Calcitonin Receptor-stimulating Peptide, a New Member of the Calcitonin Gene-related Peptide Family

ITS ISOLATION FROM PORCINE BRAIN, STRUCTURE, TISSUE DISTRIBUTION, AND BIOLOGICAL ACTIVITY*

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We isolated a novel biologically active peptide, designated calcitonin receptor-stimulating peptide (CRSP), from the acid extract of the porcine brain by monitoring cAMP production in the porcine kidney cell line LLC-PK1. Determination of the amino acid sequence and cDNA analysis encoding a CRSP precursor showed that this peptide has ~60% identity in the amino acid sequence with human calcitonin gene-related peptide type-α (αCGRP), type-β (βCGRP), and porcine CGRP. Northern blot analysis and radioimmunoassay demonstrated that CRSP is expressed mainly in the thyroid gland and the central nervous system, in which the calcitonin receptor was abundantly expressed. Synthetic CRSP elicited a potentiating stimulatory effect on the cAMP production in LLC-PK1 cells. Although it shows significant sequence similarity with CGRPs, this peptide did not elicit cAMP elevation in cells that endogenously expressed a CGRP receptor or an adrenomedullin receptor or were transfected with either of these recombinant receptors. Administration of CRSP into anesthetized rats did not alter the blood pressure but induced a transient decrease in the plasma calcium concentration. In fact, this peptide potentially increased the intracellular cAMP concentration in COS-7 cells that expressed the recombinant calcitonin receptor. These unique properties indicate that CRSP is not a porcine counterpart of βCGRP and probably elicits its biological effects via the calcitonin receptor.

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\* The abbreviations used are: AM, adrenomedullin; CRSP, calcitonin receptor-stimulating peptide; CGRP, calcitonin gene-related peptide; αCGRP, CGRP type-α; βCGRP, CGRP type-β; CT, calcitonin; CL receptor, calcitonin-like receptor; RAMP, receptor activity-modifying protein; DMEM, Dulbecco’s modified Eagle’s medium; HPLC, high performance liquid chromatography; ESF, electrospay ionization; CNS, central nervous system; IR, immunoassay; BSA, bovine serum albumin; RIA, radioimmunoassay; CM, carboxymethyl; Mops, 4-morpholinepropanesulfonic acid.

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In this paper, we report the isolation and structural determination of a novel biologically active peptide from porcine brain extract by monitoring the cAMP production in LLC-PK<sub>1</sub> cells. Although the purified peptide showed higher sequence homology to CGRP, this peptide bound to CT receptor with a higher affinity and stimulated cAMP production at a potency 1,000-fold greater than CT. Thus, we designated this peptide as calcitonin receptor-stimulating peptide (CRSP). The findings obtained in this study strongly suggest that CRSP is a candidate for the unidentified ligand to CT receptor in the CNS and may participate in physiological events by activating the central as well as peripheral CT receptor.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—** LLC-PK<sub>1</sub>, HeLa, Swiss 3T3, and COS-7 cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum supplemented with 100 μM/ml penicillin and 100 units/ml streptomycin in a humidified atmosphere of 95% air, 5% CO<sub>2</sub> at 37 °C.

**Measurement of Intracellular cAMP Production—** Cells were harvested and cultured on 48-well plates for 2 days. The cells were washed twice with DMEM/Hepes (20 mM, pH 7.4) containing 0.5 mM 3-isobutyl-1-methyl xanthine (Sigma) and 0.05% bovine serum albumin (BSA) and incubated in the same mixture to a peptide solution (CRSNLPTKGMPVPG-NH<sub>2</sub>) that corresponds to residues 24–38 of CRSP (CRSP-(24–38)) to which an N-terminal cysteine was added to facilitate cross-linking to maleimide-activated keyhole limpet hemocyanin (Pierce). New Zealand White rabbits (Japan SLC, Hamamatsu, Japan) were immunized by injecting 1 mg of the peptide-keyhole limpet hemocyanin antigen conjugate in complete Freund's adjuvant, and antibody production was boosted by 5 additional injections of the antigen in complete Freund's adjuvant at 3-week intervals.

**Northern Blot Analysis—** A CRSP probe was prepared by a PCR reaction performed on 1 ng of full-length CRSP cDNA using RPL plus polymerase and primers (CTCTCTGAGGAGGAATCACG and GAGTTCATGACTAAGCC) and TA-cloning (TACAGACTAAGAAGCTTACATGACTAAGCC) of 10 μg of porcine hypothalamus cDNA. The PCR reaction was performed with these primers and radiolabeled as a probe for screening. Porcine hypothalamic cDNA was synthesized from 3 μg of poly(A)<sup>+</sup> RNA using a Timesaver cDNA synthesis kit (Amersham Biosciences), inserted into ZAP II bacterio-
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**Results**

**Isolation of CRSP**—Crude basic peptides (SP-III fraction) extracted from porcine brain were first subjected to Sephadex G-50 gel filtration to remove any remaining proteins. The peptide fraction thus obtained was next separated by Sephadex G-25 gel filtration, and each fraction was assayed by monitoring the cAMP production in LLC-PK1 cells. Elevation of cAMP to allow for equilibration. Then 100 μl of saline containing the peptide and 0.1% BSA (n = 3) or the same amount of saline containing 0.1% BSA alone as a vehicle (n = 4) was injected through the left jugular vein. Mean arterial blood pressure and heart rate were measured 15 min before and 0, 2, 5, 10, and 30 min after the administration.

**Measurement of Plasma Calcium Concentration**—The rats were anesthetized with sodium pentobarbital (60 mg/kg; Wako, Osaka, Japan). Rectal temperature was monitored and maintained at 37 ± 1 °C during the entire experimental period, and a tracheotomy was performed to aid spontaneous breathing. The mean arterial blood pressure and heart rate were monitored through the right carotid artery using a PE 50 catheter connected to a pressure transducer (model P231D, Gould) and a polygraph (7758 B System, Hewlett-Packard), and the left jugular vein was cannulated using a PE 50 catheter for the bolus administration of peptides or vehicle. The rats were left for at least 30 min after surgery to allow for equilibration. Then 100 μl of saline containing the peptide and 0.1% BSA (n = 3) or the same amount of saline containing 0.1% BSA alone as a vehicle (n = 4) was injected through the left jugular vein. Mean arterial blood pressure and heart rate were measured 15 min before and 0, 2, 5, 10, and 30 min after the administration.

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**Measurement of Blood Pressure**—Eight-week-old male Sprague-Dawley rats were purchased from Japan SLC. All study protocols were approved by the local animal experiments and care committee. All animals were provided free access to tap water and standard chow. The rats were anesthetized by an intraperitoneal injection of thiobutabarbital sodium salt (60 mg/kg; Wako, Osaka, Japan). Rectal temperature was monitored and maintained at 37 ± 1 °C during the entire experimental procedure, and a tracheotomy was performed to aid spontaneous breathing. The mean arterial blood pressure and heart rate were monitored through the right carotid artery using a PE 50 catheter connected to a pressure transducer (model P231D, Gould) and a polygraph (7758 B System, Hewlett-Packard), and the left jugular vein was cannulated using a PE 50 catheter for the bolus administration of peptides or vehicle. The rats were left for at least 30 min after surgery.

**Co-expression of Receptor and Receptor Activity-modifying Protein (RAMP) cDNA**—Porcine CT receptor, calcitonin-like receptor (CL receptor), and three isoforms of RAMP (RAMP1, -2, and -3) cDNAs encoding their complete open reading frames were isolated from porcine lung and hypothalamus cDNA libraries and ligated into pcDNA 3.1 expression vector (Promega). The CT receptor cDNA ligated into pcDNA (pcDNA/CTR) was transfected into COS-7 cells with LipofectAMINE Plus (Invitrogen) according to the manufacturer’s protocol. The transfected cells were used for cAMP assay and competitive binding experiments 24 h after transfection.

**Expression of CT Receptor**—A partial cDNA clone of porcine CT receptor was amplified by PCR with porcine hypothalamus cDNA. Full-length porcine CT receptor was isolated from a porcine hypothalamus cDNA library synthesized as described above. The full-length clone was ligated into pcDNA 3.1 expression vector (Promega). The CT receptor cDNA ligated into pcDNA (pcDNA/CTR) was transfected into COS-7 cells with LipofectAMINE Plus (Invitrogen) according to the manufacturer’s protocol. The transfected cells were used for cAMP assay and competitive binding experiments 24 h after transfection.

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production in LLC-PK1 cells was detected in the fractions corresponding to Mr 2000–4000. These fractions were pooled and separated by CM-52 cation exchange chromatography, and an aliquot of each fraction was subjected to the cAMP production assay in LLC-PK1 cells (Fig. 1a). At least five major peaks of stimulatory activity in the cAMP production assay were observed. Among them peaks 1 and 2 were identified as CGRP (peak 2) and its methionine sulfoxide form (peak 1). Although one peptide was purified from peak 3 and found to have an N-terminal sequence identical to that of CGRP, we could not completely determine the structure of this peptide. Peaks 4 and 5 showed different chromatographic behavior from those of known biologically active peptides, and peak 5 was used for the present isolation of CRSP. The biologically active fractions corresponding to peak 5 were further subjected to CM ion exchange HPLC on a TSKgel CM-2SW column, and only one fraction elicited significant biological activity (data not shown). We next applied the biologically active fraction to reverse phase HPLC on a C18 column, and a single minute peak with absorbance at 210 nm showed compatible cAMP-increasing activity (Fig. 1b, arrow). Final purification was performed by another reverse phase HPLC on a diphenyl column, and the peptide was purified to a homogenous state (Fig. 1c). The yield of each purification step was retrospectively determined by RIA for CRSP and is summarized in Table I. At the CM-52 ion exchange chromatography step, the purification yield was greatly reduced. This reduction was probably due to the long term storage of these fractions.

Amino Acid Sequence of CRSP — Intact and tryptic fragments of the peptide purified from peak 5 were subjected to N-terminal sequence analysis using a protein sequencer (Fig. 2, arrows). Based on the sequence analysis data, its amino acid sequence was deduced up to the 37th residue (Fig. 2), except for the 2nd and 7th residues. The molecular mass of this peptide was estimated to be 4042.0 daltons (Da) from the sequence, assuming that two unidentified residues were cysteines forming an intramolecular disulfide bond. To determine the precise molecular mass of CRSP, the purified peptide was analyzed with an ESI mass spectrometer, and its molecular mass was determined to be 4130.6±17.0 Da. The difference between the molecular mass estimated from the peptide sequence and that determined by mass spectrometry was assumed to be due to the presence of two methionine sulfoxides (16.0 Da) and a C-terminal glycine amide (57.0 Da), which were hard to identify by protein sequencing.

To determine the cDNA sequence of a precursor for CRSP, we synthesized degenerated primers corresponding to the N and C termini of the sequence, and a PCR reaction was performed with porcine genomic DNA. An amplified fragment was radiolabeled for screening of a hypothalamus cDNA library.
and 12 positive clones were obtained out of $10^5$ clones. Fig. 2 shows the nucleotide and deduced amino acid sequences of a CRSP precursor. Analysis of the amino acid sequence of the precursor for CRSP showed that it had typical features of a secretory precursor protein for a biologically active peptide. The nucleotide sequence of a CRSP mRNA was isolated from various porcine tissues and probed with a 32P-labeled DNA probe. The arrow indicates ~1.0 kilobase.

**TABLE II**

Distribution of IR-CRSP in porcine tissues

| Tissue                        | IR-CRSP (pmol/g wet tissue) |
|-------------------------------|-----------------------------|
| Cerebral cortex               | 0.29 ± 0.04                 |
| Cerebellum                    | 0.18 ± 0.02                 |
| Midbrain                      | 7.5 ± 0.9                   |
| Hippocampus                   | 0.78 ± 0.16                 |
| Caudate nucleus               | 1.3 ± 0.1                   |
| Thalamus                      | 3.5 ± 0.4                   |
| Hypothalamus                  | 9.9 ± 1.2                   |
| Pons/medulla oblongata        | 2.2 ± 0.3                   |
| Spinal cord                   | 0.52 ± 0.06                 |
| Olfactory bulb                | 0.74 ± 0.22                 |
| Anterior pituitary            | 14 ± 2                      |
| Posterior pituitary           | 96 ± 15                     |
| Lung                          | 0.11 ± 0.00                 |
| Adrenal gland                 | 0.42 ± 0.05                 |
| Kidney, cortex                | 0.12 ± 0.01                 |
| Kidney, medulla               | 0.088 ± 0.039               |
| Liver                         | 0.13 ± 0.02                 |
| Spleen                        | 0.11 ± 0.01                 |
| Stomach                       | 0.29 ± 0.00                 |
| Small intestine               | 0.072 ± 0.018               |
| Pancreas                      | 0.066 ± 0.010               |
| Thyroid gland                 | 68 ± 39                     |
| Ovary                         | 0.18 ± 0.09                 |
| Cardiac atrium                | 0.20 ± 0.04                 |
| Cardiac ventricle             | 0.21 ± 0.09                 |
| Aorta, thoracic               | 0.33 ± 0.19                 |

and 12 positive clones were obtained out of $10^5$ clones. Fig. 2 shows the nucleotide and deduced amino acid sequences of a CRSP precursor. Analysis of the amino acid sequence of the precursor for CRSP showed that it had typical features of a secretory precursor protein for a biologically active peptide including a signal sequence (Fig. 2, *italics*), dibasic cleavage sites (*double-underlined*), and a cleavage/amidation site (*gray box*). This cDNA sequence supported the existence of glycine amide at the C-terminal of CRSP.

We next chemically synthesized CRSP based on the sequence and structure (C-terminal amidation and intramolecular disulfide linkage) thus determined. Synthetic CRSP or its methionine sulfoxide form elicits chromatographic and mass spectrometric behavior quite similar to that of the native peptide.

Both mature and prepro-CRSP sequences were utilized in a BLAST search of the DDBJ/GenBank™/EBI protein database, and this peptide and its precursor were found to show sequence similarity with CGRP and its related peptides (Fig. 3). To further find out a corresponding peptide and its precursor in other species, we searched the EST database and identified one candidate gene in horse in addition to horse CGRP. The candidate gene had 77% identity with porcine CRSP, and the amino acid sequence from the C terminus was highly conserved (see “Discussion”). In human and mouse, no corresponding gene has been identified that has significant similarity with CRSP except for α- and β-CGRP and amylin in both databases.

**Tissue Distribution of CRSP**—Messenger RNA levels for CRSP in various porcine tissues were estimated by Northern blot analysis (Fig. 4). Approximately 1.0 kilobase of CRSP mRNA was detected predominantly in the CNS, particularly in the hypothalamus and pons/medulla oblongata, but a faint band was observed in the spinal cord, where CGRP was abundantly expressed (24). Another strong band was detected in the thyroid gland, although no band was observed in other peripheral tissues.

To measure the concentrations of CRSP in various tissues, we raised a polyclonal antibody against CRSP-(24–38). Both CRSP-(24–38) and CRSP-(1–38) elicited comparable affinity to this antibody. Half-maximal inhibition of radiodinated ligand binding to the antibody by these peptides was observed at 10 pmol/tube, and no significant cross-reactivity was observed with porcine CT and CGRP (<0.001%). Table II shows the concentrations of immunoreactive (IR) CRSP in porcine tissues. The posterior pituitary was found to contain the highest concentration of IR-CRSP. CRSP was also abundant in the CNS, particularly in the midbrain, thalamus and hypothalamus (> 3.0 pmol/g wet weight). In the peripheral tissues, the thyroid gland contained an extremely high concentration of IR-CRSP (68 ± 39 pmol/g wet weight), but other peripheral tissues contained low levels of IR-CRSP (<0.5 pmol/g wet weight).

**Effects of CRSP on cAMP Production in Various Cells**—We next measured the effects of CRSP on cAMP production in LLC-PK1 cells along with porcine CT, porcine CGRP, and hu...
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reported to be stimulated predominantly by CGRP and AM, respectively (27). Although CGRP and AM increased cAMP concentration in Hs68 and Swiss 3T3 cells, CRSP did not alter the cAMP level in either cell line (Figs. 5, b and c).

Systemic Pharmacological Effects of CRSP—Because CRSP shares structural similarity with CGRP, CRSP was expected to elicit a vasodilatory effect comparable with that of CGRP. CRSP was injected into anesthetized rats up to 16 nmol/kg, and the blood pressure was measured. However, no significant decrease in the blood pressure was observed by bolus administration of CRSP. In the same system, CGRP induced a potent depressor effect (Fig. 6a).

We next examined the effects of CRSP on the plasma calcium levels and compared them to those of salmon and porcine CT (Fig. 6b). Bolus administration of CRSP to anesthetized rats at a dose of 16 nmol/kg resulted in a significant decrease in the plasma calcium level. The plasma calcium-reducing effect of CRSP was detectable up to 1 h and almost diminished 3 h after injection. On the other hand, injection of salmon or porcine CT caused a sustained reduction in the plasma calcium level even 3 h after injection.

Effects of CRSP and Its Related Peptides on Recombinant CT Receptor—Porcine CT receptor was transiently expressed in COS-7 cells, and we evaluated the effect of CRSP and its related peptides on these cells. In Fig. 7a, a dose-dependent production of cAMP was observed by stimulation of CRSP, salmon CT, porcine CT, porcine CGRP, and human AM. The production of cAMP was most strongly enhanced with salmon CT (a median effective dose (ED50) of 0.03 nM), which is known to stimulate mammalian CT receptor more potently than the respective endogenous CT. On the other hand, CRSP stimulated the CT receptor (ED50 of 0.20 nM) with a potency more than 350 times higher than that of porcine CT (ED50 = 71 nM), although its effect was weaker than that of salmon CT. None of these peptides stimulated the cAMP production in mock-transfected COS-7 cells (data not shown).

To investigate the binding activity of CRSP to CT receptor, 125I-labeled porcine CT and 10 pM to 10 nM CRSP, salmon CT, or porcine CT as a competitor were incubated with COS-7 cells that transiently expressed the CT receptor (Fig. 7c). The binding of 125I-labeled porcine CT to CT receptor in the COS-7 cells was abolished by the addition of an excess amount of each peptide. The median inhibition concentration (IC50) of CRSP in this binding assay system was found to be 1.3 nM, which was ~2-fold greater than that of salmon CT (0.61 nM).

Effect of CRSP on CT Receptor or CL Receptor with or without Co-transfection of RAMP—A recent report revealed that AM and CGRP receptors are composed of CL receptor and RAMPs with a single transmembrane domain protein, although CL receptor belongs to the family B of G-protein-coupled receptors. The CGRP receptor consisted of CL receptor and RAMP1, whereas the AM receptor consisted of CL receptor and RAMP2 or RAMP3 (28). High affinity amylin receptor was also shown to be constituted by co-expression of CT receptor and RAMPs (29, 30). On the basis of these findings, CT receptor or CL receptor was expressed in COS-7 cells with one of the RAMPs or a blank expression vector and stimulated with 100 nM CRSP, porcine CT, CGRP, or human AM to examine through which receptor-RAMP complex CRSP can stimulate cAMP production. Porcine CT stimulated the cAMP production more than 10-fold in the COS-7 cells expressing CT receptor with or without RAMP but not in those expressing CL receptor (data not shown). Porcine CGRP or human AM stimulated the cAMP production ~5-fold in the COS-7 cells expressing CL receptor with RAMP1, -2, or -3, respectively (data not shown). These results were compatible with those reported previously.
(25). On the other hand, ~10-fold enhancement of the cAMP production was observed by stimulation of CRSP in the COS-7 cells expressing CT receptor with or without RAMPs, whereas this peptide did not alter the cAMP level in the COS-7 cells expressing CL receptor with or without RAMPs (Fig. 7b).

**DISCUSSION**

In the present study, we isolated a novel peptide, CRSP, from a porcine brain extract by monitoring the cAMP production in LLC-PK1 cells. Amino acid sequence analysis and molecular cloning of CRSP and its precursor protein showed that this peptide shows significant sequence similarity to CGRP. CGRP is a potent vasodilatory peptide, and two isoforms have to date been reported in humans and rodents (31–34). CGRP type-α was generated from tissue-specific alternative splicing of mRNA transcribed from the CT gene (32). On the other hand, βCGRP gene encodes a CT-like sequence that is not transcribed into mRNA (34). Both genes have striking sequence similarity to each other. Because one form of CGRP corresponding to oCGRP has been identified in the pig, we first considered that CRSP was a porcine counterpart of the βCGRP. However, this peptide has a distinct structure, function, and distribution compared with those of α- and βCGRP as described below. Compared with the amino acid sequence of porcine CGRP, this peptide has 90% identity in the 10-amino acid sequence from the N terminus, and two cysteines that are deduced to form an intramolecular disulfide bond are conserved. On the other hand, only 40% identity between CRSP and CGRP was observed in the 10-amino acid sequence from the C terminus. In contrast, the amino acid sequences of human and porcine CGRPs are highly conserved throughout the molecule (Fig. 3). CRSP stimulated cAMP production in LLC-PK1 cells about 300-fold more potently than porcine CGRP (Fig. 5a). CRSP also stimulated the cAMP production in COS-7 cells expressing recombinant CT receptor in a manner similar to that in LLC-PK1 cells. On the other hand, this peptide did not augment the cAMP production at all in either Hs68 or Swiss 3T3 cells, in which it was stimulated by CGRP and adrenomedullin. Both α- and βCGRP are reported to be abundantly expressed in the rat spinal cord (24). On the other hand, Northern blot analysis data shows that CRSP is expressed predominantly in the hypothalamus and poorly in the spinal cord (Fig. 4). Although CRSP has structural similarity to CGRP, several lines of evidence listed above indicate that CRSP has characteristics distinct from those of CGRP and elicits its effects via the CT receptor. Thus, we designated this peptide as calcitonin receptor-stimulating peptide (CRSP).

By searching CRSP or related peptides in the EST database, one candidate peptide, named CGRP-I, was found in horse. In horse, another peptide named CGRP-II has been identified, which has 97, 87, and 89% identity with porcine and human α- and βCGRP, respectively, and this peptide is considered to be a counterpart of CRSP. The equine CGRP-I gene encodes a peptide having 77% identity with porcine CRSP, which is ~10% higher than that with porcine CGRP (67%). In particular, the sequence identity between equine CGRP-I and porcine CRSP is 80% in the 10-amino acid sequence from the C terminus, whereas that between equine CGRP-I and porcine CGRP is only 30%. The high identity in the N-terminal region and low identity in the C-terminal region is one of the features that differentiates CRSP from CGRP as described above. Because no corresponding gene has so far been found in humans and rodents by database searching, the peptides having high identity with CRSP might be limited to species evolutionarily close to pig. Further biochemical and pharmacological analyses using synthetic equine CGRP-I should be performed to determine whether it actually elicits CRSP-like effects.

CGRP, CT, AM, and amylin, belonging to the CT and CGRP superfamily, are widely distributed in the CNS as well as in the peripheral tissues and induce multiple biological effects, including vasodilation, calcium resorption in bone, and reduction in nutrient intake (31). However, their receptor systems are more complex than other biologically active peptides. Recent studies have verified that the functional AM receptor and CGRP receptor consist of two membrane proteins, CT receptor, and one of three RAMPs (30). CGRP is considered to stimulate cAMP production via a CL receptor-RAMP1 complex (30), whereas AM is shown to increase the intracellular signal via a CL receptor-RAMP2 or a CL receptor-RAMP3 complex (28). Furthermore, later studies showed that the expression of RAMP3 facilitated amylin to bind and activate CT receptor (29, 30). Although CRSP was found to stimulate CT receptor, these previous data indicated that the CRSP receptor should be characterized in detail in endogenous as well as recombinant systems. As shown in Fig. 5, CGRP and AM predominantly stimulated cAMP production in Hs68 and Swiss 3T3 cells, respectively, but CRSP did not alter the cAMP concentration in either of these cell lines. As clearly shown in Fig. 7b, CRSP did not stimulate cAMP production at all via porcine CL receptor in the presence or absence of porcine RAMPs in the COS-7 cell
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expression system. These findings demonstrate that CRSP does not stimulate endogenous and recombinant AM/CGRP receptors, although this peptide has high structural similarity to CGRP. However, CRSP stimulated cAMP production in the COS-7 cells expressing the recombinant CT receptor more potently than porcine CT (Fig. 7a), and co-expression of RAMPs did not alter the cAMP production level induced by CRSP stimulation (Fig. 7b). Binding of 125I-labeled porcine CT to the COS-7 cells expressing the recombinant CT receptor was prominently displaced by CRSP (Fig. 7c). The evidence described above indicate that both CRSP and CT can stimulate the CT receptor, although a detailed analysis of ligand-receptor interaction is required to understand how two structurally distinct peptides can bind to the same receptor.

Bolus injection of CRSP into rats transiently reduced the plasma calcium concentration, although its effect was weaker than that of salmon and porcine CT (Fig. 6). At least two mechanisms have been elucidated for the plasma calcium reduction. 1) CT receptor is abundantly expressed on osteoclast cells (35). Treatment of resorbing bones with CT increases loss of the ruffled border of the osteoclast cells and decreases the release of lysosomal enzymes from them, which results in a reduction in the plasma calcium level (16, 17). 2) Renal tubular epithelia express CT receptor. The excretion of plasma calcium through the kidney is stimulated by CT by inhibiting renal tubular calcium reabsorption (18). As both osteoclast and renal epithelial cells express CT receptor, CRSP is deduced to activate CT receptor on these cells and decrease the plasma calcium concentration. In contrast to the potent effects of CRSP on cAMP production on LLC-PK1 cells and COS-7 cells expressing CT receptor, the plasma calcium-reducing activity of CRSP is much weaker than that of porcine CT. The difference in the biological potency of CRSP in the in vivo and in vitro system may indicate the presence of unidentified physiological effects and mechanisms distinct from those of CT.

The CT receptor is also expressed in the CNS at a high level (36) in addition to the peripheral system. Although CT receptor in the CNS has been recognized to be involved in the regulation of appetite (37), gastric acid secretion (38), and analgesic effect (39), endogenous ligands for the CT receptor in the brain remain unidentified. In fact, CT is mainly synthesized and secreted from the thyroid gland but is almost undetectable in the CNS. The CT secreted from the thyroid and circulating in the blood is not considered to be incorporated into the CNS beyond the blood brain barrier. Several attempts have been made to identify an endogenous ligand that stimulates cAMP production via CT receptor in the CNS. Fischer et al. (22) reported that human calcitonin- and C-terminal adjacent peptide-like immunoreactivities were found in extracts of the human periventricular mesencephalic region. Sexton and Hilton (23) detect salmon CT-like immunoreactivity in rat brain using an anti-salmon CT antibody. This finding was supported by CT bioassays including cAMP production and receptor binding to culture cells and brain membranes. In 1998, these authors reported the successful purification of brain CT-like peptide with a molecular mass of 3267 Da, which was N-terminally blocked but had a unique 6-amino acid sequence of EKQSP, in the molecule (24). Unfortunately, no subsequent study has been reported for this peptide. In our study, the structure of CRSP was determined by direct analysis of the peptide purified from porcine brain extracts, and the sequence of prepro-CRSP was deduced from cDNA cloning and analysis. The CT-like biological activity was confirmed by the data showing that synthetic CRSP stimulated cAMP production via recombinant CT receptor and reduced the rat plasma calcium concentration by bolus administration. These findings suggest that this pep-

![Fig. 7. Effects of CRSP and its related peptides on the expression system of CT receptor alone, or CT receptor or CL receptor with RAMPs.](image)

- a. dose-dependent stimulation of adenylyl cyclase activity via porcine CT receptor. COS-7 cells expressing porcine CT receptor were stimulated with the indicated concentrations of CRSP (closed circle), salmon CT (closed square), porcine CT (open square), porcine CGRP (open circle), and human AM (open triangle).
- b. effect of CRSP on cAMP production via porcine CT receptor or CL receptor in the presence or absence of RAMPs. COS-7 cells were co-transfected with CT receptor or CL receptor cDNA and one of RAMP1, -2, -3 cDNA or pcDNA. The cells were stimulated with 100 nM of CRSP. c. inhibition of 125I-labeled porcine CT binding to the CT receptor by unlabeled peptides. Binding of 125I-labeled porcine CT to porcine CT receptor expressed in COS-7 cells was measured in competition against unlabeled CRSP (closed circle), salmon CT (closed square), and porcine CT (open square) at the indicated concentrations. COS-7 cells were transfected with cDNAs using Lipo-jectAMINE Plus, cultured for 24 h, and then used for the experiments. Each point represents the mean ± S.E. of three (a and b) and four (c) separate determinations.
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tide with low amino acid sequence similarity with CT is a
cognate ligand for CT receptor in the CNS, although it is
necessary to examine whether a unique CRSP receptor that is
more specific than CT receptor is present or not. Identification
of CRSP in other species as well as the elucidation of its
physiological effects and expression profiles in the CNS and
peripheral system will clarify the relationship between CRSP
and the previously reported CT-like peptide.

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