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Urotensin II Is the Endogenous Ligand of a G-Protein-Coupled Orphan Receptor, SENR (GPR14)

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Two molecular species of urotensin II (UII) were isolated from porcine spinal cords and identified as the endogenous ligands of a G-protein-coupled orphan receptor, SENR (sensory epithelium neuropeptide-like receptor), which is identical to GPR14. We established a CHO cell line stably expressing the rat SENR and investigated several tissue extracts to evoke the response mediated by the SENR. Extract from porcine spinal cords showed an activity of arachidonic acid metabolites release from SENR-expressing cells and was purified using HPLC. Two active substances were isolated and their sequences were determined as GPTSECFWKYCV and GPPSECFWKYCV, which were revealed to be porcine UII. Synthetic UII peptides caused arachidonic acid metabolites release activity in the rat SENR-expressing cells with an EC_{50} value of 1 nM. Three cDNAs encoding the precursor proteins of porcine UII were cloned from a porcine spinal cord cDNA library; 2 consist of 121 amino acid residues and the other, which seemed to be a splicing variant, consist of 85 residues.

Many orphan G-protein-coupled receptors (GPCRs), which are characterized by their seven membrane-spanning regions, were recently identified as the result of rapid progress in genetic analysis technologies and an exponential increase in genomic information. Their unidentified endogenous ligands are potential targets for innovative drug discovery research since they may be involved in biological events that have yet to be investigated. This so-called "reverse pharmacology" [1] approach has yielded valuable data and is one of the most interesting techniques in current pharmacogenomics studies. Some ligands of orphan GPCRs, which play a significant physiological role in various biological systems, have been discovered by several laboratories [2–6]. Recently, motilin [7] and melanin-concentrating hormone (MCH) [8–12] were identified as the endogenous ligands of the orphan receptors, GPR38 and SLC-1, respectively. While these peptides were previously known from their biological activity, their receptor was not known. MCH is considered an important peptide in obesity, and mice lacking the MCH gene show a phenotype of leanness due to hypophagia and an increased metabolic rate [13]. The identification of MCH as the ligand of the SLC-1 receptor was independently, and almost simultaneously reported by 5 groups, including our laboratory [8–12].

SENR (sensory epithelium neuropeptide-like receptor), or GPR14, is an orphan GPCR which was obtained from the rat genome by Tal *et al.* [14] and Marchese *et al.* [15], independently. This receptor showed homology to somatostatin receptor subtype 4 and to the κ, δ, or μ opioid receptors [15]. RNase protection analysis demonstrated that the mRNA of the receptor is expressed in neural and sensory tissues such as the retina, circumvallate papillae, and olfactory epithelium [14]. We established a CHO cell line to express the rat SENR to investigate the ligand in several tissue extracts which could evoke secondary signaling in the cells. We found that the peptide fraction prepared from porcine spinal cord caused a release of arachidonic acid metabolites from SENR-expressing CHO cells. This finding allowed us to isolate and identify urotensin II (UII) as the endogenous ligand of the SENR.

MATERIALS AND METHODS

Preparation of the CHO cell line expressing the rat SENR. Rat SENR cDNA was obtained using PCR from the reverse-transcriptase product of the poly $(A)^+$ RNA derived from rat whole brain extract (Clontech) as the template using the primers 5′-GTCGACATGGCT-

Abbreviations used: UII, urotensin II; SENR, sensory epithelium neuropeptide-like receptor; EC_{50}, median effective concentration; GPCR, G-protein-coupled receptor; TFA, trifluoroacetic acid.

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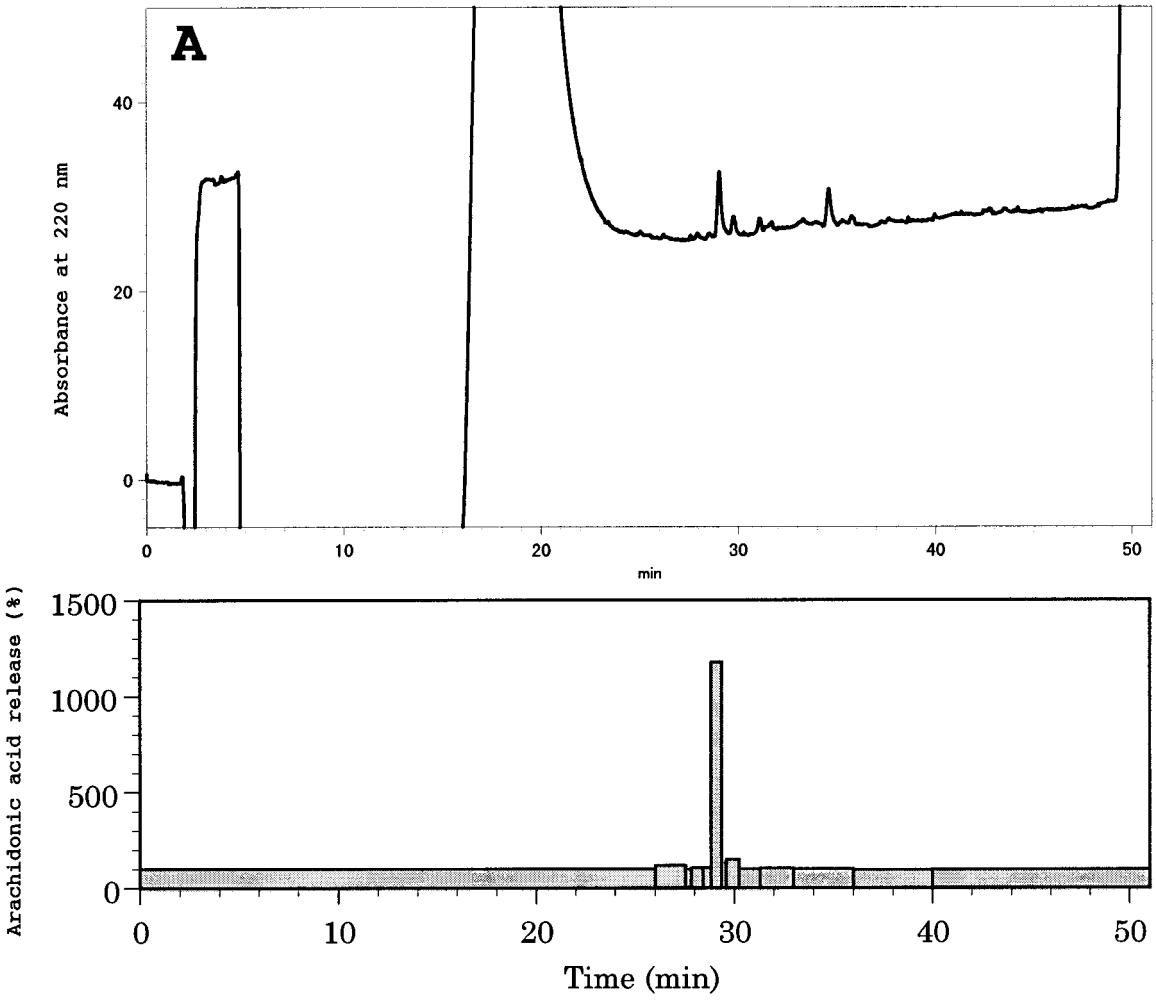

FIG. 1. HPLC profiles of the final purification step on a semi-microbore C-18 column (Wakosil-II 3C18HG). The eluate was manually fractionated. The activity was recovered as peaks at 32.2% (purified from fr. 31, A) or 32.5% (purified from fr. 32, B) of acetonitrile.

CTGAGCCTGGAGTCTACAAC-3′ and 5′-ACTAGTATTGCACAGT-GCACTCTCAGAGAAGG-3′, which were designed to amplify the full coding region of the rat SENR cDNA [14] and to add extension sequences at the 5′- and 3′-ends which were recognized by *Sal*I and *Spe*I, respectively. The 945th nucleotide from the initiation codon was reported to be G in GPR14 [17], although it was C in the cDNA amplified in the present study and in the reported sequence of the SENR [16]. The rat SENR cDNA was then introduced into an expression vector plasmid, pAKKO-111H [16], which equipped the SRα promoter and *dhfr* gene as the selection marker, using the *Sal*I and *Spe*I sites. The constructed vector was then transfected to CHO (dhfr$^-$) cells by the calcium phosphate method using a CellPhect transfection kit (Amersham Pharmacia Biotech). The CHO cells expressing the rat SENR receptor were selected and established using a selection medium deficient in nucleotides.

Assay for arachidonic acid metabolites release. CHO cells expressing the rat SENR were plated on 24-well plates at 5×10^4 cell/well and incubated for 24 h and 9.25 kBq/well of [^{3}H]-arachidonic acid (NEN Life Science Products) was incorporated into the cells. After incubation for 16 h, the cells were washed with Hank's buffered salt solution (HBSS) supplemented with 0.05% bovine serum albumin (BSA) and were exposed to the sample dissolved in 500 μl of HBSS supplemented with 0.05% BSA. After incubation for 30 min, 350 μl of culture supernatant was mixed with scintillation cocktail and the released radioactivity was measured using a scintillation counter.

Extraction and isolation of the endogenous ligand of the rat SENR receptor from the porcine spinal cord. One kilogram of spinal cords from 50 pigs obtained from a slaughterhouse were homogenized in 10 L of 70% acetone containing 1.0 M acetic acid and 20 mM HCl on the same day in which the animals were sacrificed. The homogenate was centrifuged to obtain the supernatant and 10 L of 70% acetone containing 1.0 M acetic acid and 20 mM HCl was added to the precipitates. The latter mixture was stirred overnight and centrifuged. The supernatants obtained were combined and concentrated to remove the solvent, and were then extracted with diethylether to eliminate the lipids. The solution was then applied to a C-18 column (YMCgel ODS-AM 120-S50, 30 × 240 mm (YMC)) and the column was eluted with 500 ml of 60% acetonitrile in 0.1% TFA. The eluate was concentrated and lyophilized and the lyophilized powder (*ca.* 1.9 g) was dissolved into 10% acetonitrile in 0.1% TFA and applied to HPLC with a C-18 column (TSKgel ODS-80Ts, 21.5 × 300 mm (Tosoh)) and a gradient elution of 10% to 60% acetonitrile in 0.1% TFA for 80 min after elution with 10% acetonitrile in 0.1% TFA for 10 min. Elution was at a flow rate of 5.0 ml/min and 10-ml fractions (2 min) were collected from 10 to 130 min. Each fraction was assayed and the activity was detected in 2 fractions (fr. 31 and fr. 32). Each fraction was separately purified using the following procedure. The

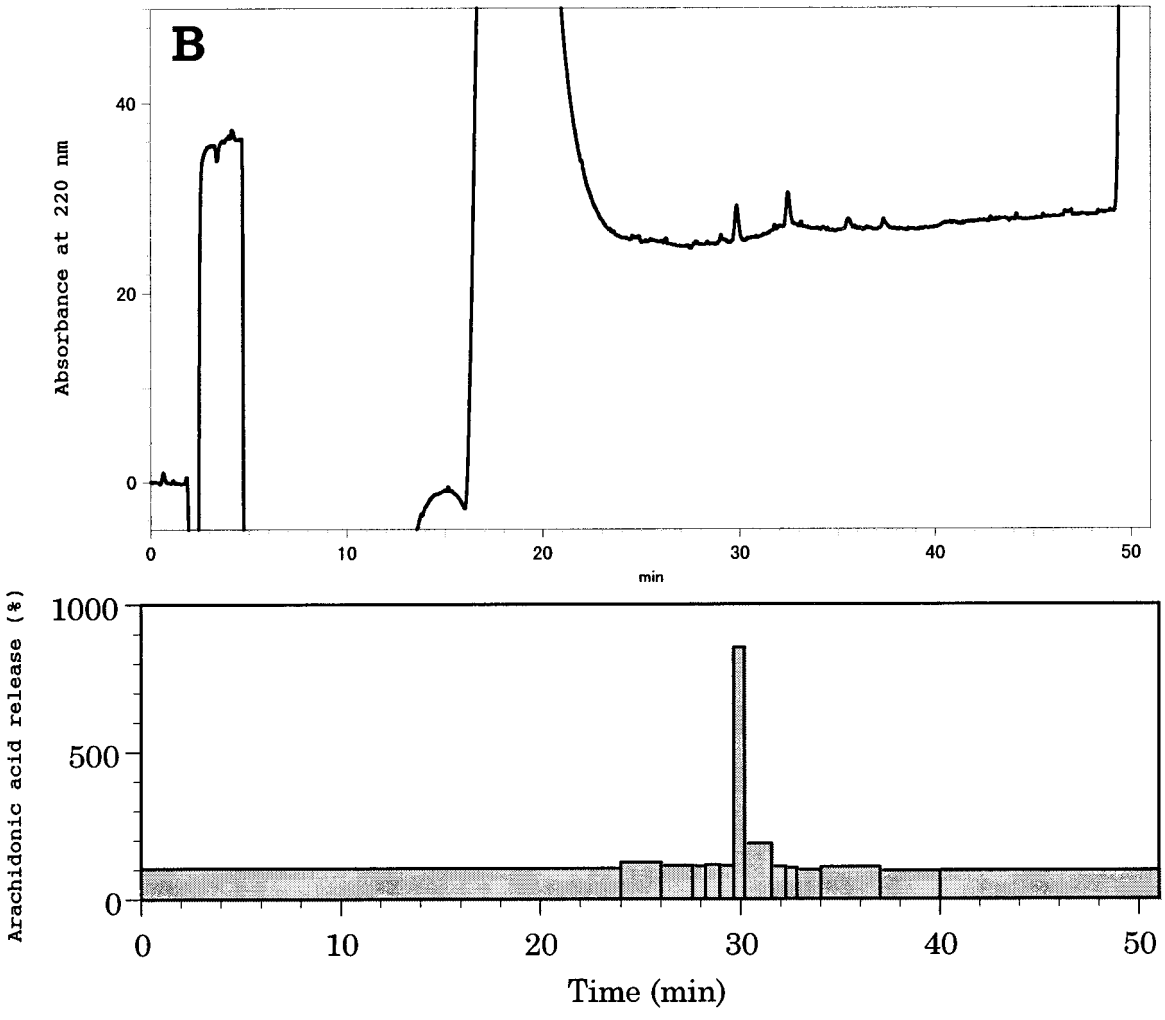

FIG. 1—*Continued*

fractions were lyophilized, reconstituted with 10 mM ammonium formate in 10% acetonitrile, and processed using cation exchange chromatography on an SP-5PW column (20 × 150 mm, Tosoh). The column was eluted with a gradient of 10 mM to 300 mM ammonium formate in 10% acetonitrile for 30 min at a flow rate of 5.0 ml/min. The active fractions eluted with about 140 mM ammonium formate were lyophilized and dissolved into 10% acetonitrile in 0.1% TFA. The solutions were further purified using a diphenyl column (Vydac 219-TP54, 4.6 × 250 mm (Separation Group)) and a gradient of 26% to 31% acetonitrile in 0.1% TFA for 20 min at a flow rate of 1.0 ml/min. The active substances from 2 fractions were eluted when the concentrations of acetonitrile reached 27.1% and 27.6% and were then lyophilized. The lyophilized materials were dissolved with 100 μl of dimethylsulfoxide and 400 μl of 10% acetonitrile in 0.1% TFA and were subjected to a cyanopropyl column on a Develosil CN-UG-5 (4.6 × 250 mm, Nomura Chemical). The column was eluted with a gradient of 28.5% to 33.5% of acetonitrile in 0.1% TFA for 20 min at a flow rate of 1.0 ml/min and the eluate was manually fractionated by peaks. The activity was recovered with peaks which appeared at 29.7% or 29.9% of acetonitrile. The eluate containing these active peaks were diluted 2-fold with water and applied to a C18 semi-microbore column (Wakosil-II 3C18HG, 2.0 × 150 mm (Wako Pure Chemical)). The column was eluted with a gradient of 30% to 35% acetonitrile in 0.1% heptafluorobutyric acid for 20 min at a flow rate of 0.2 ml/min. The activity was recovered as single peaks at 32.2% (from fr. 31) or 32.5% (from fr. 32) of acetonitrile.

Sequence analysis of the purified ligands. The amino terminal amino acid sequences of the active materials were determined using a Procise 494 Protein Sequencer (PE Applied Biosystems).

Synthesis of porcine and human UII. Porcine UII-1 and -2 and human UII were chemically synthesized using an ABI 430A peptide synthesizer (PE Applied Biosystems) utilizing the Boc-strategy. After deblocking all the protecting groups by hydrogen fluoride treatment, the peptides were air-oxidized to form intramolecular disulfide bonds between two cysteines.

Cloning and sequencing of cDNAs encoding porcine UII precursor proteins. PCR using porcine genomic DNA (Clontech) as the template and primers 5′-GATTTCTCTGGACAAGATCC-3′ and 5′-ACGAAGACCTTTATGACACAGACT-3′, which were designed according to the reported cDNA sequence of human prepro-UII [17], yielded a genomic sequence encoding a part of porcine prepro-UII. The full length cDNA encoding porcine UII precursor protein was cloned using a probe derived from this partial sequence.

Poly $(A)^+$ RNA fraction was purified from total RNA extracted from a porcine spinal cord and a cDNA library was established using the SuperScript λ System for cDNA Synthesis and λ ZipLox Cloning

(Gibco BRL), and using the Gigapack III Gold packaging extract (Stratagene). The cDNA library was then screened by hybridization using a probe corresponding to the above-mentioned partial DNA sequence and 9 positive plaques were independently obtained. Plasmids containing cDNAs encoding porcine prepro-UII were excised from the positive plaques using the Cre/*lox* P system and DNAs were purified from *E. coli* transformed with the plasmids. The purified DNAs were sequenced using an ABI Prism 377 DNA Sequencer (PE Applied Biosystems) and a DyeDeoxy Terminator Cycle Sequence Kit (PE Applied Biosystems). Seven of 9 clones covered the full length of the open reading frame and 3 nucleotide sequences encoding prepro-UII were obtained.

RESULTS

Isolation and identification of UII as the endogenous ligand of the rat SENR. Using the established CHO cells stably expressing the rat SENR, we examined the intracellular secondary signaling evoked by the extracts prepared from several tissues and found that the extract of porcine spinal cords showed release activity for arachidonic acid metabolites form the cells. Starting with 50 porcine spinal cords, we purified the active substances using a combination of HPLC processes. In the first step using a semi-preparative C18 column, the activity was recovered in 2 successive fractions (fr. 31 and fr. 32). Each fraction was separately subjected to the same subsequent chromatographic procedure and 2 active substances, which showed different behavior in each purification step, were obtained (Fig. 1). The isolated materials, estimated at about 10 pmol, were then subjected to N-terminal amino acid sequence analysis using a protein sequencer.

The active substance from fractions 31 and 32 afforded the sequences, GPTSEXFWKYXV and GPPSEXFWKYXV, respectively. The 2 Xs in the sequences indicate that no amino acids were detected in the corresponding sequencing steps. Although the 6th and 11th residues remained unidentified, we assumed that both Xs were cysteines forming an intracellular disulfide bond, since these sequences showed homology to fish or human UII [17, 18]. Thus the sequences of the active substances were determined as GPTSECFWKYCV (porcine UII-1) and GPPSECFWKYCV (porcine UII-2), that is, the ligands of the rat SENR in porcine spinal cord are two molecular species of porcine UII (Fig. 2).

Biological activity of synthetic UII. Porcine UII-1 and -2 were synthesized utilizing a solid phase peptide synthesizer. Since the chromatographic behaviors of the active substances isolated from spinal cords were indistinguishable from those of the synthetic peptides, the ligand structures of the SENR were unambiguously confirmed. The synthetic porcine UII-1 and -2 evoked a release of arachidonic acid metabolites from the SENR-expressing CHO cells in a dose-dependent manner, with an estimated EC_{50} value of 1.0 nM (Fig. 3). Human, frog and fish UII were also tested and showed a release activity for arachidonic acid metabolites similar in intensity to that for porcine UII (Fig. 3). Somatostatin or cortistatin, which share the partial amino acid sequence with UII, Phe-Trp-Lys in a Cys-Cys ring structure, failed to show any activity.

Porcine-1	**GPT-SECFWKYCV**
Porcine-2	**GPP-SECFWKYCV**
Human	**ET-PDCFWKYCV**
Frog	**AGNLSECFWKYCV**
Goby	**AGT-ADCFWKYCV**
Trout	**GGN-SECFWKYCV**
Sucker A	**GSG-AECFWKYCV**
Sucker B	**GSN-TECFWKYCV**
Carp α	**GGG-ADCFWKYCV**
Carp β1	**GGN-TECFWKYCV**
Carp β2	**GSN-TECFWKYCV**
Carp γ	**GGG-ADCFWKYCI**
Flounder	**AGT-TECFWKYCV**
Sturgeon	**GST-SECFWKYCV**
Paddlefish	**GST-SECFWKYCV**
Skate	**NNF-SDCFWKYCV**
Dogfish	**NNF-SDCFWKYCV**
Petromyzon	**NNF-SDCFWKYCV**
Lamptera	**NNF-SDCFWKYCV**

FIG. 2. Comparison of amino acid sequences of UII peptides.

Cloning of cDNAs encoding porcine UII precursor proteins. We employed PCR using primers derived from the reported nucleotide sequence of human UII and porcine genome as a template and obtained a fragment of genomic DNA sequence that encodes a part of the precursor protein of UII. Using this partial sequence as a probe, 3 cDNAs encoding precursor proteins of porcine UII were cloned by plaque hybridization using a cDNA library established from a porcine spinal cord. Two of the 3 sequences were of the same length in the open reading frame with 1 nucleotide change, which caused no amino acid change, and encoded a precursor protein of 121 amino acids (Fig. 4). Another cDNA seemed to be a splicing variant encoding an 85 amino acid protein. The mature UII peptide was located at the C-terminus of the precursor proteins, as is the case of human, frog, and carp UII [17, 19]. All the cDNA analyzed gave the sequences of the precursor proteins for porcine UII-2.

DISCUSSION

UII is known as a piscine neuropeptide distributed in urophysis of teleost fish and is reportedly involved in significant regulatory functions, such as cardiovascular regulation, osmoregulation concerning seawater adaptation, and regulation of lipid metabolism [20]. Biochemical or immunohistochemical studies have demonstrated UII in skate, lamprey, and frog, indicating its broad physiological significance in lower vertebrates [20]. Furthermore, the occurrence of UII-like immunoreactivity in the gastropod *Aplysia californica* has been reported [21], suggesting some physiological functions of UII or a UII-like substance in invertebrates.

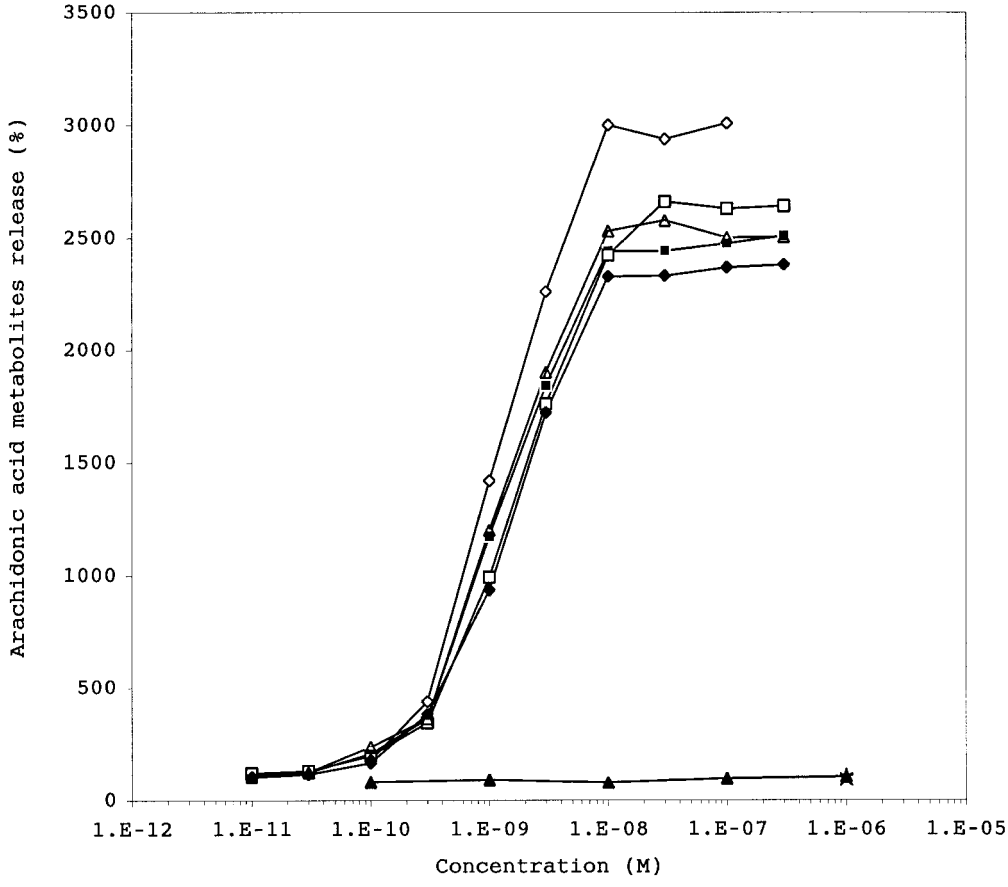

FIG. 3. Arachidonic acid metabolites release activity of UII peptides from the rat SENR-expressing CHO cells. Porcine UII-1 (■) and -2 (□), human UII (◇), frog UII (◆), and fish UII (△) were applied to the cells. Somatostatin-14 (+) and cortistatin-14 (×) were also challenged (1×10^{-6} M). CHO cells expressing the rat SLC-1 receptor [8] were treated with porcine UII-2 (▲) as a control. The activity was indicated as percentages of the released radioactivity from cells compared with that of cells to which no samples were applied.

A cardiovascular activity of fish UII in mammals, by exogenous administration, has also been reported. Goby UII was intravenously injected in rats and showed a hypotensive effect that seemed to be mediated by nitric oxide or prostaglandins [22, 23]. The complex effect of fish UII on isolated vascular smooth muscle, which is relaxative in low doses and contractile in high doses, was also demonstrated [24]. These observations suggest that UII or a related peptide is produced in mammalian tissues. A cDNA encoding human UII precursor protein was recently cloned [17], indicating that mammalian UII is present and functions as a neuropeptide similar to its piscine counterpart. In the present study, we isolated the porcine UII from spinal cords as the endogenous ligand of the SENR, indicating that the processed UII peptide actually exists in mammalian tissues and plays several physiological roles that involve the SENR.

The deduced precursor protein of porcine UII consists of a typical signal sequence spanning 19 amino acids, a long intervening sequence, and, at the C-terminus, the mature UII peptide flanked by 3 basic amino acids (Lys-Lys-Arg) as a processing site (Fig. 4). A shorter cDNA, probably a splicing variant, was isolated and also encodes a precursor protein of the same UII peptide. All the cDNA obtained from the positive plaques encoded the prepro-form of porcine UII-2. The diversity of the 3rd residue in UII-1 and -2 is probably due to the polymorphism at this position. It is noteworthy that the UII peptides were isolated from the spinal cords of 50 pigs whereas the cDNA library was established from just 1 spinal cord. The amino acid sequence of porcine prepro-UII exhibits high overall identity to human prepro-UII (61.6%) but low identity to that of carp UII-α (9.9%). The highly conserved C-terminal region of UII (CFWKYCV) was also completely preserved in porcine UII. Unlike human UII, the N-terminal region of porcine UII peptides also shows high homology to several fish UII peptides (Fig. 2). For example, porcine UII-1 differs from sturgeon UII [18] in only 1 residue at the 2nd position. In addition, porcine UII is composed of 12 amino acid residues, which is equal to the fish UII peptides but not to frog or human UII.

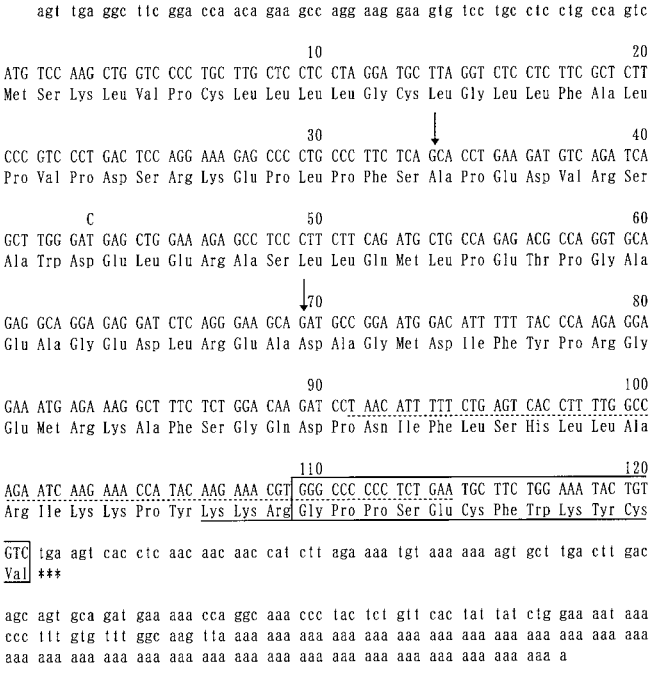

FIG. 4. Nucleotide and deduced amino acid sequences of the porcine prepro-UII. The noncoding sequence is shown in lowercase. Dotted line indicates the nucleotide sequence employed as a probe. The assumed splicing sites are indicated by arrows. The 3rd codon of Asp^{43} was C in 3 clones and T in 4 out of 7 clones. Box indicates the mature UII peptide. Underline indicates the processing site flanking the mature peptide. Asterisks indicate the termination codon.

Itoh *et al.* reported that radiolabeled goby UII showed specific binding to the membrane from the rat thoracic aorta with a dissociation constant of 5.9×10^{-9} M, suggesting the presence of a functional receptor of UII [25]. Itoh *et al.* also reported that the C-terminal region of goby UII is essential for the contractile effect on the rat artery [25]. Our results suggested that all the UII peptides tested induced a similar intensity of release activity of arachidonic acid metabolites in the rat SENR-expressing CHO cells regardless of their structural diversity in the N-terminal region, which is consistent with their observations. Thus, it is conceivable that the SENR may function as the receptor of UII in this system.

The cDNA of human UII is reportedly abundant in the spinal cord and in situ hybridization studies have shown that the UII gene is expressed in motoneurons [17]. On the other hand, Tal *et al.* demonstrated that the SENR mRNA is distributed in neural and sensory tissues [14]. These reports regarding the distribution of the ligand and its receptor do not necessarily imply specific biological functions of UII, nor do they satisfactorily interpret the cardiovascular effects of the peptide. The presence of UII in mammals has only been revealed recently and its physiological significance in the mammalian system remains to be identified. The identification of the SENR as the receptor of UII will be a contribution to the understanding and ultimate resolution of many of these issues.

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Note added in proof. During preparation of the manuscript, the identification of UII as an agonist for the GPR14 (=SENR) was published [26].

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