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Identification and Characterization of a Novel Human Cortistatin-like Peptide[1]

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Expressed sequence tags (ESTs) that showed significant homology to rat cortistatin (CST) were found in a human fetal brain cDNA library. A protein coded by the cDNA showed 55% identity to rat preprocortistatin in amino acid. Similarly in the generation of mature peptides from rat preprocortistatin, it was expected that cleavage at dibasic amino acids in the C-terminal portion of the coded protein might produce at least two different sizes of mature peptides with 29 and 17 amino acid residues, respectively. We chemically synthesized the predicted mature peptide with 17 amino acid residues (hCS-17) and examined its biological activities. It bound to all human somatostatin receptor (SSTR) subtypes in almost the same manner as rat CST-14. It also inhibited cAMP production induced by forskolin through SSTRs. Administration of hCS-17 to the cerebral ventricle showed flattening of cortical and hippocampal electroencephalograms in rats. These results indicate that a bioactive peptide encoded by the cDNA is a human counterpart corresponding to rat CST.

Rat cortistatin (CST) which exhibits structural similarity to somatostatin (SST), has been reported by Lecea *et al* [1]. It binds to somatostatin receptors (SSTRs) on a pituitary GH4 cell line and inhibits cAMP increase in the cells in almost the same manner as SST. It is also reported that rat CST depresses neuronal electrical activity and regulates paradoxical sleep in slightly different manner from SST.

To investigate a human homologue of rat CST, we searched through a human expressed sequence tag (EST) database derived from a human brain cDNA library, and found several ESTs that showed significant homology to rat preprocortistatin cDNA. On the basis of the EST sequences, we isolated a cDNA (phCSP6) having a complete coding region from human brain poly(A)$^+$RNA by reverse transcription polymerase chain reaction (RT-PCR), and then confirmed its sequence. We chemically synthesized one of the predicted mature peptides and its derivatives. Here we show the primary structure, mRNA distribution, and biological activities of a human cortistatin-like peptide.

MATERIALS AND METHODS

Isolation of human cortistatin-like peptide cDNA. EST analysis of cDNA clones derived from a human cDNA library (oligo(dT)-primed and constructed in the λ ZapII vector (Stratagene, USA)) identified two clones demonstrating significant homology to rat preprocortistatin. One set of oligonucleotide primers (CS-2 5′-ACAAGATGCCATTGTCCCCCGGCCTCCT-3′ and CS-7 5′- TTCAGGTCTGTAATTAAACTTGCGTGA-3′) was synthesized on the basis of their sequences. First-strand cDNA was synthesized in a total reaction volume of 20 μl on 5 μg of human brain poly(A)$^+$RNA (Clontech, USA), 50 ng random hexamers, 200 units of Superscript TM II RNaseH$^-$Reverse Transcriptase (Gibco BRL, USA), 10 mM dithiothreitol, and 1 mM deoxynucleotide triphosphate. The mixture was incubated for 60 min at 42°C. The reaction was stopped by incubation at 90°C for 5 min. The resultant cDNA was purified by ethanol precipitation. To perform PCR, one thirtieth of the cDNA was admixed with 2.5 units of Ex Taq polymerase (Takara, Japan), its PCR buffer, 10 pmol each primer, 1 mM deoxynucleotide triphosphate, and water in a total of 50 μl. The reaction mixture was covered with mineral oil and incubated in a thermalcycler (Perkin Elmer DNA thermal cycler 480, ABI, USA) programmed to repeat 35 cycles under the following conditions: 30 seconds at 95°C, 60 seconds at 65°C, and 30 seconds at 72°C. About 300 bp PCR product was recovered after agarose gel electrophoresis, and subcloned with a TA cloning kit (Invitrogen, USA). The nucleotide sequences of subcloned cDNA fragments were analyzed with an ABI 377 sequencer using a dideoxyterminator cycle

[1] The nucleotide sequence data reported in this paper will appear in the DDBJ, EMSL and GenBank nucleotide sequence databases with the following accession number: AB000263.

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Abbreviations: EST = expressed sequence tag, CST = cortistatin, SST = somatostatin, SSTR = somatostatin receptor, RT-PCR = reverse transcription polymerase chain reaction, EEG = electroencephalogram, EMG = electromyogram, SWS1 = light slow wave sleep, SWS2 = deep slow wave sleep, PS = paradoxical sleep.

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sequence kit (ABI, USA). A cDNA clone, phCSP6, was obtained thereby.

Northern blot analysis. Human multiple tissue northern blot filters (Clontech, USA) were used for northern blot analysis. A 300-bp cDNA fragment excised from phCSP6 was labelled with [^{32}P]dCTP (Dupont/NEN, UK), and then used as a probe. Hybridization was done with the probe at 68°C in ExpressHyb Hybridization Solution (Clontech, USA) for 1 hr. The filter was washed at 65°C in 0.1 × SSC containing 0.1% SDS for 40 min, and then exposed for three weeks to an X-ray film AR (Kodak, USA) at −70°C with an intensifying screen. After removing the probe, the same filter was rehybridized with a human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA probe as an internal control.

Chemical synthesis of human cortistatin-like peptides. hCS-17 (DRMPCRNFFWKTFSSCK), hCS-15 (MPCRNFFWKTFSSCK), and hCS-13 (CRNFFWKTFSSCK) were synthesized by solid phase method using Boc HOBT/NMP chemistry. The Boc amino acids with the following side chain protection were used: Lys(Cl-Z), Cys(MeBzl), Ser(Bzl), Thr(Bzl), Trp(CHO), Arg(Tos), and Asp(OcHex). The protected peptide resins were treated with HF/p-cresol/1,4-butanedithiol. After gel filtration chromatography on Sephadex G-25, the desired dithiol peptides were subjected to air-oxidation reaction in 0.01 M $AcONH_4$ buffer (pH8). The peptides were purified by reverse phase chromatography, CM chromatography, and gel filtration chromatography. Amino acid analysis of purified peptides gave values that agreed well with the theoretical ones. Sequencing analysis showed the desired results. The molecular weights measured by FAB mass spectroscopy were 2150.9460, 1879.7610 and 1651.5830 for hCS-17, hCS-15 and hCS-13, respectively. These values agreed well with the theoretical ones (2150.9730, 1879.7850, and 1651.7510, respectively).

Receptor binding assays. Five types of human SSTR (SSTR1, SSTR2, SSTR3, SSTR4 and SSTR5) cDNAs cloned by PCR based on the published sequence [2, 3] were inserted into an expression vector pAKKO-111, which had SR α promoter and a dehydrofolate reductase gene (*dhfr*) as a marker, respectively. Each vector was then introduced into *dhfr*$^-$CHO cells by calcium phosphate-mediated transfection [4]. Transformed CHO cells were cultured in a selection medium, and then a single colony expressing high levels of each receptor subtype was isolated.

Transformed CHO cells were grown in a medium in 150-cm^2 flasks for 3 days, and then dispersed in phosphate-buffered saline (PBS) containing 0.2 mM EDTA. The cells were washed with the same buffer, and then suspended in 10 mM sodium carbonate buffer containing 1 mM EDTA, 0.25 mM PMSF, 20 μg/ml of leupeptin, 10 μg/ml of phosphoramidon, and 1 μg/ml of pepstatin. After the cells were homogenized with a polytron homogenizer, the homogenates were centrifuged at 1000 × g for 10 min. The resultant supernatants were ultracentrifuged twice at 100,000 × g for 60 min. The pellets were then suspended in a buffer containing 25 mM Tris-HCl (pH.7.4), 1 mM EDTA, 0.5 mM PMSF, 20 μg/mm of leupeptin, 10 μg/ml of phosphoramidon, and 1 μg/ml of pepstatin, and then used as membrane fractions.

```
  1  ACAAGATGCCATTGTCCCCCGGCCTCCTGCTGCTGCTGCTCTCCGGGGCCACGGCCACC    59
  1        M  P  L  S  P  G  L  L  L  L  L  L  S  G  A  T  A  T    18

 60 GCTGCCCTGCCCCTGGAGGGTGGCCCCACCGGCCGAGACAGCGAGCATATGCAGGAAGCG   119
 19  A  A  L  P  L  E  G  G  P  T  G  R  D  S  E  H  M  Q  E  A    38
       ▲
120 GCAGGAATAAGGAAAAGCAGCCTCCTGACTTTCCTCGCTTGGTGGTTTGAGTGGACCTCC   179
 39  A  G  I  (R)(K) S  S  L  L  T  F  L  A  W  W  F  E  W  T  S    58

180 CAGGCCAGTGCCGGGCCCCTCATAGGAGAGGAAGCTCGGGAGGTGGCCAGGCGGCAGGAA   239
 59  Q  A  S  A  G  P  L  I  G  E  E  A  R  E  V  A (R)(R) Q  E    78

240 GGCGCACCCCCCCAGCAATCCGCGCGCCGGGACAGAATGCCCTGCAGGAACTTCTTCTGG   299
 79  G  A  P  P  Q  Q  S  A (R)(R) D  R  M  P  C  R  N  F [F][W]   98

300 AAGACCTTCTCCTCCTGCAAATAAAACCTCACCCATGAATGCTCACGCAAGTTTAATTAC   359
 99 [K][T] F  S  S  C  K  *                                        105

360 AGACCTGAA                                                      368
105                                                                105
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FIG. 1. DNA and predicted amino acid sequences of human cortistatin-like peptide cDNA (phCSP6). The numbers on the right refer to the amino acid and nucleotide positions of phCSP6. The arrowhead indicates the postulated cleavage site of the signal sequence. The tandem dibasic amino acids are circled. FWKT which is reported to be critical to SSTR binding, is boxed.

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h somatostatin      1 MLSCRLQCAL AALSIVALG CVTGAPSDPR LRQFLQKSLA   40
r cortistatin       1 .....MGGCS TRGKRPSALS LLLLLLLSGI AASALPLESG   35
h cortistatin-like  1 .......... ..MPLSPGLL LLLLSGATAT AALPLEGGPT   28

h somatostatin     41 AAAGKQELAK YFLAELLSEP NQTENDALEP EDLSQAAEQD   80
r cortistatin      36 PTGQDSVQDA TGGRRTGLLT FLAWWHEWAS QDSSSTAFEG   75
h cortistatin-like 29 GRDSEHMQPS AGIRKSSLLT FLAWWFEWTS QASAGPLIGE   68

h somatostatin     81 EMELELQRSA NSNPAMAPRE RKAGCKNFFW KTFTSC....   116
r cortistatin      76 GTPELSKRQE RPPLQQPHR DKKPCKNFFW KTFSSCK...   112
h cortistatin-like 69 DAREVARRQE GAPPQQSARR DRMPCRNFFW KTFSSCK...   105
                                                  hCS-17
                                        hCS-29
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FIG. 2. Amino acid sequence alignment of the preproform of human cortistatin-like peptide, rat cortistatin and human somatostatin. The upper sequence is for human somatostatin, the middle is for rat cortistatin, and the lower is for human cortistatin-like peptide. The amino acid residues identical in at least two sequences are boxed. Predicted mature peptide regions of human cortistatin-like peptide (hCS-29 and hCS-17) are underlined.

Each cell membrane fraction (1 μg protein was used for SSTR1 and 0.5 μg protein for SSTR2-5) was incubated in a total of 200 μl with 50 pM [^{125}I]-[Tyr^{11}]-somatostatin-14 (SST-14)(25,000 cpm)(Amersham, UK) and increasing concentrations of SST-14 (Peptide Research Inc., Japan), hCS-17, hCS-15, hCS-13, and CST-14 (Peptide Research Inc., Japan) for 60 min at room temperature. Nonspecific binding was defined as [^{125}I]-[Tyr^{11}]-SST-14 binding in the presence of 1 μM SST-14. The binding reaction was terminated by rapid filtration through Whatman GF/B glass fiber filters (Whatman, UK) soaked in 0.2% polyethylenimine, followed by washing three times with 300 μl of 25 mM Tris-HCl (pH7.4). The radioactivity retained in the filters was determined with a Packard auto-gamma counter (Packard, USA). The concentration of a competing peptide causing 50% inhibition of specific binding (IC_{50}) was determined as described elsewhere [5].

Assay of intracellular cAMP. The CHO cells expressing human SSTR2, 3, 4, and 5 were separately grown in the medium in 24-well plates. The cells were washed twice with the incubation buffer consisting of Dulbecco's modified Eagle's medium containing 20 mM HEPES (pH7.5), 0.2% BSA, and 0.2 mM IBMX, and then incubated with 400 μl of the same buffer at 37°C for 60 min. Then each sample (50 μl) at various concentrations was added with 50 μl forskolin (final concentration 10 μM) to the well, and then the cells were incubated at 37°C for 30 min. After aspirating the buffer, the cells were washed twice with the incubation buffer and lysed in 20% perchloric acid. The intracellular cAMP was determined using a cAMP EIA assay system (Amersham, USA) according to the instructions of the supplier.

Electroencephalogram (EEG) and electromyogram (EMG) recording. Rats (Jcl:Wistar, male) were purchased from Japan Clea Inc. (Japan), and used for experiments when weighing 200-250 g at the surgery. To perform EEG and EMG recording, rats were mounted on a stereotaxic apparatus under sodium pentobarbitone (40 mg/kg i.p.) anesthesia. Electrodes were implanted bilaterally in the cortex

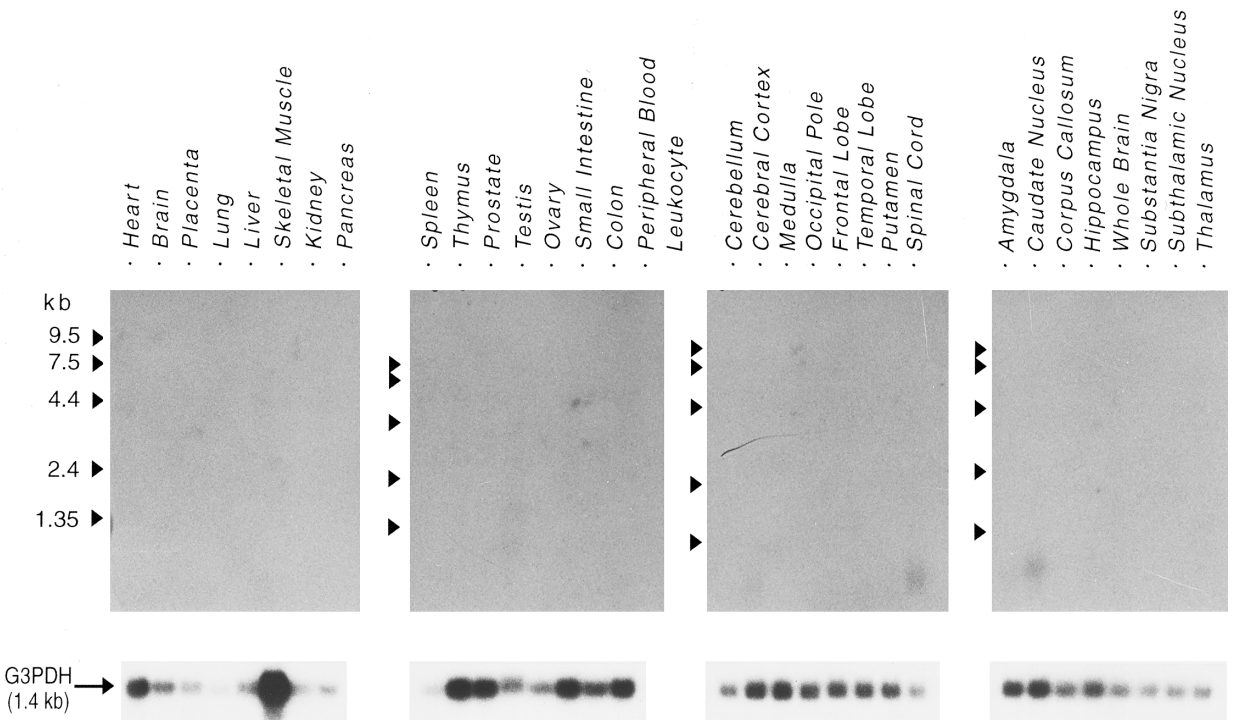

FIG. 3. Northern blot analysis of the human cortistatin-like peptide. Poly(A)+RNA (2 μg) from indicated tissues were blotted on filters, and hybridized with [^{32}P]dCTP labeled phCSP6 (upper panel). Numbers on the left indicate the position of the RNA molecular weight marker in kb. For internal control, human G3PDH is rehybridized (lower panel).

and hippocampus. Muscle activity was recorded from stainless-steel wires fixed in the dorsal neck muscle. The rats were allowed to recover from surgery prior to being acclimated to the test chamber and EEG recording cables. EEGs and EMG were recorded for a total of 5 hours (from 1 hour before treatment to 4 hours after treatment). EEG and EMG were recorded before and after the intracerebroventricular injection of hCS-17 and analyzed as described elsewhere[6]. The criteria for sleep-wakefulness stages were also judged as described elsewhere [6].

RESULTS AND DISCUSSION

Sequence Analysis of cDNA Coding for a Human Cortistatin-like Peptide

Fig. 1 shows the DNA and predicted amino acid sequences of phCSP6 isolated by means of RT-PCR. A protein coded by phCSP6 was thought to start with a 21-residue apparent signal sequence and might be secreted after cleavage. There also existed three dibasic amino acid repeats (^{42}R^{43}K, ^{75}R^{76}R, and ^{87}R^{88}R) in the protein, suggesting a possibility that these sites might be received in the process of cleavage. As reported in the case of rat CST [1], it was predicted that the cleavage of two dibasic amino acid pairs at the C-terminal portion would produce mature peptides, hCS-29 and hCS-17, which corresponded to CST-29 and CST-14, respectively. Although the entire amino acid sequence has about 55% identity against rat preprocortistatin, the predicted mature peptides were well conserved between rat CST and human cortistatin-like peptide (Fig. 2). The most apparent difference was the presumed cleavage sites between hCS-17 and CST-14. The putative cleavage site of CST-14 is reported to be between ^{97}K^{98}K and ^{99}P. However, it should be noticed that this site might not be cleavaged enzymatically because proline is the iminoacid. To determine exact cleavage sites, it is necessary to confirm the sequence of natural peptides derived from tissues. hCS-17 shared 13 of 17 residues with rat CST-14 and 10 of 17 with SST-14 including two cysteines that might cause the peptide to cyclize, and the FWKT sequence (boxed in Fig. 1) that is critical for SST binding to its receptor [7]. These results indicated that phCSP6 coded for a human counterpart corresponding to rat CST.

Tissue Distribution of Human Cortistatin-like Peptide mRNA

We determined the distribution of human cortistatin-like peptide mRNA by northern blotting. As shown in Fig. 3, a single band of about 1 kb was apparently detected in the spinal cord and caudate nucleus. In addition, we thought that very faint bands might exist in the cerebral cortex, hippocampus and testis. Lecea *et al.* have reported that rat CST mRNA is expressed in the brain (cerebral cortex and hippocampus) when determined by *in situ* hybridization [1]. Further study will

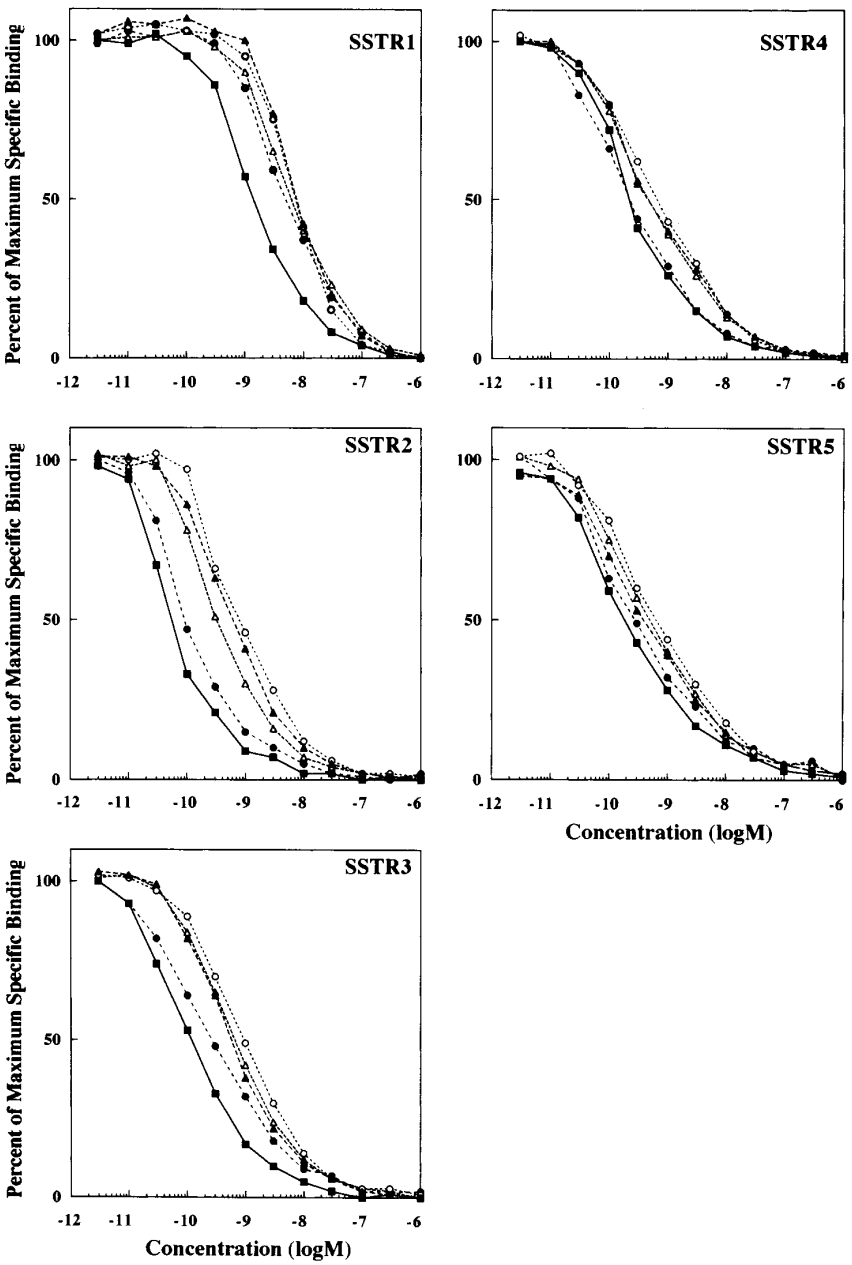

FIG. 4. Inhibition of [^{125}I]SST-14 binding activity by human cortistatin-like peptides to SSTR subtypes. [^{125}I]SST-14 binding to each SSTR subtype was performed in the presence of the indicated concentration of SST-14 (■), hCS-17(▲), hCS-15(○), hCS-13(△), and CST-14 (●), respectively. The maximum binding without a competitor was 4120 cpm (SSTR1), 5580 cpm (SSTR2), 4580 cpm (SSTR3), 5940 cpm (SSTR4), and 2900 cpm (SSTR5); nonspecific binding in the presence of 1 μM cold SST-14 was 480 cpm (SSTR1), 380 cpm (SSTR2), 350 cpm (SSTR3), 350 cpm (SSTR4), and 350 cpm (SSTR5). Results are expressed as the mean values in duplicate experiments.

be necessary to determine whether tissue distribution of mRNA is identical or not between rat CST and human cortistatin-like peptide. Our results suggested that human cortistatin-like peptide might play important roles mainly in the central nervous system.

Binding Activity of Synthetic Human Cortistatin-like Peptides to SSTR Subtypes

To ascertain the biological activities of the predicted peptides, we chemically synthesized hCS-17 and its derivatives (hCS-15 and hCS-13), and then compared their binding activities with those of SST-14 and CST-14 against human SSTR subtypes expressed in CHO cells. The binding of radiolabelled SST-14 was inhibited by SST-14, hCS-17, hCS-15, hCS-13, and CST-14, respectively, in a dose-dependent manner (Fig. 4). The IC_{50} values of each peptide against SSTR subtypes were summarized in Table 1. In SST-14, IC_{50} value against SSTR1 was higher than those against the other subtypes. Similar tendency was observed in hCS-17. However, hCS-17

TABLE 1

Binding Activities of Human Cortistatin-like Peptides to SSTR Subtypes

Ligand	IC_{50} (nM)				
	SSTR1	SSTR2	SSTR3	SSTR4	SSTR5
SST-14	1	0.05	0.1	0.2	0.2
hCS-17	7	0.6	0.6	0.5	0.4
hCS-15	7	0.8	0.9	0.6	0.6
hCS-13	6	0.3	0.7	0.5	0.5
CST-14	5	0.09	0.3	0.2	0.3

IC_{50} values are calculated through each competition curve in Fig. 4 and expressed as the mean values in duplicate experiments.

showed somewhat higher IC_{50} values as a whole than SST-14. The inhibitory activity of hCS-17 was almost comparable to hCS-15 and hCS-13, indicating that N-terminal portion of hCS-17 was not so important for binding to SSTR subtypes. The inhibitory activities of hCS-17 against SSTR subtypes were almost comparable to those of CST-14. These results demonstrated that hCS-17 had activity similar to CST-14 in respect to SSTR binding.

Inhibition of cAMP Production through SSTR Subtypes

As shown in Fig. 5, SST-14, hCS-17 and hCS-15 inhibited forskolin-stimulated cAMP production in CHO

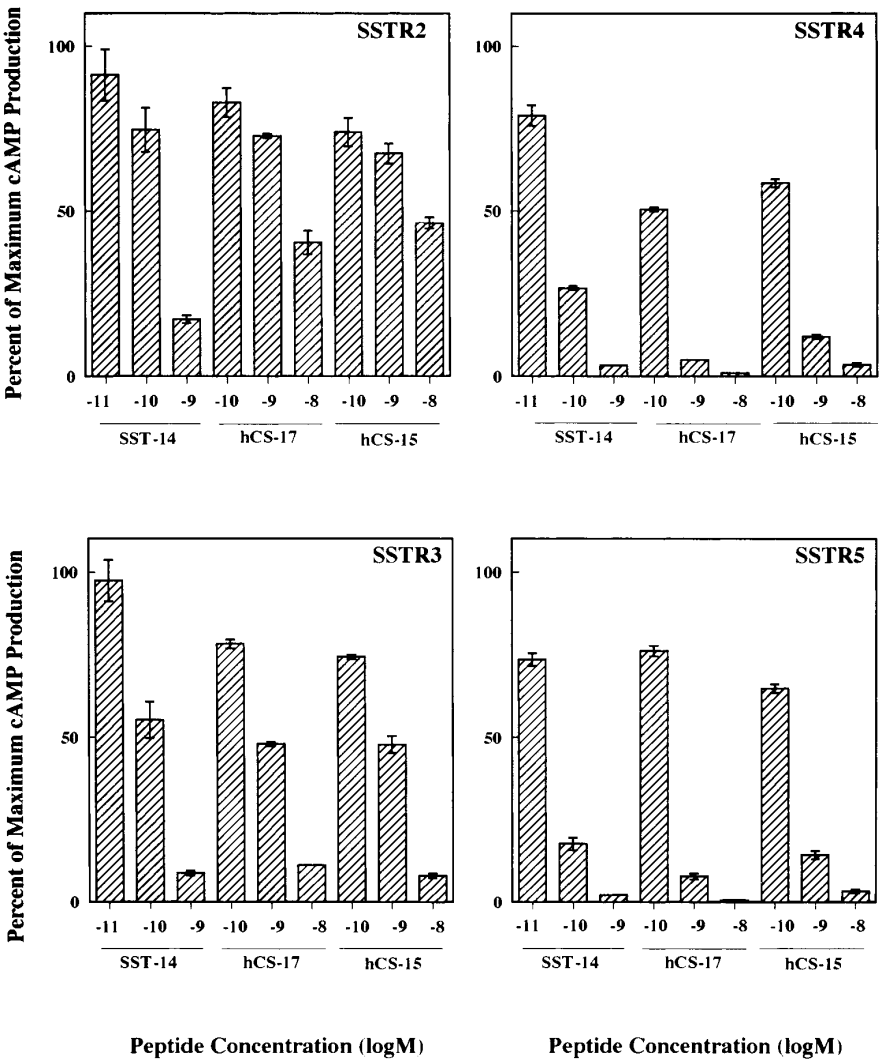

FIG. 5. Inhibition of cAMP production by human cortistatin-like peptides. The inhibition of forskolin-stimulated cAMP increase in CHO cells expressing each SSTR subtype was measured in the presence of the indicated peptides. Under our experimental conditions, the basal level concentration of cAMP was 14 fmol/well (SSTR2), 17 fmol/well (SSTR3), 34 fmol/well (SSTR4), and 29 fmol/well (SSTR5); the maximum level in the presence of 10 μM forskolin was 330 fmol/well (SSTR2), 460 fmol/well (SSTR3), 320 fmol/well (SSTR4) and 560 fmol/well (SSTR5). Results were expressed as the mean values±S.E. in triplicate experiments.

TABLE 2

Inhibition of cAMP Production by Human Cortistatin-like Peptides

Ligand	ED_{50} (nM)			
	SSTR2	SSTR3	SSTR4	SSTR5
SST-14	0.3	0.1	0.04	0.03
hCS-17	5	1	0.1	0.2
hCS-15	7	1	0.2	0.2

ED_{50} values are calculated through each dose–response curve in Fig. 5 and expressed as the mean values in triplicate experiments.

cells expressing SSTR2-5, respectively, in a dose-dependent manner. The ED_{50} values of SST-14, hCS-17 and hCS-15 estimated from Fig. 5 were summarized in Table 2. SST-14 inhibited cAMP production more efficiently through SSTR4 and SSTR5 than through SSTR2 and SSTR3. Similar tendency was observed in hCS-17 and hCS-15. However, the ED_{50} values of hCS-17 and hCS-15 against each SSTR subtype were higher than that of SST-14. In the binding assays, there was not much difference in the IC_{50} values of these peptides between SSTR2 or SSTR3 and SSTR4 or SSTR5. This discrepancy might imply that SSTR2 and SSTR3 closely coupled to the other second messenger system (e.g., phospholipase C, Ca^{2+}, or tyrosine phosphatase) as well as cAMP. Our results demonstrated that hCS-17 and hCS-15 were able to transduce the inhibition of cAMP production through SSTR2-5, although their activities were slightly weaker than that of SST-14.

Effect of hCS-17 on EEG and EMG in Rats

Lecea *et al.* have reported that CST-14 showed regulatory effects on sleep in rats. We examined whether hCS-17 showed similar effects by intracerebroventricular injection of hCS-17 on sleep and wakefulness pattern in free-moving rats by recording EEG of the cerebral cortex and hippocampus as well as EMG. As shown in Fig. 6, hCS-17 (1 nmol/brain) caused flattening of the cortical and hippocampal EEG, which lasted 3-5 min after the injection. hCS-17 (0.1-1 nmol/brain) decreased light slow wave sleep (SWS1) and increased deep slow wave sleep (SWS2) in a dose-dependent manner when analyzed for 4 hours after the injection (Fig. 7). The peptide also reduced the occurrence of paradoxical sleep (PS) consistent with an earlier report by Lecea *et al.* In their report, it is also demonstrated that CST-14 produces a significant increase in SWS2 and a decrease in PS in rats. The efficacy of hCS-17 appeared to be almost comparable to that of CST-14 reported by Lecea *et al.* These results suggested that hCS-17 had sleep modulating activity similar to CST-14.

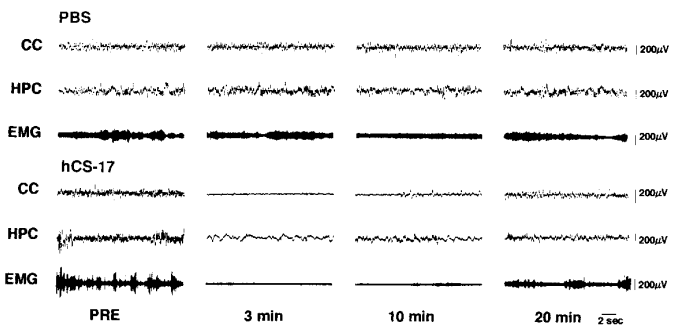

FIG. 6. Effects of hCS-17 on electroencephalogram and electromyogram in free moving rats. CC and HPC mean electroencephalogram in the cerebral cortex and hippocampus. EMG means electromyogram.

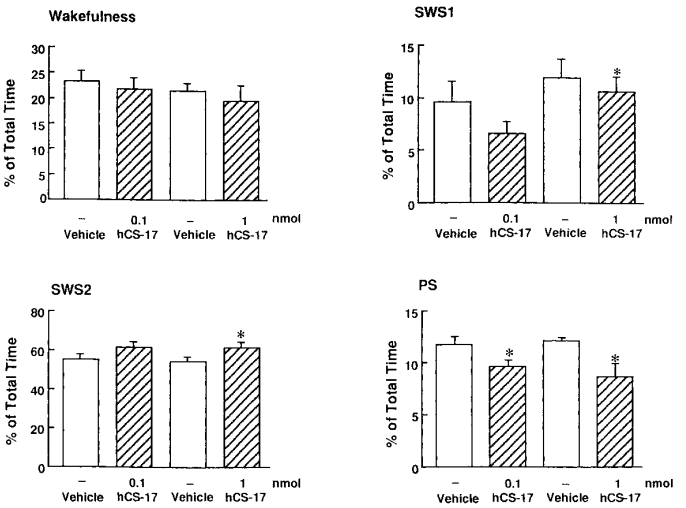

FIG. 7. Effects of hCS-17 on sleep and wakefulness in free-moving rats. SWS1, SWS2, and PS mean light slow wave sleep, deep slow wave sleep, and paradoxical sleep, respectively. Vehicle means PBS control. Data are expressed as the mean±S.E. of at least five different experiments. *$P<0.05$, are compared with the vehicle control (paired t-test).

In this paper we demonstrated the structure of a human cortistatin-like peptide and its biological activities. We believe that our report will facilitate studies on the physiological functions of cortistatin-like peptide in human.

ACKNOWLEDGMENTS

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