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Identification of Urotensin II as the Endogenous Ligand for the Orphan G-Protein-Coupled Receptor GPR14

Qingyun Liu,*[,1] Sheng-Shung Pong,† Zhizhen Zeng,* Qing Zhang,* Andrew D. Howard,† David L. Williams Jr.,‡ Michael Davidoff,* Ruiping Wang,* Christopher P. Austin,* Terrence P. McDonald,* Chang Bai,* Susan R. George,§ Jilly F. Evans,* and C. Thomas Caskey*

**Department of Human Genetics, Merck Research Laboratories, WP26A-3000, West Point, Pennsylvania 19486; †Department of Metabolic Disorders, Merck Research Laboratories, RY80Y-265, P.O. Box 2000, Rahway, New Jersey 07065; ‡Department of Pharmacology, Merck Research Laboratories, WP46-300, West Point, Pennsylvania 19486; and §Departments of Pharmacology and Medicine, University of Toronto, Medical Sciences Building, Toronto, Ontario M5S 1A8, Canada*

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Urotensin II (UII) is a neuropeptide with potent cardiovascular effects. Its sequence is strongly conserved among different species and has structural similarity to somatostatin. No receptor for UII has been molecularly identified from any species so far. GPR14 was cloned as an orphan G protein-coupled receptor with similarity to members of the somatostatin/opioid receptor family. We have now demonstrated that GPR14 is a high affinity receptor for UII and designate it UII-R1a. HEK293 cells and COS-7 cells transfected with rat GPR14 showed strong, dose-dependent calcium mobilization in response to fish, frog, and human UII. Radioligand binding analysis showed high affinity binding of UII to membrane preparations isolated from HEK293 cells stably expressing rat GPR14. *In situ* hybridization analysis showed that GPR14 was expressed in motor neurons of the spinal cord, smooth muscle cells of the bladder, and muscle cells of the heart. The identification of the first receptor for UII will allow better understanding of the physiological and pharmacological roles of UII.

Urotensin II (UII) is a cyclic 12-amino acid residue peptide first isolated from the urophysis of the caudal neurosecretory system of the teleost fish goby, *Gillichthys mirabilis* (1), and then from various other teleost fish species. Sequence analysis of the UII peptides revealed that C-terminal cyclic hexapeptide has been perfectly conserved whereas the N-terminal sequences are highly variable (2–5). The cyclic hexapeptide of UII: -Cys-Phe-Trp-Lys-Tyr-Cys- is structurally similar to the biologically important region of somatostatin-14: -Phe-Trp-Lys-Thr- (1, 6). However, molecular cloning and sequence analysis of the carp preprourotensin II gene has demonstrated that UII and somatostatin are not derived from the same ancestor (7).

UII was long regarded as a product only found in the teleost urophysis. Recent studies, however, have shown that UII also exists in other species of fish and even in tetrapods (5, 8, 9). A gene encoding the putative human UII has been cloned and characterized (10). The predicted human UII peptide contains only 11 amino acid residues with a cyclic hexapeptide completely identical to those found in fish and frog UIIs (10). Both the human and frog genes are abundantly expressed in the motor neurons of spinal cord (10).

Even though the existence of UII in tetrapods was accepted only recently, fish UII has been shown to have various physiological and pharmacological effects in mammals. Injection of goby UII into rats resulted in a sustained fall in arterial blood pressure without change in heart rate (11, 12). UII was also shown to be capable of developing relaxations of carbachol-induced tone of the mouse anococcygeus muscle (13). In addition, specific binding sites for UII have been characterized in rat blood vessels (14). However, no receptor for UII has been cloned from any species.

GPR14 was first cloned from rat as an orphan G protein coupled receptor (15). It was also cloned from rat circumvallate papillae cells and called SENR (sensory epithelia neuropeptide-like receptor) (16). The predicted protein sequence of rat GPR14/SENR is most similar to members of the somatostatin and opioid receptors with an overall identity of 27% to the somatostatin receptor SSTR4. RNase protection analysis showed the gene was most abundantly expressed in neural and sensory tissues including retina, heart muscle, olfactory epithelium, cerebellum, and choroid

[1] To whom correspondence should be addressed. Fax: (215)652-2075. E-mail: Jim_liu@merck.com.

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plexus (16). However, no natural ligand for GPR14 has been reported. Here we show that UII is an endogenous ligand of GPR14 and, therefore designate GPR14 the UII-R1a receptor.

EXPERIMENTAL PROCEDURES

Plasmid construction. The complete coding sequence of rat GPR14 (Genbank Accession No. U32673) was amplified by PCR from GPR14 plasmid DNA (15) using these two primers: forward primer-5′-CTCCTGCGGCCGCGCCACCATGGACAACGCCTCGTTCTCTCG-3′ and reverse primer-5′-CTCGGGATCCAGGAGTGGCGCGCAGTT-GCGGAGCC-3′. The forward primer contained an *Not*I site and consensus Kozak (GCCACC) sequence for translation immediately upstream of the initiation codon. The reverse primer contained a *Bam*HI site downstream of the stop codon. PCR reactions were carried out using the DNA polymerase PFU Turbo (Stratagene) following the conditions suggested by the enzyme supplier. The PCR product was purified, digested with *Not*I plus *Bam*HI, and subcloned into the vector pIRESpuromycin (Clontech). Clones containing GPR14 in the correct construct were verified by complete sequencing of the GPR14 insert to ensure the lack of PCR-introduced errors.

Generation of GPR14-expressing cells. HEK 293 cells stably expressing the jellyfish protein aequorin (HEK293/aeq17) (17) were transfected with plasmid DNA of rat GPR14-pIRESpuromycin using Lipofectamine (GIBOR-BRL). Three days after transfection, the cells were trypsinized and plated out by 1:5 dilution in complete culture medium plus puromycin at 0.5 μg/ml. Cells were incubated at 37°C/5% CO_2 and changed to fresh medium twice per week. Two weeks after transfection, puromycin-resistant cells were trypsinized, pooled, and assayed.

Aequorin assay. Aequorin bioluminescence assays were performed as described previously with minor modification (17). Cells at 80–90% confluency were charged with 8 μM coelenterazine for one hour. The cells were then washed once with versene, detached using enzyme-free dissociation buffer (GIBCO-BRL), and resuspended in ECB (Ham's F12 medium plus 0.3 mM $CaCl_2$, 0.1% fetal bovine serum, 25 mM HEPES, pH 7.3). The cells were then washed once with ECB and resuspended in ECB at a density of ~500,000 cells/ml. Goby fish and frog (*Rana ridibunda*) UII peptides were purchased from Peninsula Laboratories. Human UII peptide was custom-synthesized by Phoenix Pharmaceuticals. Ligands were prepared in ECB as 2× concentrate and dispensed into 96-well assay plates at 0.1 ml/well. Cells in suspension were injected at 0.1 ml per well and light emission was read and integrated for 20 seconds immediately after injection of cells using a Dynex MLX luminometer.

FLIPR assay. COS-7 cells were transiently transfected with rat GPR14-pIRESpuromycin using Lipofectamine-2000 (GIBCO-BRL). Two days after transfection, cells were detached with enzyme-free dissociation buffer and seeded into 96-well plates at ~15,000 cells/well. The next day, cells were loaded with Fluo-3 in the presence of 2.5 mM probenicid. After washing, the cells were treated with varying concentrations of UII. Fluorescence output was measured by a Fluorometric Imaging Plate Reader (FLIPR, Molecular Devices) and normalized to the amount of fluorescence in the presence of the calcium ionophore A23187.

Cell membrane preparation. HEK-293/aeq17 cells stably expressing rat GPR14 (3 T-175 tissue culture flasks, ~3 × 10^8 cells) were harvested by scraping, washed once in 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, and centrifuged at 2,000× g for 15 min. All procedures were conducted on ice. Cell pellets were homogenized in a tissue grinder with a PTFE pestle (25 strokes). Crude cell membranes were then isolated by centrifugation of the cell lysate at 13,000× g for 30 min. Membrane pellets were resuspended at a protein concentration of 2.8 mg/ml in 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$.

Radioligand binding. Frog UII peptide (*Rana ridibunda*) was labeled at the N-terminal NH_3^+ group of the Ala residue (Woods Assay, Portland, OR) to a specific activity of ~2,000 Ci/mmol. The binding solution (0.5 ml in 12 × 75 mm borosilicate glass tubes) contained 46 μg GPR14 expressing cell membrane, 0.1 nM ^{125}I-UII in 25 mM Tris-HCl, pH 7.4, buffer with 2 mM EDTA, 10 mM $MgCl_2$ and 100 μg/ml bacitracin. After incubation for 1 hour at room temperature, binding reactions were filtered through GF/C filters (Whatman; presoaked for 1 hour in 1% polyethylenimine) on a 48-well cell harvester (Brandel), washed 3 × 3 ml with ice-cold 50 mM Tris-HCl, pH 7.4, buffer with 10 mM $MgCl_2$ and 0.02% Triton X-100. Radioactivity on the filters was quantitated by gamma counting.

Northern analysis. Human multi-muscle tissue Northern blot was purchased from Clontech. The entire coding sequence of rat GPR14 was radio-labeled using [α-^{32}P]dCTP (ReadyPrime kit, Amersham). Hybridizations were carried out in ExpressHyb buffer (Clontech) overnight at 60°C. The blot was then washed at moderate stringency (30 min at 45°C in 0.2× SSC, 0.1% SDS) and exposed to X-ray film for one week.

In situ hybridization analysis. A 500 bp fragment covering the 3′ half of the rat GPR14 coding region was generated by PCR using primers anchored with a T7 promoter sequence (the sense primer) and a T3 promoter sequence (the antisense primer). Sense and antisense probes were prepared using DIG RNA Labeling Kit from Boehringer Mannheim. Tissue sections were prepared from a mouse perfused with 4% paraformaldehyde/PBS before the tissues were harvested. Slides were hybridized overnight at 65°C with digoxigenin-labeled probe at a final concentration of 2 pmol/ml in Hybridization Cocktail buffer from AMRESCO. After incubation, the slides were rinsed in 1× SSC, treated with RNase A (20 μg/ml) for 30 min at 37°C, and then washed 3 × 15 min in 50% formamide/2× SSC at 65°C, 1 × 10 min in 0.2× SSC at 70°C, and 2 × 15 min in TBST buffer (Sigma) at room temperature. For immunocytochemical detection of digoxigenin-labeled probes, slides were treated with blocking solution containing 2% normal rabbit serum (NRC) and Avidin D (Vector) in TBST. Slides were washed 3 × 10 min in TBST and incubated in 1:2500 sheep anti-digoxigenin antibody, 1% NRC, and biotin in TBST overnight at 4°C. After washing 3 × 15 min in TBST, slides were incubated in 1:2000 biotin-SP-conjugated rabbit anti-sheep IgG F(ab′)$_2$ fragment specific (Jackson ImmunoResearch) in 1% NRC in TBST for 2 hours at room temperature. Detection was performed with Vectastain *Elite* ABC Kit (Vector), TSA–Indirect (ISH) Kit (NEN Life Science Products, Inc.), and DAB Substrate Kit for Peroxidase (Vector) following the manufacturer's protocols.

RESULTS AND DISCUSSION

Activation of GPR14 by UII leading to calcium mobilization. The complete coding sequence of rat GPR14 was subcloned into the vector pIRESpuromycin and transfected into HEK293/aeq17 cells. A pool of drug resistant colonies (bulk stables) were collected and used for assays. The majority of cells from such bulk stables were expected to express the receptor gene since most drug-resistant cells also express the gene inserted upstream of the drug resistance gene (18). Northern analysis of RNA isolated from the bulk stables showed a strong expression of GPR14 while untransfected HEK293/aeq17 cell showed no signal (data not shown).

The aequorin assay is a sensitive method to measure Ca^{2+} mobilization based on bioluminescence of jelly fish aequorin upon Ca^{2+} binding (17). In the initial screening, we found that cells expressing GPR14 responded to fish and frog UII (data not shown).

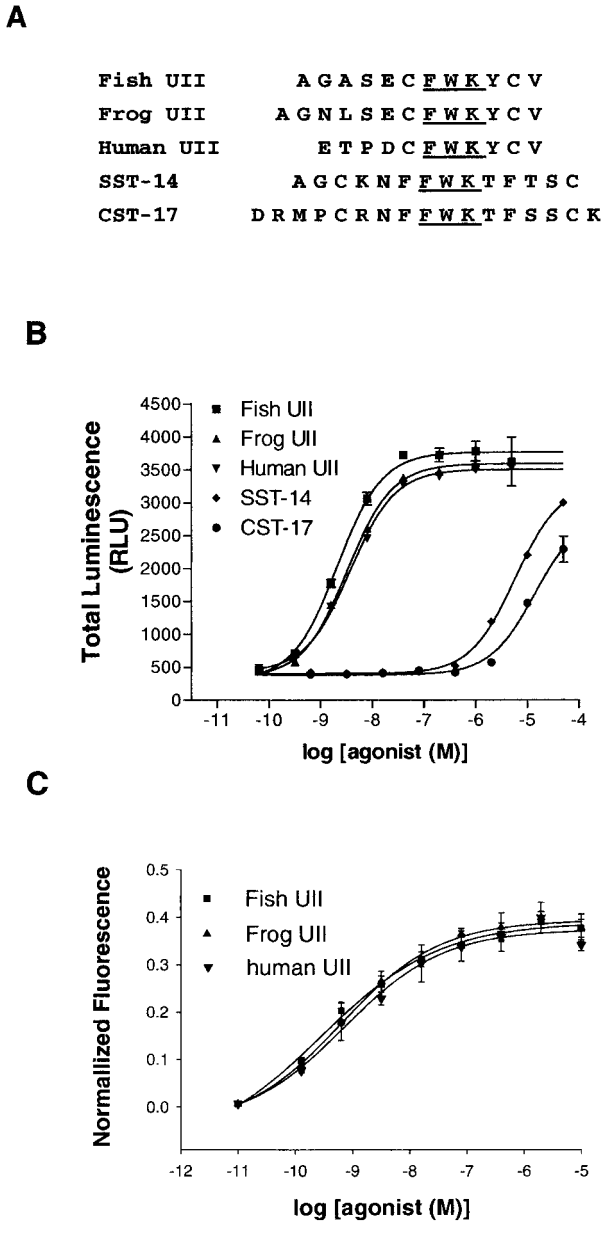

FIG. 1. Activation of GPR14 by urotensin II (UII) and somatostatin. (A) Sequence alignment of UII, somatostatin-14 (SST-14), and human cortistatin-17 (human CST-17). The shared tri-amino acid motif –Phe-Trp-Lys- (FWK) is underlined. (B) Activation of GPR14 by UII in the aequorin assay. HEK293/aeq17 cells stably expressing rat GPR14 were assayed against 5-fold serial dilutions of UII peptides, somatostatin, and cortistatin-17, as described under Experimental Procedures. (C) Activation of GPR14 by UII in the FLIPR assay. COS-7 cells transiently transfected with rat GPR14 were assayed against 5-fold serial dilutions of UII peptides, as described under Experimental Procedures. Both results are shown as the means (±SEM) of triplicate determinations.

HEK293/aeq17 cells expressing rat GPR14 showed a robust, dose-dependent response to fish, frog, and human UII peptides (Fig. 1B). The effective half maximum concentration (EC_{50}) of fish, frog, and human UII in these cells are 5.0, 5.6, 7.2 nM, respectively, indicating that UII is a high affinity ligand for GPR14. Cells that were mock-transfected (vector only) or transfected with plasmids expressing other G-protein coupled receptors cloned into the same vector did not show any response to UII (data not shown). Thus, the data indicate that UII of fish, frog, and human can activate GPR14 with approximately equal potency, most likely through the Gαq/11 pathway. Furthermore, we also tested whether GPR14 can be activated by somatostatin and cortistatin given the structural similarity between the two peptides and UII (Fig. 1A). As shown in Fig. 1B, GPR14 can also be activated by both somatostatin and cortistatin but at much lower efficacy. The EC_{50} of somatostatin and cortistatin is estimated to be more than 1 μM.

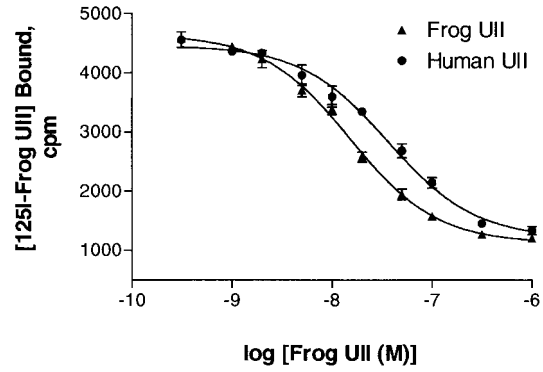

FIG. 2. Radio-ligand binding analysis of ^{125}I-labeled frog UII. Cellular membranes were prepared from HEK293/aeq17 cells stably expressing rat GPR14. Competition analysis was carried out in the presence of 0.1 nM [^{125}I] frog UII. Binding data were analyzed by a nonlinear curve-fitting program (Prism, version 2.0; GraphPad Software, San Diego, CA). Results shown are the means (±SEM) of triplicate determinations.

To confirm that UII causes Ca^{2+} mobilization in cells expressing GPR14, COS-7 cells were transiently transfected with rat GPR14/pIRESpuromycin and then assayed by FLIPR (Fluorometric Imaging Plate Reader)

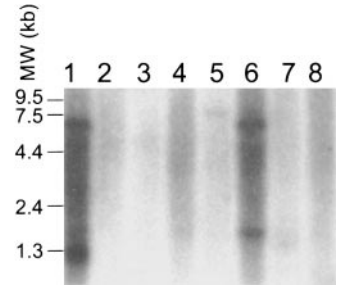

FIG. 3. Multi-tissue Northern analysis of UII-R1a. Human multi-tissue blot was probed with the entire coding sequence of rat UII-R1a. Lanes 1, skeletal muscle; 2, uterus (no endometrium); 3, colon (no mucosa); 4, small intestine; 5, bladder; 6, heart; 7, stomach; 8, prostate. The high background is probably due to the fact that only moderate stringency can be used in washing since the human mRNA blot was probed by a rat gene.

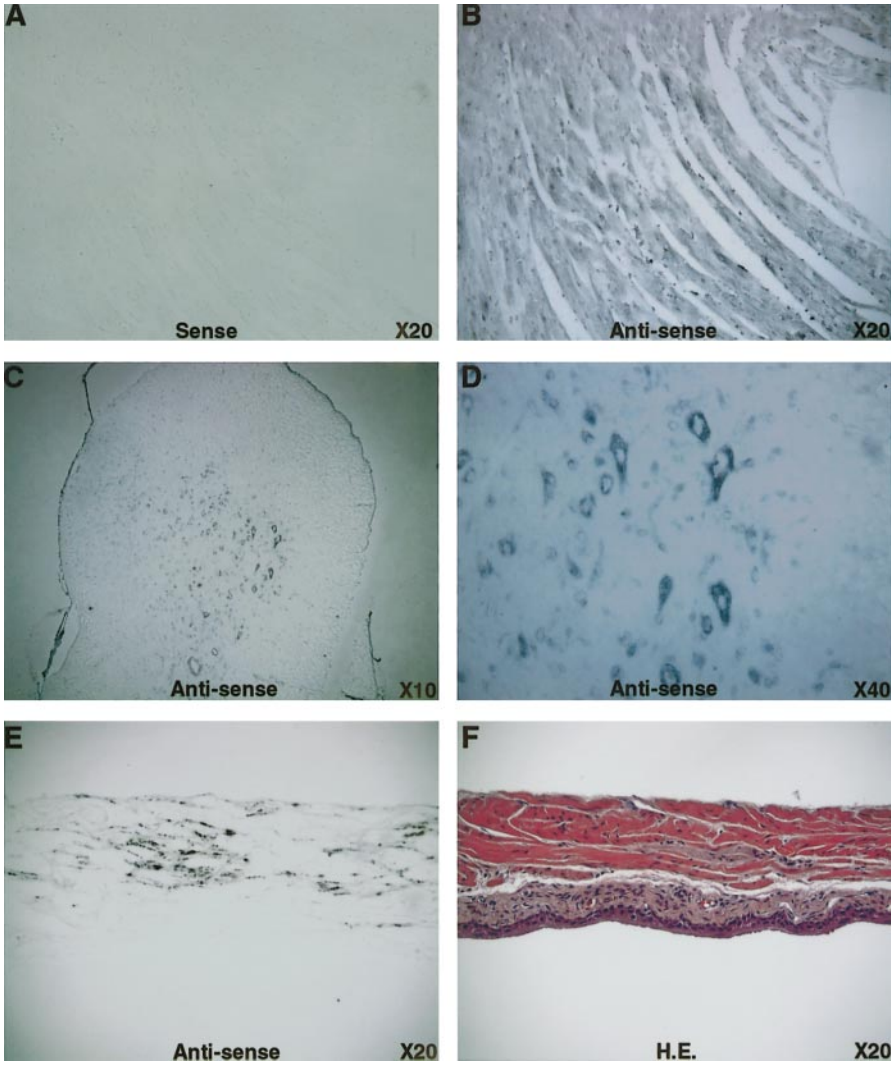

FIG. 4. *In situ* hybridization analysis of UII-R1a expression in the mouse. Sense and antisense riboprobes were prepared from the 3′ half of rat GPR14 coding sequence. (A) Hybridization by the sense probe in the heart. (B) Hybridization by the antisense probe in the heart. Specific hybridization is detected by the antisense probe in cardiac myocytes. (C) Hybridization by the antisense probe in the spinal cord, showing expression in the anterior horn of the grey matter. (D) Higher magnification (×40) of (C) showing the expression in motor neurons. (E) Hybridization of the antisense probe in the bladder, showing expression in the smooth muscle layer. (F) H.E. (Hematoxylin–Eosin) staining showing the different layers (from top to bottom: smooth muscle, loose connective tissue, and epithelium) of the bladder. UII-R1a transcripts were detected only in the smooth muscle layer.

which monitors changes of intracellular calcium concentration in real time. Cells transfected with GPR14 showed a dose-dependent response to fish, frog, and human UII (Fig. 1C). The EC_{50} of fish, frog, and human UII is 0.28, 0.63, 0.72 nM, respectively, confirming that UII is a natural ligand of GPR14.

Binding of UII to GPR14. To Further confirm that UII is a high affinity ligand of GPR14, radioligand binding assays were performed. ^{125}I-labeled frog UII was prepared and competition binding analysis was performed using cellular membranes prepared from HEK293/aeq17 cells stably transfected with GPR14. Both frog UII (IC_{50} = 15 nM) and human UII (IC_{50} = 36 nM) displayed high affinity binding to GPR14-transfected cells (Fig. 2). However, the apparent affinity determined by the binding assay is somewhat lower than what was observed in the functional assays, probably due to the incorporation of the ^{125}I-atom at the N-terminus. Current efforts are in progress to synthesize a more efficacious radioligand. Based on the data shown above, it is concluded that GPR14 is a receptor for UII and therefore designated UII-R1a.

Expression pattern of GPR14. Previously GPR14 in the rat was shown to be expressed in the olfactory epithelium, retina, cerebellum, choroid plexus, and cardiac muscle by RNase protection assay (16). Since the UII peptide has been shown to be active in muscle contraction assays, we surveyed the expression of UII-R1a in various

muscle tissues. Relative strong expression was observed in skeletal muscle, bladder, and heart (Fig. 3). Observation of different transcript sizes in various tissues suggests that UII-R1a is alternatively spliced (Fig. 3).

To determine the cellular localization of UII-R1a transcripts, *in situ* hybridization analysis was carried out in various tissues of the mouse using the rat UII-R1a gene as probe. In the heart, UII-R1a was expressed only in the cardiac myocytes (Fig. 4B), which may account for the hypotensive effect of UII observed previously (11, 12). In the spinal cord, UII-R1a transcripts were detected in the anterior horn throughout the grey matter (Fig. 4C), with particularly strong expression in cells with motor neuron morphology (Fig. 4D). Thus, both the UII ligand and receptor are expressed in motor neurons of the spinal cord, suggesting that UII-R1a may function as an autoreceptor in motor neurons. In the bladder, UII-R1a was detected only in the smooth muscle layer (Fig. 4E). Since UII was previously shown to cause spasmogenic actions on frog bladder smooth muscle (19), it is likely that UII is spasmogenic in the mammalian bladders as well. In each *in situ* analysis, no specific signal was detected using the sense probe of the same fragment with one example shown in Fig. 4A.

In conclusion, we have demonstrated that GPR14 is a receptor for UII and designated it UII-R1a. The receptor most likely couples to the Gαq/11 pathway since activation by UII leads to strong calcium mobilization. Human, fish, and frog UII displayed almost equal potency on the UII-R1a receptor, indicating the cyclic hexapeptide at the C-terminus is critical for the function of UII. Interestingly, the UII ligand is similar in structure to somatostatin and GPR14/UII-R1a is most similar to members of the somatostatin receptor family. For somatostatin receptors, the Asp residue in transmembrane domain-3 interacts directly with Lys-9 of somatostatin-14 (20, 21). Since GPR14 also has an Asp residue at the same position of transmembrane domain-3, it is predicted that this Asp residue interacts directly with Lys-8 of human UII. The identification and characterization of the first mammalian receptor for UII adds further credence to the existence of the peptide UII in mammals and will facilitate further studies of the physiology and pharmacology of this neuropeptide in mammals and other species.

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Note added in proof. During the preparation of the manuscript, two reports (Ames *et al.* (1999) *Nature* **401,** 282–286; Nothacker *et al.* (1999) *Nature Cell Biol.* **1,** 383–385) were published which described the identification of urotensin II as the ligand of GPR14. Our data independently confirmed those results.

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