

**Calcitonin receptor-stimulating peptides,
new members of the calcitonin gene-related peptide superfamily**

Takeshi Katafuchi and Naoto Minamino

Department of Pharmacology, National Cardiovascular Center Research Institute,

5-7-1 Fujishirodai, Suita 565-8565, Osaka, Japan

(e-mail: katafuch@ri.ncvc.go.jp)

Introduction:

The calcitonin gene-related peptide (CGRP) superfamily is composed of CGRP and its structurally related peptides, i.e., amylin (AMY), adrenomedullin (AM) and calcitonin (CT), is also called "the calcitonin (CT)/CGRP family" or "the CGRP family" (Fig. 1). Each member of the superfamily possesses a ring structure formed by an intramolecular disulfide linkage and a C-terminal amide structure, which are the common structural features of the CGRP superfamily. Although these peptides have amino acid sequence similarity, each member has a distinct biological activity. For example, CT regulates plasma calcium concentration(1,2); CGRP and AM induce vasodilation (3-7); AMY inhibits insulin-stimulated glucose uptake and glycogen synthesis in skeletal muscle (8).

In 2003, we isolated a novel member of the CGRP superfamily from the acid extracts porcine brain, by monitoring cAMP production in the porcine renal epithelial cell line LLC-PK₁ and designated it calcitonin receptor-stimulating peptide-1 (CRSP-1) (9). By the cDNA cloning, two other members having the similar structure were identified and designated CRSP-2 and CRSP-3 (10). As shown in Fig. 1, all CRSPs possess the six-amino acid ring structure and C-terminal amide, which are shared by the members of the CGRP superfamily. Although CRSPs show the highest amino acid sequence similarity with CGRP in the CGRP superfamily, CRSP and CGRP elicit completely different biological activity. CRSP-1 was also identified in the cow and dog, and CRSP-2 was identified in the dog, but none have the C-terminal amide structure. In this short review, we describe the current status of CRSP research in the structure, receptor, tissue distribution and function of these peptides.

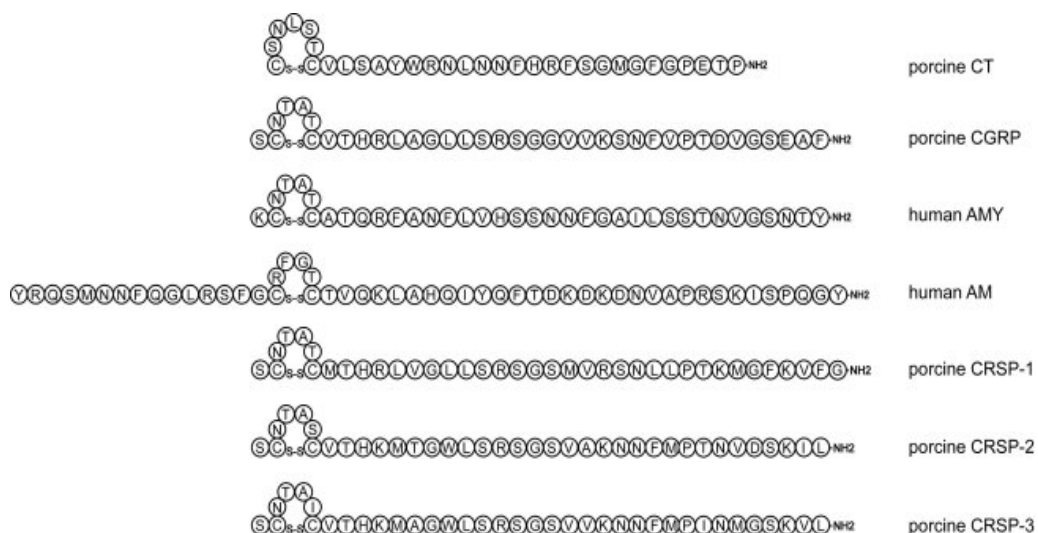


Figure 1. Amino acid sequences of the CGRP superfamily members; porcine calcitonin (CT), porcine calcitonin gene-related peptide (CGRP), human amylin (AMY), human adrenomedullin (AM), porcine calcitonin receptor-stimulating peptide (CRSP-1), porcine CRSP-2 and porcine CRSP-3.

Structure:

CRSP-1, CRSP-2 and CRSP-3 are 37 and 38 amino acids in length, and include two cysteines that form an intramolecular disulfide linkage and a C-terminal amide structure (Fig. 1). CRSP-1 was the first identified member and was purified from porcine brain by monitoring cAMP levels in LLC-PK₁ cells (9). The cDNA clone of CRSP-1 was isolated from the porcine hypothalamus cDNA library. By the cDNA sequence analysis, the CRSP-1 precursor was shown to have the typical features of a peptide hormone precursor including a signal sequence, prohormone convertase (PC) cleavage sites (11), and a cleavage/amidation site (Fig. 2). As shown in Fig. 3B, the amino acid sequence of mature CRSP-1 shows the highest identity with that of equine CGRP-I (77%) (12). Equine CGRP-I was later verified to be a counterpart of CRSP-1 in the horse. Porcine CRSP-1 shows a high amino acid sequence identity with porcine and human CGRP (~60%) and a low identity with human AMY, porcine CT and human AM (< 37%). Furthermore, porcine CRSP-1 has only 33% identity with porcine CGRP in the C-terminal 12-residue sequence, while the remainder of CRSP-1 has 85% identity with that of porcine CGRP. CGRP(27-37) is the shortest fragment which elicits significant affinity for CGRP₁ receptor (13,14), and was known to include key residues for interaction with the receptor (15-17). Thus, the low amino acid sequence identity in the C-terminal 12-residue sequence is considered to change the receptor specificity of CRSP-1.

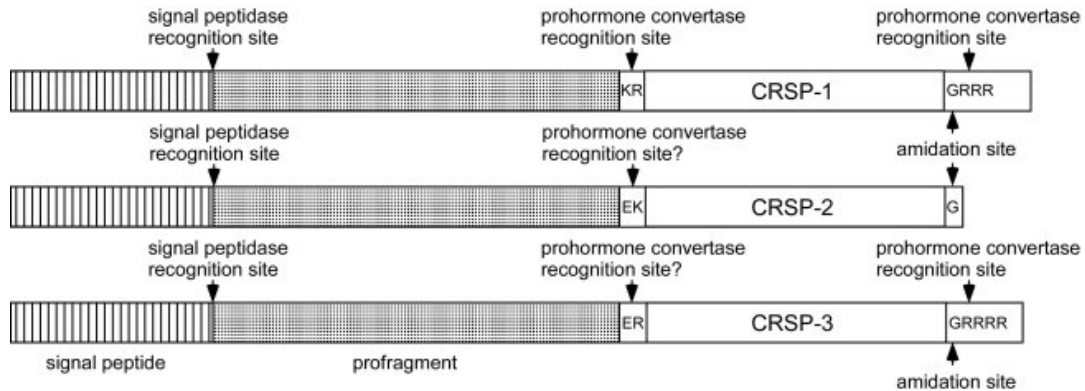


Figure 2. Schematic presentation of three porcine CRSP precursors. Signal peptide, profragment, prohormone convertase recognition site, biologically active unit and C-terminal fragment are shown in each precursor. Signal peptidase recognition site, prohormone convertase recognition sites, and amidation sites are indicated by arrows.

CRSP-2 and CRSP-3 cDNAs were identified by screening of the porcine hypothalamus cDNA library using a cDNA probe of CRSP-1 (10). The deduced mature CRSP-2 and CRSP-3 peptides show high sequence identity with those of CRSP-1 and CGRP, but amino acid replacements are frequently observed in their C-terminal region (Fig. 1).

Amino acid sequences of CRSP-2 and CRSP-3 precursors are well conserved as compared with those of CRSP-1 and CGRP, but the sequences dividing the profragments and mature peptides of CRSP-2 and CRSP-3 are -Glu-Arg- and -Glu-Lys-, respectively, which are not typical recognition sequences for PC, while that of CRSP-1 is its typical cleavage sequence, -Lys-Arg- (Fig. 2). The 37-residue peptides of CRSP-2 and CRSP-3 shown in Fig. 1 and Fig. 3D have not yet been identified as their mature and endogenous forms.

On the other hand, the cDNA sequence encoding the 5'-non-coding region and signal peptide of CRSP-2 are almost identical with that of CRSP-1 (> 98% identity), while the nucleotide and the deduced amino acid sequences of the remainder of CRSP-2 shows a higher similarity with those of CRSP-3 than those of CRSP-1. This fact suggests the possibility that these three CRSPs were evolutionarily generated from a common ancestral gene.



Figure 3. Alignment of amino acid sequences of CRSPs and their related peptides. (A) Amino acid sequence of porcine CRSP-1 (pCRSP-1) is aligned with those of porcine CGRP (pCGRP), human α CGRP (haCGRP), human β CGRP (hbCGRP). (B) Amino acid sequence of pCRSP-1 is aligned with those of bovine CRSP-1 (bCRSP-1), canine CRSP-1 (cCRSP-1) and equine CGRP-I (eCGRP-I). (C) Amino acid sequences of haCGRP and hbCGRP are aligned with those of pCGRP, chicken CGRP (chCGRP), frog CGRP (frCGRP) and salmon CGRP (saCGRP). (D) Amino acid sequence of deduced mature porcine CRSP-2 (pCRSP-2) is aligned with those of porcine CRSP-3 (pCRSP-3), pCRSP-1 and pCGRP. The amino acid residues identical to pCRSP-1 (A), pCRSP-1 (B), haCGRP (C) and pCRSP-2 (D) are shaded.

In order to identify CRSPs in mammals other than the pig, we first screened rat and

human brain cDNA and human genome libraries with three CRSP cDNA probes, and searched the EST and genome databases using the query engine. However, no cDNA or gene having significant nucleotide sequence identity with any of the three CRSP cDNAs has been identified, except for the cDNAs and genes of rat, human and mouse α and β CGRP, and amylin. To identify CRSPs in mammals evolutionarily close to the pig, bovine and canine thyroid cDNA libraries were screened using cDNA probes of three porcine CRSPs, and bovine and canine CRSP-1, and canine CRSP-2 cDNAs were isolated (18). In contrast to the structural difference between CRSP-1 and porcine CGRP, the 27th-36th residues of CRSP-1 are well-conserved among porcine, bovine and canine CRSP-1, and equine CGRP-I, and their amino acid identities are 90%. However, the amino acid residues after the 36th residue are distinct in these four peptides, and bovine and canine CRSP-1 further lack the C-terminal amide (Fig. 3B). The deduced amino acid sequences of bovine and canine CRSP precursors do not have a glycine for the C-terminal amide structure (18,19). These peptides are the first members lacking the C-terminal amide in the CGRP superfamily. The C-terminal residue of the other CGRP superfamily members are completely conserved from fishes to mammals; Pro-amide for CT, Phe-amide for CGRP, Tyr-amide for AMY and Tyr-amide for AM are, but the 37th residues of CRSP-1 in these species are inconsistent with each other (Figs. 3B and 3C), which is recognized as another structural feature of CRSP-1. On the other hand, canine CRSP-2 has low amino acid sequence identity (36%) with porcine CRSP-2, and these peptides have no characteristic structural feature between them (Fig. 3D).

Receptors:

CRSP-1 was purified by monitoring the enhancement of the cAMP level in LLC-PK₁ cells, and synthetic CRSP-1 enhanced the cAMP production in LLC-PK₁ cells in a dose-dependent manner. On the other hand, CRSP-1 did not increase the cAMP level in Hs68 or Swiss 3T3 fibroblasts expressing the CGRP receptor or the AM receptor, respectively. This fact indicates that LLC-PK₁ cells express the CRSP-1 receptor, which enhances the cAMP level in the cells. As LLC-PK₁ cells are known to express the CT receptor (20), we hypothesized that the enhancement of cAMP production by CRSP-1 was mediated by the activation of the CT receptor. To examine the effect of CRSP-1 on the CT receptor, COS-7 cells were transfected with porcine CT receptor cDNA, and stimulated with CRSP-1 and related peptides. CRSP-1 dose-dependently enhanced the cAMP production in COS-7 cells expressing porcine CT receptor, and its potency was more than 100-fold greater than that of porcine CT. The effects of CRSPs on recombinant receptors for the CGRP superfamily members were evaluated by administration of the peptides to the porcine core receptor (CT or CT-like (CL) receptor) and porcine receptor activity-modifying protein (RAMP) co-expression system (21). Co-expression of CT or CL receptor with one of three RAMPs

was reported to generate a specific receptor for each member of the CGRP superfamily, as shown in the upper portion of Table 1 (21-24). On the other hand, CRSP-1 strongly stimulated the cAMP production only via the CT receptor, and co-expression of RAMP with the CT receptor did not alter the stimulatory effect of CRSP-1 (the middle portion of Table 1).

Table 1. Magnitude of cAMP-producing activity of the CGRP superfamily in COS-7 cells co-expressing core receptor (CT or CL receptor) and one of the RAMPs of pig origin.

	CT receptor				CL receptor			
	none	RAMP1	RAMP2	RAMP3	none	RAMP1	RAMP2	RAMP3
pCT	++	++	++	++	-	-	-	-
pCGRP	+	++	+	+	-	+++	+	+
hAM	(+)	(+)	(+)	(+)	-	+	+++	+++
hAMY	+	+	+	++	-	-	-	-
pCRSP-1	+++	+++	+++	+++	-	-	-	-
pCRSP-2	(+)	(+)	(+)	(+)	-	-	-	-
pCRSP-3	(+)	(+)	(+)	(+)	-	-	-	-
bCRSP-1	+++	+++	+++	+++	-	-	-	-
cCRSP-1	+	+	+	+	-	-	-	-
eCGRP-I	++	++	++	++	-	-	-	-
cCRSP-2	-	-	-	-	-	-	-	-

+++, $ED_{50} < 10^{-9}$ M; ++, 10^{-9} M $< ED_{50} < 10^{-8}$ M; +, 10^{-8} M $< ED_{50} < 10^{-7}$ M; (+), $ED_{50} > 10^{-7}$ M; -, no significant alteration in the cAMP concentration. p, Porcine; h, human; b, bovine; c, canine; e, equine.

As mature forms of CRSP-2 and CRSP-3 have not yet been identified, 37 amino acid-long peptides corresponding to mature CRSP-1 were tentatively synthesized and their activity was examined. CRSP-2 and CRSP-3 weakly enhanced the cAMP level in LLC-PK₁ cells at high doses. CRSP-2 and CRSP-3 weakly stimulated the cAMP production via the CT receptor, and did not stimulate it via the CL receptor at all, even in the presence of RAMPs (the middle portion of Table 1).

Effects of bovine and canine CRSP-1, canine CRSP-2 and equine CGRP-I for the core receptor and RAMP co-expressing cells were shown in the lower portion of Table 1. Bovine and canine CRSP-1 as well as equine CGRP-I dose-dependently enhanced cAMP production in the cells expressing CT receptor, but canine CRSP-2 did not stimulate it at all. Co-expression of one of three porcine RAMPs with the CT receptor did not alter the stimulatory effects of these peptides. The rank order of their potency was porcine CRSP-1 \geq bovine CRSP-1 \geq equine CGRP-I $>$ canine CRSP-1 \gg canine CRSP-2. Porcine, bovine and

canine CRSP-1s, equine CGRP-I and canine CRSP-2 did not enhance the cAMP level via the porcine CL receptor even in the presence of RAMP. These results indicate that bovine and canine CRSP-1s and equine CGRP-I share biological characteristics with porcine CRSP-1. Taken together, equine CGRP-I is concluded to be an orthologue of CRSP-1 in the horse. It is important that the amide structure at the C-terminus of CRSP-1 is not essential for the activation of the CT receptor, while CT requires the C-terminal amide structure for eliciting its activity (25). The C-terminal amino acid sequence of CRSP-1 has low sequence identity with that of CGRP (Fig. 3A), but is highly conserved in four species, pig, cow, dog and horse (Fig. 3C). Thus, the C-terminal sequence of CRSP-1 is crucial for recognition of the CT receptor, but its very C-terminal end is not considered to interact with the receptor.

Tissue distribution and expression:

CRSP-1, CRSP-2 and CRSP-3 mRNAs were detected in various tissues as shown in Table 2, and their expression profiles were similar to that of CGRP. By Northern blot analysis, mRNAs of three CRSPs were detected in the CNS, pituitary and thyroid gland. Low levels of each CRSP mRNA were detected by RT-PCR in several peripheral tissues. On the other hand, CT mRNA is found in the adrenal and thyroid glands, and is not detected in the CNS at all, as reported previously (26). These facts suggest that three CRSPs regulate physiological events both in the CNS and in the peripheral tissues, while the physiological action of CT is not elicited in the CNS. The radioimmunoassay using the antiserum raised against porcine CRSP-1 indeed showed that the midbrain, hypothalamus, pituitary and thyroid gland contained high concentrations (> 5 pmol/g wet tissue) of immunoreactive CRSP-1, and this result was in agreement with those obtained by Northern blot and RT-PCR analyses.

Table 2. Tissue expression levels of three CRSPs, CGRP and CT in the pig.

tissue	CRSP-1	CRSP-2	CRSP-3	CGRP	CT
cerebral cortex	med	med	med	-	-
cerebellum	-	-	-	-	-
midbrain	high	high	high	high	-
thalamus	med	med	med	med	-
hypothalamus	high	high	high	high	-
pons/medulla oblongata	med	med	med	med	-
spinal cord	low	low	low	med	-
pituitary	med	med	med	-	-
lung	low	-	low	-	-
adrenal gland	low	-	-	-	low
kidney	-	-	-	-	-
spleen	-	-	-	-	-
liver	low	-	-	-	-
small intestine	low	-	-	-	-
thyroid gland	high	high	high	high	high
ovary	low	-	low	-	-
cardiac atrium	-	-	-	-	-
cardiac ventricle	-	-	-	-	-
thoracic aorta	-	-	-	-	-

-, not detectable.

Physiological effects:

Although CRSP-1 has high amino acid sequence identity with CGRP, it is a specific ligand for the CT receptor and its potency is far higher than that of CT. Thus, it was an interesting point whether CRSP-1 induced its effect on blood pressure or plasma calcium concentration when injected into rats. The blood pressure was significantly reduced by bolus administration of CGRP into anesthetized rats, but it was not altered by that of CRSP-1. On the other hand, the plasma calcium concentration was significantly reduced by bolus administration of CRSP-1. These results indicate that CRSP-1 in systemic circulation stimulates the CT receptor in bone and kidney, and regulates the plasma calcium concentration by the mechanism similar to that of CT.

In the CNS, CRSP-1 is expressed and synthesized mainly in the midbrain and hypothalamus. The CT receptor is abundantly expressed in the paraventricular nucleus of the hypothalamus (27), and the intracerebroventricular injection of CT has been reported to reduce food intake (28,29) and gastric acid secretion (30), and to induce analgesia (31,32). However, only a few reports have been published that indicate the possible existence of a CT-like molecule in the brain; Fischer *et al.* reported that human calcitonin- and katecalcin-like immunoreactivities were detected in the extracts of the human periventricular mesencephalic region(26); Sexton *et al.* observed salmon CT-like immunoreactivity in rat brain using a guinea pig anti-salmon CT antibody (33). Hilton *et al.* purified a brain CT-like peptide, which was an N-terminally blocked peptide with an internal six amino acid sequence of EKSQSP in the molecule, but this partial sequence had no significant similarity with CRSP-1 (34). As shown in Table 2, CT mRNA was not detected in any brain region, even by highly sensitive RT-PCR analysis. Thus, CRSP-1 is considered to be the endogenous ligand for the central CT receptor that had not been identified for years, and is expected to regulate food intake, gastric acid secretion and nociception through the central CT receptor.

Conclusion:

The discovery of CRSPs and elucidation of their biological properties revealed new structural and biological features of the CGRP superfamily. Porcine CRSP-1 stimulates the porcine CT receptor 100-fold greater than porcine CT, but does not activate the porcine CGRP receptor at all, although it has a high structural similarity with CGRP. Porcine CRSP-1 and CGRP have higher structural identity as a whole molecule, but there are a relatively large number of substitutions in the 27th-36th amino acid sequence of CRSP-1. In this region, the amino acid sequence of porcine, bovine, canine and equine CRSP-1s are highly conserved. It is noteworthy that the 27th, 28th and 32nd residues in CRSP-1 are completely conserved in the four species. On the other hand, these three residues are Phe, Val and Val in CGRP, respectively, which are conserved from fishes to mammals. The amino acid residues at the

37th position and thereafter, including the C-terminal amidation, are not conserved in the four CRSP-1s so far identified. These results indicate that the receptor specificity of CRSP-1 is defined by the limited number of residues in the C-terminal sequence but not by its very C-terminal end.

It is highly probable that CRSP-1 is the endogenous ligand for the central CT receptor and may regulate appetite and nociception in the brain. To address this issue, it is necessary to examine whether intracerebroventricular injection of CRSP-1 inhibits appetite as well as gastric acid secretion, and induces analgesia. Furthermore, *in situ* identification of CRSP-1-secreting cells and CT receptor-expressing cells will facilitate the elucidation of the physiological function of CRSP-1 in the CNS.

As a limited number of reports have been published since the discovery of porcine CRSP-1, two important questions remain to be answered to understand the biological properties of the three CRSPs. The first question is about the presence of CRSP or CRSP-like peptide in humans and rodents. Even by experimental screening and database search, the counterparts of three CRSPs have not yet been identified in humans or rodents, suggesting that human and rodent CRSP-1 have a low structural similarity with porcine CRSP-1. The second question is about the receptors of CRSP-2 and CRSP-3, as these peptides failed to activate the known receptor/RAMP system. One possibility is that the tentatively synthesized peptides are not endogenous forms. Another possibility is that CRSP-2 and CRSP-3 have their own receptors. In fact, the C-terminal regions of CRSP-2 and CRSP-3 have low amino acid sequence identity with CRSP-1 and CGRP. In any case, it is essential to identify the endogenous and mature molecular forms of CRSP-2 and CRSP-3.

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