microcomputer recording system based on a multichannel interface unit (Grafitek SP9) and an IBM-PC compatible computer (Tandon PCA).

2.5. Measurement of the effect of human NPAF on spontaneous activity in lumbar dorsal roots

Suction electrodes were attached to lumbar dorsal roots in the same preparation as described above. Recordings of spontaneous extracellular action potentials were made in 30 successive 15-s bins using a spike discriminator, before and during perfusion with human NPAF dissolved in ACSF.

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CATGAAGTCCTGGGGGCGCCATGGGAGGAGATCCCAGGTGGCTCCTAATGAGCCCTGCAT   60

TTCATTTGCCTGCTCTAGATTCCCCTAAGGCTACTGTGAGGCTGGGGGTGGGGGAACAGC  120

AGGTATAAGAGGTTGGGGTGGCTGTAGGAGGGTAGGTGGCAGCATGGATTCTAGGCAGGC  180
                                          M  D  S  R  Q  A    6

TGCTGCACTGCTGGTGCTGCTGCTGTTAATAGACGGGGGCTGTGCTGAAGGGCCAGGAGG  240
  A  A  L  L  V  L  L  L  L  I  D  G  G  C  A  E  G  P  G  G   26

CCAGCAGGAAGACCAGCTCTCCGCGGAGGAAGACAGCGAACCCCTCCCACCACAGGATGC  300
  Q  Q  E  D  Q  L  S  A  E  E  D  S  E  P  L  P  P  Q  D  A   46

CCAGACCTCTGGGTCACTGTTGCACTACCTGCTCCAGGCAATGGAGAGACCTGGCCGGAG  360
  Q  T  S  G  S  L  L  H  Y  L  L  Q  A  M  E  R  P  G  R  S   66

CCAAGCCTTCCTGTTTCAGCCCCAGAGGTTTGGCAGAAATACCCAGGGATCCTGGAGGAA  420
  Q  A  F  L  F  Q  P  Q  R  F  G  R  N  T  Q  G  S  W  R  N   86

TGAATGGCTGAGTCCCCGGGCTGGAGAGGGGCTGAATTCCCAGTTCTGGAGCCTGGCTGC  480
  E  W  L  S  P  R  A  G  E  G  L  N  S  Q  F  W  S  L  A  A  106

CCCTCAACGCTTTGGGAAGAAGTGACATGTCATCCCTTGATATGTCTGCATGCAAGGTCC  540
  P  Q  R  F  G  K  K  *                                      113

ACACCCAAAAGTGTCAATGTTTGCCCCCCAAATAAAATTGTCTGGCTTCTG(A)22      591
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Fig. 1. Primary structure of the human FMRFamide-like peptide precursor. A cDNA clone of 591 bp encoding an 113-residue open reading frame was isolated from a human testis cDNA library. The deduced translation of the cDNA clone revealed structures of two probable FMRFamide-related peptides (underlined). Both peptides are flanked by single or pairs of basic amino acids (bold) which act as endopeptidase cleavage sites. The precursor is preceeded by a hydrophobic leader sequence (residues 1–16) with a predicted cleavage site after Cys-20 (arrow head). Nucleotide and amino-acid (underlined) positions are given on the right.

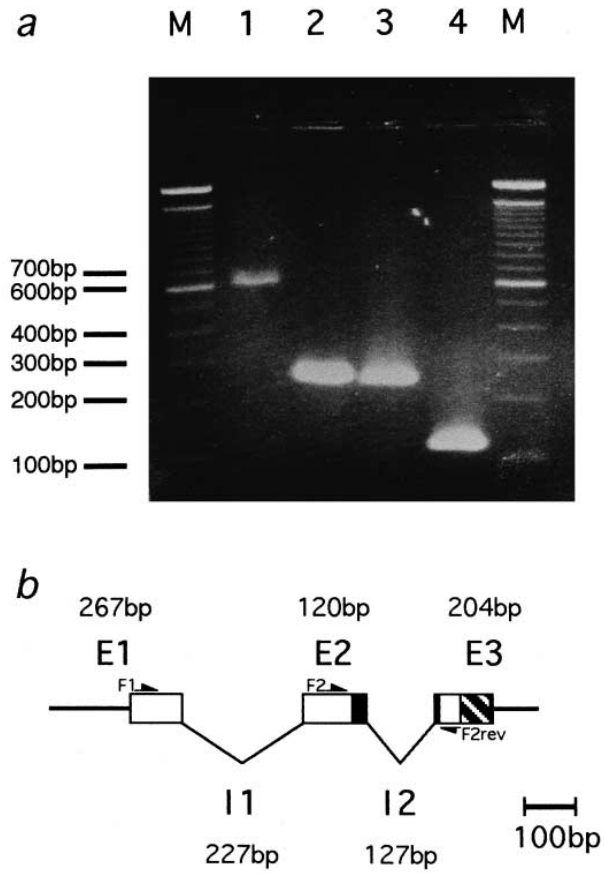
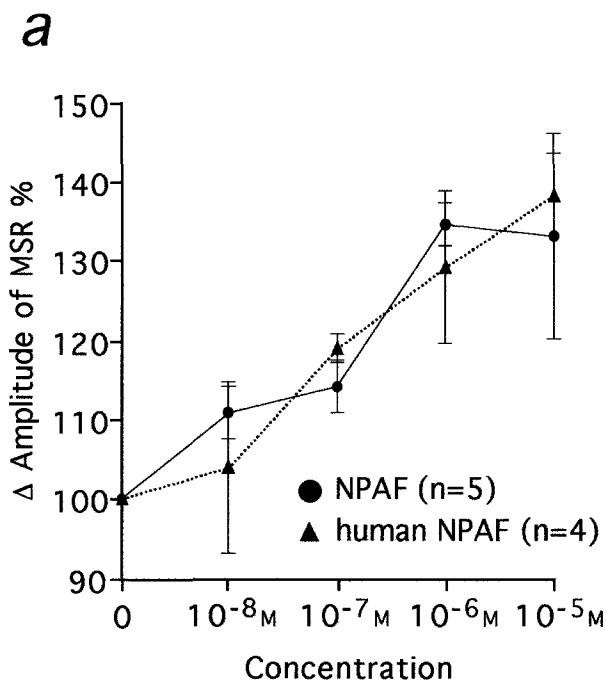


Fig. 2. The genomic organization of the human FMRFamide-like peptide gene. a: PCR products amplified from two primer pairs on both human genomic DNA and an isolated cDNA clone were separated by agarose gel electrophoresis. Primer set F1/F2rev amplified products of 612 bp from genomic DNA (lane 1) and 258 bp from the cDNA sequence (lane 2). Primer set F2/F2rev amplified products of 246 bp from genomic DNA (lane 3) and 120 bp from the cDNA sequence (lane 4). Marker (M) 100-bp ladder (Gibco-BRL). b: PCR products from Fig. a were purified and subjected to automated cycle sequencing. Half arrows represent positions of PCR primers used in Fig. a. The deduced structure of the gene contains 3 exons of 267 bp (E1), 120 bp (E2) and 204 bp (E3) and 2 introns of 227 bp (I1) and 127 bp (I2) within the open reading frame (white bars); the 2nd intron interrupts the shorter predicted peptide sequence, NPSF (black bars). The longer peptide (diagonal striped bar) is encoded entirely by E3.

3. Results

3.1. The human FMRFamide-related peptide gene

The site of synthesis of mammalian FMRFamide-related peptides is not known. In order to identify FMRFamide-related transcripts, a human expressed sequence tag (EST) database was searched for sequences homologous to the bovine peptides. From a screen of >300 000 ESTs, 2 potential positives were identified. Both sequences were found in a cDNA library constructed from human epididymus mRNA. Full-length cDNAs (591 bp) were subsequently isolated by screening a human testis library (Clontech) using the ESTs as hy-



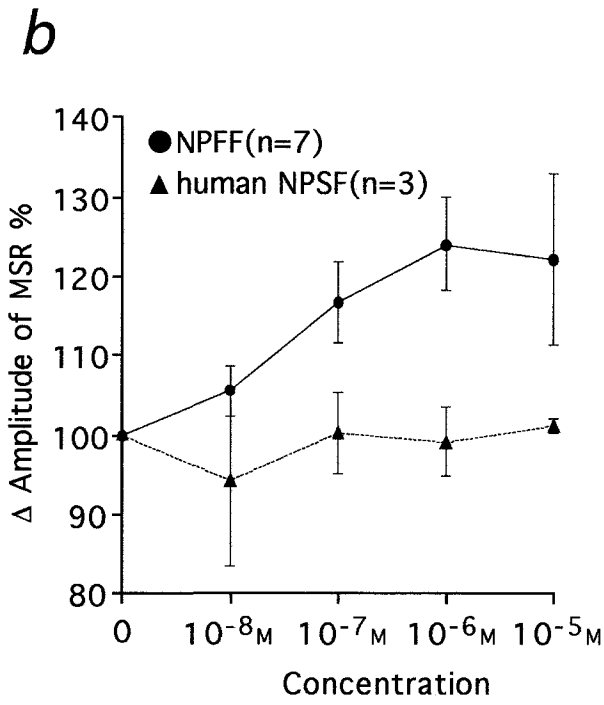


Fig. 3. Comparison of the effects of (a) bovine NPAF vs. human NPAF and (b) bovine NPFF vs. human NPSF. The amplitude of the monosynaptic component of the reflex response evoked in lumbar ventral roots in isolated rat spinal cord preparations was determined in response to dorsal root stimulation. Either three or four dorsal roots were mounted on silver wire stimulating electrodes and recordings were made from ventral roots using a suction electrode.

bridization probes. The sequence of one such clone was determined (Fig. 1). This was found to contain an open reading frame encoding a 113-residue polypeptide (164–502 bp) containing 2 FMRFamide-like peptides both related to biochemically isolated bovine peptides [4]. The 6th amino acid (Ser) of the longer bovine peptide (NPAF) is replaced by Asn in the predicted human sequence, and the 8th amino acid Pro by Gln. Proteolytic processing of the neuropeptide probably occurs by cleavage at Arg-92 and Lys-112 with amidation occurring at Gly-111. The smaller bovine peptide, 8th amino-acid neuropeptide FF (NPFF), appears to be N-terminally extended by 3 amino acids (SQA) in the predicted 11 amino-acid human neuropeptide SF (NPSF). It is likely that this peptide is cleaved from the precursor at Arg-65 and Arg-78; Gly-77 is then available for amidation. As expected for neuropeptide precursors, the predicted precursor contains a hydrophobic leader sequence of 11 amino acids at the N-terminal end, with its predicted cleavage site after Cys-20.

3.2. *Genomic organization of the human FMRFamide-related peptide gene*

PCR amplification of the gene from human genomic DNA was performed using primer pairs designed to the 5′ and 3′ untranslated regions (5′UTR and 3′UTR) of the cDNA clone. A product of ≈1000 nucleotides was obtained, and further PCR analysis with internal primer pairs (F1/F2rev and F2/F2rev) indicated the presence of 2 introns (Fig. 2). DNA sequencing of these genomic fragments confirmed the presence of 2 introns, containing classical 5′ and 3′ splice consensus sequences, at positions 267 bp (I1, 227 bp in length) and 387 bp (I2, 127 bp in length) and branch site sequences. The 2nd intron lies within the sequence encoding the smaller peptide

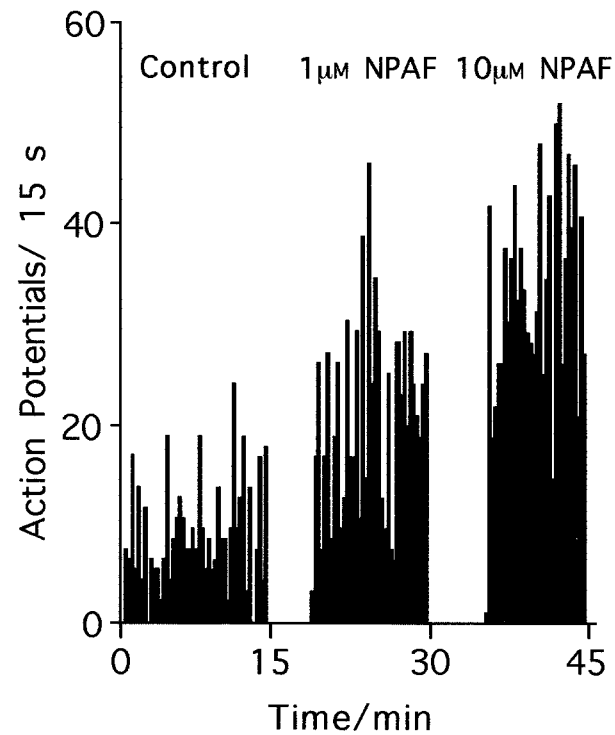


Fig. 4. The effects of human NPAF upon the frequency of spontaneous dorsal root action potentials recorded from an isolated rat spinal cord preparation. Each bar represents the number of action potentials made in 30 successive 15-s time epochs under control conditions (mean 8.4±0.7 S.E.M.) and with 10^{-6} M (mean 20.4±1.4 S.E.M.) and 10^{-5} M (mean 32.4±1.5 S.E.M.) human NPAF dissolved in oxygenated ACSF.

NPSF, the other between the hydrophobic leader and the 1st peptide. Both introns contain translational stop codons in all 3 reading frames, eliminating the possibility of alternative splicing producing different peptides.

3.3. Comparison of the effects of the human and bovine peptides on rat spinal cord activity

Rat NPFF modulates opioid functions via specific receptors localized in the spinal cord and the brain [4,6,9,16]. This biological system was used to test the physiological effects of the 2 novel human peptides. Changes in the amplitude of the monosynaptic component of the reflex response (MSR) evoked in lumbar ventral roots, by dorsal root stimulation in response to perfusion with the peptides were recorded (Fig. 3). Rat spinal cord preparations were perfused with bovine and human RFamide peptides at concentrations between 10 nM and 10 μM. The amplitude of the MSR was shown to increase significantly on application of bovine and human NPAF at concentrations of >10 nM, in a dose-dependent manner (Fig. 3b). The effect on the MSR by the human peptide was indistinguishable from that of the bovine peptide. Bovine NPFF also caused a significant increase in the amplitude of the MSR in a dose-dependent manner although this effect was generally weaker than that of the bovine and human NPAF peptides. However, human NPSF showed no effect on the amplitude of the MSR at any of the concentrations tested (Fig. 3a).

3.4. Effect of human NPAF on spontaneous activity in lumbar dorsal roots

Spontaneous action potentials are detectable within the lumbar dorsal roots of isolated rat spinal cords. In order to determine whether human NPAF effects the level of this spontaneous activity, the frequency of the action potentials during perfusion with human NPAF, were recorded. A dose-dependent increase in the frequency at concentrations of 1 μM (20.4 ± 1.4 S.E.M.) and 10 μM (32.4 ± 1.5 S.E.M.) from the basal level of activity (8.4 ± 0.7 S.E.M.) was observed (Fig. 4).

4. Discussion

The cloning of the human FMRFamide-related peptide gene has revealed the structures of 2 novel neuropeptides. Their structures differ from those already known from bovines [4] by the substitutions of 2 amino acids in the longer peptide, NPAF, and the N-terminal extension by 3 residues of the shorter peptide, NPFF (human NPSF). Two introns within the gene were identified by PCR from genomic DNA using primer sites present within the cDNA sequence. Neither intron contained open reading frames or splice sites other than those used to produce the transcript identified from the isolated cDNA clone. This eliminated the possibility of alternative transcripts being produced that may encode peptides of different sequences, as is the case in some molluscan FMRFamide genes [17], as failure to remove either of the introns would produce truncated prepropeptides.

The sequence similarity of human NPAF and NPSF to those isolated from bovines and rats, suggested that the novel human peptides may have similar functions in modulation of nociception in the spinal cord. To test this, their effects on the dorsal root–ventral root reflex response (DR-VRR) were compared to bovine NPAF and NPFF. As can be seen in Fig. 3a, the monosynaptic component of the ventral root reflex response evoked by dorsal root stimulation increased in amplitude with the application of human NPAF. This increase paralleled that observed with the bovine peptide demonstrating that the physiological consequences of the change in the 2 amino acids that differentiate the human from bovine peptide are not significant. Human NPAF peptide also produced a dose-dependent increase in spontaneous extracellular action potentials in lumbar dorsal roots at concentrations of 1 and 10 μM. The importance of these observations is that the 2 NPAF peptides appear to increase neuronal activity in regions known to be involved in nociception. The change in the DR-VRR indicates that reflex activity in the spinal cord is enhanced, and the spontaneous dorsal root activity is known to originate in the superficial layers of the dorsal horn [18], an area associated with the processing of nociceptive signals. This supports previous data indicating that i.v. injection of FMRFamide-related peptides modulates morphine-induced analgesia [4]. In contrast, the shorter human peptide NPSF has no effect on the amplitude of the monosynaptic component of the ventral root reflex response (Fig. 3b) although a clear increase in amplitude is seen with the addition of the bovine NPFF. The sequence of human NPSF is identical to bovine NPFF, except for the N-terminal extension of 3 amino acids. These data suggest that either the human peptide is inactive and requires proteolytic cleavage to activate it, or the rat NPFF receptor does not respond to the human peptide. Convincing HPLC and RIA data suggests that a peptide with the 3 N-terminal acids deleted exists at low concentrations in human serum [5]. The arginine at position 65 (Fig. 1) is most likely a cleavage site for a neuropeptide precursor-processing enzyme, and the removal of the 3 most N-terminal amino acids to produce the functional 8-residue peptide NPFF may occur by the action of an aminopeptidase. Such N-terminal trimming by a tripeptidyl aminopeptidase is observed in the metabolism of CCK-8 [19] and by the endopeptidase neprilysin in the nematode peptide AF1 in *Ascaris suum* [20]. In the latter example, it is striking that cleavage of this FMRFamide-related peptide specifically precedes a phenylalanine residue as would also be the case during maturation of human NPSF to NPFF. Alternatively, a propeptide-processing enzyme family, the dipeptidylaminopeptidases (DPAP), exist in many species (reviewed in [21]). These enzymes specifically cleave amino-acid pairs with the structure X-Ala or X-Pro from the N-termini of propeptides. Such an amino-acid pair exists at positions 2 and 3 of the predicted human NPSF peptide, and thus could be removed from the 11-mer peptide to form the biologically active 8-mer NPFF. This processing event, however, would require the previous removal of Ser at position 1 to allow the action of a DPAP enzyme. Such N-terminal 'clipping' of neuropeptides has been described elsewhere [22] and, in conjunction with DPAP activity, could be responsible for this processing.

The biological effects of vertebrate RFamide peptides include the regulation of heart rate and blood pressure [7] and the modulation of morphine-induced antinociception [4,6]. The association of these neuropeptides with the vertebrate male reproductive system has not previously been reported although FMRFamide is known to function in the molluscan reproductive organs [23]. It has been speculated that NPFF detected in human serum is the result of neural 'leakage' [24], however, the production of FMRFamide-like peptides by the

testis and their subsequent release into the circulatory system seems a more plausible explanation.

The first FMRFamide-like peptides to be discovered were in molluscs where they were identified by their biological activity on the isolated clam heart [1]. It is interesting to note that the molluscan FMRFamide peptides show a similar effect on molluscan heart rate as they do on the vertebrate heart, and that the vertebrate FMRFamide-like peptides also increase heart rate. Such conservation of biological activity suggests that the control of heart rate in both vertebrates and invertebrates is a process in which FMRFamide peptides play an important role.

The isolation of the human gene encoding 2 FMRFamide-like neuropeptides, described here, will greatly help advance the study of this important group of vertebrate peptides. We have shown the predicted peptide structures to be very similar to previously isolated peptides from bovines and rat. One of the peptides, NPAF, has indistinguishable activity on rat spinal cord as its bovine homologue while NPSF appears to be wholly inactive. These findings will allow the extrapolation of results using rat and bovine peptides in physiological studies to their probable effects in humans.

Note added in proof: The cDNA sequence is available on GenBank, accession no. AF005271.

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