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Identification, structural determination, and biological activity of bovine and canine calcitonin receptor-stimulating peptides☆

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Abstract

We have recently identified in porcine brain a series of new peptides, designated calcitonin receptor-stimulating peptide-1 (CRSP-1), CRSP-2, and CRSP-3, but failed to find their counterparts in humans and rodents by either database searching or experimental cross-hybridization. In this study, we isolated cDNAs encoding precursors of bovine CRSP-1, canine CRSP-1, and canine CRSP-2 from their thyroid cDNA libraries. Although the deduced mature amino acid sequences of bovine and canine CRSP-1s and canine CRSP-2 showed identity with their respective porcine CRSP counterparts, none of them had a C-terminal amide structure. In LLC-PK_1 cells endogenously expressing the calcitonin (CT) receptor, bovine and canine CRSP-1s enhanced the cAMP production, while canine CRSP-2 did not stimulate it at all. Equine CGRP-I had a high identity in its amino acid sequence with porcine CRSP-1 and stimulated LLC-PK_1 cells at a potency comparable to that of porcine CT. None of these CRSPs or equine CGRP-I stimulated the CT-like receptor, even in the presence of receptor activity-modifying proteins. These results demonstrate that CRSP-1, a new class of biologically active peptide, is present in animals evolutionarily close to pigs and induces its activity through the calcitonin receptor, suggesting a wide existence and common properties of this peptide in mammals.

Keywords: Calcitonin receptor-stimulating peptide; Calcitonin; Calcitonin gene-related peptide; Calcitonin receptor; Calcitonin-like receptor; Receptor activity-modifying protein

Calcitonin (CT) receptor-stimulating peptide-1 (CRSP-1) was isolated from porcine brain extracts as a candidate ligand for brain CT receptor [1]. To date, second and third members of the porcine CRSP family have been identified in the cDNA library of the hypothalamus and designated CRSP-2 and CRSP-3 [2]. Northern blot and RT-PCR analyses revealed that these peptides are expressed mainly in the central nervous system and thyroid gland, and that their expression profiles were similar to each other. These three peptides have a C-terminal amide, as well as a ring structure formed by a disulfide linkage, and show higher sequence identity with calcitonin gene-related peptide (CGRP) than with CT. However, these peptides did not stimulate cAMP production via a functional CGRP receptor that was composed of a CT-like (CL) receptor and receptor activity-modifying protein 1 (RAMP1) [3,4]. On the other hand, CRSP-1 stimulated the cAMP production via an endogenous or recombinant CT receptor [5]. CRSP-2 and CRSP-3 did not stimulate cAMP production via either the CT receptor or CL receptor, and co-expression of RAMPs did not alter their effects [2].

Although porcine CRSPs have remarkable features in their structure and activity, counterparts of CRSPs should be present in other mammals as endogenous ligands for the brain CT receptors. By the cross-hybridization experiments using porcine CRSP cDNA as probes, we failed to detect the CRSP counterparts in human and rodent cDNA and genomic DNA libraries. By searching the genome and expressed sequence tag (EST) databases in humans and mice, only α and βCGRP [6,7], and amylin (AMY) [8,9] were found to show significant similarity with CRSPs. In the databases

☆ The nucleotide sequence reported in this paper has been submitted to the DDBJ/GenBank/EBI Data Bank with Accession Nos. AB125101 for bovine CRSP-1, AB125102 for canine CRSP-1, and AB125103 for canine CRSP-2.

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of other animals, the amino acid sequence encoded by equine CGRP-I cDNA showed the highest similarity (77%) with that of porcine CRSP-1 [10]. We assumed that equine CGRP-I is a counterpart of porcine CRSP-1 and that the CRSP system at least exists in species that are evolutionarily close to the pig. Unfortunately, as a relatively small number of sequences have been registered in the databases of non-human and non-rodent animals, we constructed bovine and canine thyroid cDNA libraries, screened them using pig CRSP-1, CRSP-2, and CRSP-3 as probes, and identified bovine CRSP-1, canine CRSP-1, and canine CRSP-2 cDNAs. In this paper, we report on the cDNA and the deduced amino acid sequences of CRSPs in the dog and cow, along with their biological activities.

Materials and methods

Materials. An Oligotex-dT$_{30}$ mRNA purification kit was purchased from Takara (Osaka, Japan). A Timesaver cDNA synthesis kit was obtained from Amersham–Pharmacia Biotech (Uppsala, Sweden). A Lambda ZAP II bacteriophage vector and a Giga Pack Gold III in vitro packaging extract were purchased from Stratagene (La Jolla, CA, USA). Synthetic porcine CRSP-1, CT, CGRP, and human adrenomedullin (AM) were obtained as reported previously. Bovine and canine CRSP-1, equine CGRP-I, and canine CRSP-2 were prepared by Peptide Research Institute (Osaka, Japan). A mammalian expression vector pcDNA3.1(+) was purchased from Promega (Madison, WI, USA). LipofectAMINE Plus transfection reagent was obtained from Invitrogen (Carlsbad, CA, USA).

Isolation of bovine and canine CRSP-1 and canine CRSP-2 cDNA by screening of a thyroid cDNA library. Total RNAs of bovine and canine thyroid were extracted by the acid guanidium thiocyanate/phenol/chloroform procedure [11], and poly(A)$^+$ RNA was purified using an Oligotex-dT$_{30}$ mRNA purification kit. Bovine and canine cDNAs were synthesized from the poly(A)$^+$ RNAs using a Timesaver cDNA synthesis kit, inserted into a λZAP II, and packaged using a Giga Pack Gold III packaging extract. Approximately 2×10^5 independent clones were screened using ^{32}P-labeled full-length cDNAs of porcine CRSP-1, CRSP-2 or CRSP-3. Filter lifts were hybridized in a buffer containing 20% formamide, 0.09 M sodium citrate (pH 7.0), 0.9 M NaCl, 0.5% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone, and 0.5% SDS at 42 °C for 16 h. Filters were rinsed twice at room temperature in 0.03 M sodium citrate (pH 7.0), 0.3 M NaCl, and 0.1% SDS for 5 min and twice at 55 °C in 7.5 mM sodium citrate (pH 7.0), 75 mM NaCl, and 0.1% SDS for 1 h. Isolated positive clones were rescued as pBluescript SK$^-$, and the DNA sequence was determined by the dideoxynucleotide chain termination method using ABI 373A DNA sequencer (Applied Biosystems).

Measurement of cAMP production in LLC-PK$_1$ cells. Cells were harvested and cultured on 48-well plates for 2 days. The cells were washed twice with Dulbecco's modified Eagle's medium (DMEM)/Hepes (20 mM, pH 7.4) containing 0.5 mM 3-isobutyl-1-methyl xanthine (Sigma) and 0.05% bovine serum albumin (fraction V, Sigma), and incubated in the same medium for 30 min at 37 °C. The incubation medium was then replaced with 200 μl of medium in which the sample of interest was dissolved and further incubated at 37 °C for another 30 min. Aliquots (100 μl) of the incubation media were succinylated, evaporated, and then submitted to radioimmunoassay for cAMP as reported previously [1].

Co-expression of receptor and RAMP cDNA in COS-7 cells and measurement of cAMP production. COS-7 cells cultured as described previously [1] were harvested and seeded in a 48-well plate at a density of 2×10^6/well. Following 24-h cultivation, the cDNAs of porcine CT or CL receptor and one of the three RAMPs ligated into pcDNA3.1(+) expression vector [12] were co-transfected (cDNA ratio 1:1) into the COS-7 cells by a LipofectAMINE Plus reagent according to manufacturer's protocol. The transfected cells were used for cAMP assay 48 h after transfection.

Results

Complementary DNA and deduced amino acid sequences of bovine and canine CRSP-1

Bovine and canine CRSP-1 cDNAs were isolated from their thyroid cDNA libraries using a porcine CRSP-1 cDNA probe. Their cDNA sequences and the deduced amino acid sequences are shown in Figs. 1A and B. The mature peptides of bovine and canine CRSP-1

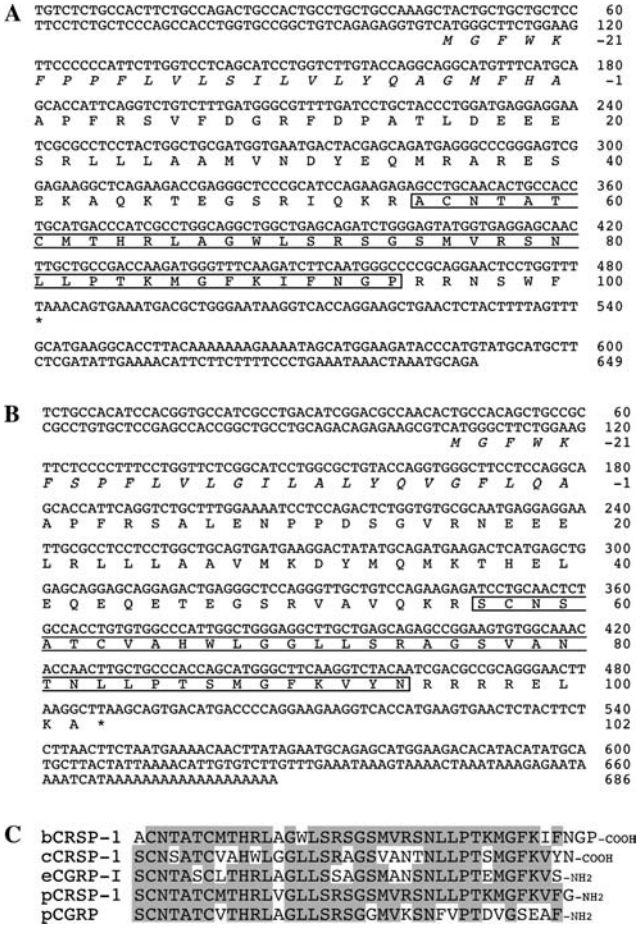

Fig. 1. Nucleotide and deduced amino acid sequences of bovine (A) and canine (B) CRSP-1, and alignment of deduced mature peptides of bovine and canine CRSP-1 with equine CGRP-I, porcine CRSP-1, and porcine CGRP (C). (A,B) Nucleotide and amino acid numbers are shown on the right. Putative signal peptides are shown in italics. The mature amino acid sequence of each CRSP-1 is boxed. (C) The deduced mature amino acid sequences of bovine (bCRSP-1) and canine CRSP-1 (cCRSP-1) are aligned with those of equine CGRP-I (eCGRP-I), porcine CRSP-1 (pCRSP-1), and porcine CGRP (pCGRP). The amino acids identical to porcine CRSP-1 are shaded.

were deduced to be 40 and 39 amino acids in length, respectively, and to have two cysteines forming an intramolecular disulfide linkage. Unlike the porcine counterpart, a glycine for a C-terminal amidation does not exist at the C-terminus of each CRSP-1 [13]. The deduced mature bovine and canine CRSP-1s were aligned with porcine CRSP-1, equine CGRP-I, and porcine CGRP in Fig. 1C. The mature amino acid sequences of bovine and canine CRSP-1 showed 87% and 66% identity with that of porcine CRSP-1, 70% and 70% with that of equine CGRP-I, and 68% and 51% with that of porcine CGRP, respectively. The amino acid sequence identity around the C-terminal region of CRSP-1s in these species is higher than that between CRSP-1 and CGRP in each species.

Complementary DNA and deduced amino acid sequences of canine CRSP-2

Fig. 2A shows the cDNA sequence and its deduced amino acid sequence of canine CRSP-2. The cDNA of canine CRSP-2 was isolated from the canine thyroid cDNA library by screening with a mixture of porcine CRSP-2 and CRSP-3 cDNA probes under low stringent conditions. Fig. 2B shows the amino acid sequence alignment of mature canine CRSP-2, CRSP-1, and CGRP, and porcine CRSP-2, CRSP-3, and CRSP-1. The mature amino acid sequences of canine CRSP-2 showed 30% and 53% identity with those of canine CRSP-1 and canine CGRP, and 47%, 42%, and 40% with those of porcine CRSP-2, CRSP-3, and CRSP-1, respectively. As the precursor of canine CRSP-2 does not possess a typical prohormone convertase (PC) cleavage site on either end of the deduced biologically active sequence unit [14], mature CRSP-2 is considered to consist of 47 amino acids, which are longer than other members of CRSP-related peptides. No other clone encoding the cDNA of a CRSP-related peptide was detected in the bovine or canine cDNA libraries under the present conditions.

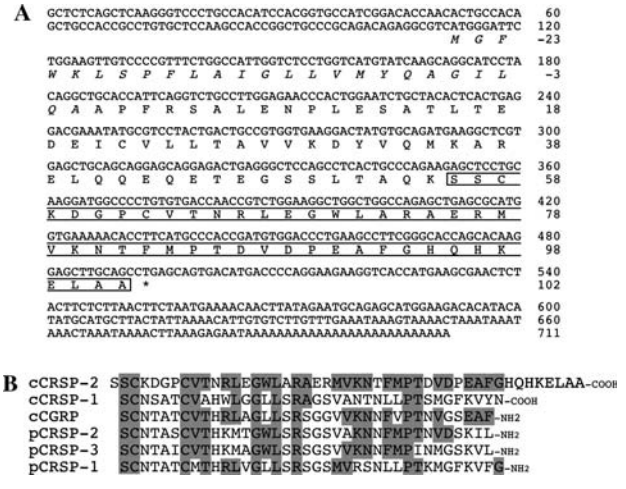

Fig. 2. Nucleotide and deduced amino acid sequences of canine CRSP-2 (A), and alignment of deduced mature peptide of canine CRSP-2 with canine CRSP-1 and CGRP, and porcine CRSP-2, CRSP-3, and CRSP-1 (B). (A) Nucleotide and amino acid numbers are shown on the right. Putative signal peptide is shown in italics. The mature amino acid sequence of canine CRSP-2 is boxed. (B) The deduced amino acid sequences of mature canine CRSP-2 (cCRSP-2) are aligned with those of canine CRSP-1 (cCRSP-1), canine CGRP (cCGRP), porcine CRSP-2 (pCRSP-2), porcine CRSP-3 (pCRSP-3), and porcine CRSP-1 (pCRSP-1). The amino acids identical to canine CRSP-2 are shaded.

Effects of bovine and canine CRSP-1, equine CGRP-I, and canine CRSP-2 on cAMP production in LLC-PK$_1$ cells

We next measured the effects of bovine and canine CRSP-1, equine CGRP-I, and canine CRSP-2 on cAMP production in LLC-PK$_1$ cells, along with porcine CRSP-1 and CT (Fig. 3). We isolated porcine CRSP-1 by monitoring the cAMP production in this cell line, which expresses a high level of the CT receptor [1]. We thought that these newly identified CRSPs would stimulate the cAMP production in LLC-PK$_1$ cells via the CT receptor. Porcine CRSP-1 stimulated the cAMP production most potently, and bovine CRSP-1 and equine CGRP-I stimulated the cAMP production approximately 3- and 10-fold less potently than that of porcine CRSP-1, respectively. Canine CRSP-1 stimulated the cAMP production at a potency approximately 100-fold weaker than that of porcine CRSP-1, while canine CRSP-2 did not stimulate it at all.

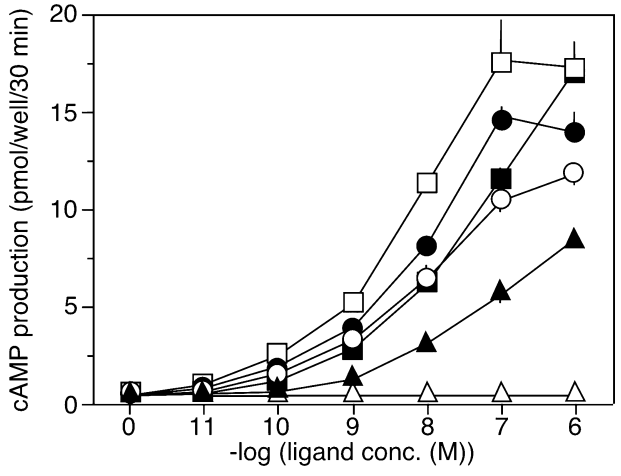

Fig. 3. Dose–response elevation of cAMP production in the culture medium of LLC-PK$_1$ cells. LLC-PK$_1$ cells were stimulated with the indicated concentrations of bovine CRSP-1 (closed circle), canine CRSP-1 (closed triangle), equine CGRP-I (open circle), porcine CRSP-1 (open square), porcine CT (closed square), and canine CRSP-2 (open triangle). Each point represents the mean ± SEM of three separate determinations.

Effects of bovine and canine CRSP-1, equine CGRP-I, and canine CRSP-2 on CT receptor or CL receptor in the absence or presence of RAMP

Recent studies have reported that expression levels of RAMPs dynamically regulate affinities of CGRP and AMY for the CT receptor, as well as those of CGRP and AM for the CL receptor [4,15–17]. On the basis of these findings, the CT receptor or CL receptor was expressed in COS-7 cells with or without one of the RAMPs, stimulated with 100 nM porcine, bovine, or canine CRSP-1, equine CGRP-I, canine CGRP-2, porcine CT, CGRP or human AM, and the cAMP production level in each combination was measured (Fig. 4). Porcine, bovine and canine CRSP-1, equine CGRP-I, and porcine CT stimulated the cAMP production more than 5-fold in the COS-7 cells expressing the CT receptor without RAMPs (Fig. 4A). CGRP and AM also stimulated cAMP production, but canine CRSP-2 did not stimulate it at all even at a concentration of 100 nM. While the enhancement of the cAMP production by stimulation of each peptide was not modified by co-expression of RAMP1 or RAMP2, it was significantly attenuated by co-expression of RAMP3 (Figs. 4B–D). A similar observation was obtained in our previous experiments [12], although the relative potency of these peptides was not altered under the RAMP3-co-expressed conditions. As was in the case with porcine CRSP-1, bovine CRSP-1, canine CRSP-1, and equine CGRP-I as well as canine CRSP-2 did not stimulate cAMP production via the CL receptor (Fig. 4E), and co-expression of RAMPs did not enhance the affinity of these peptides for the CL receptor (Figs. 4F–H).

Discussion

In a recent study, we identified three members of the CRSP family in the porcine brain. By database search, we found equine CGRP-I as a candidate for equine CRSP-1, but failed to identify human or rodent counterparts of CRSPs. To search out CRSPs in animals except for the pig, we tried to isolate the cDNAs of CRSPs from bovine and canine thyroid cDNA libraries because these animals are considered to be evolutionarily closer to pigs than humans and mice.

The cDNA sequences and the deduced amino acid sequences of bovine and canine CRSP-1 are shown in Figs. 1A and B, and the mature amino acid sequences deduced for bovine and canine CRSP-1 are aligned and compared with porcine CRSP-1, equine CGRP-I, and porcine CGRP in Fig. 1C. The mature peptides of bovine and canine CRSP-1s are not thought to have the C-terminal amide structure, because the donor glycine for C-terminal amidation does not exist in each precursor. The amino acid sequences neighboring the C-termini of mature CRSP-1s of cow and dog origin apparently have higher identity with that of porcine CRSP-1 than that of porcine CGRP. The activity of bovine CRSP-1 in the enhancement of cAMP production in LLC-PK_1 cells is approximately 3-fold weaker than that of porcine CRSP-1, and 3-fold stronger than that of porcine CT (Fig. 3). Equine CGRP-I also stimulated cAMP production in LLC-PK_1 cells, and its activity was approximately 10-fold weaker than that of porcine CRSP-1, and was comparable to that of porcine CT. The three identified CRSP-1s and equine CGRP-I stimulated cAMP production in the COS-7 cells expressing the CT receptor (Fig. 4), and their dose–response curves were more closely correlated with each other (data not shown). None of these CRSP-1s stimulated cAMP production in the COS-7 cells expressing the CL receptor, even in the presence of RAMPs. Equine CGRP-I did not increase cAMP production in the CL receptor–expression system, as was in the case with CRSP-1s. These results indicate that bovine and canine CRSP-1s, as well as equine CGRP-I, have biological characteristics similar to those of porcine CRSP-1. Thus, equine CGRP-I is concluded to be a counterpart of CRSP-1 in the horse. Based on the alignment of mature porcine, bovine, equine, and canine CRSP-1s, the amino acid sequence around the C-terminal region, residue 27–36, is crucial for the recognition of the CT receptor. On the other hand, amino acids at the very C-terminal end and C-terminal amidation in the CRSP-1 structure are not essential for eliciting its activity (Fig. 1C). These two features are now recognized to be distinguishing features between CRSP-1 and CGRP.

Although CRSP-2 was identified in the dog, we failed to detect bovine CRSP-2 and CRSP-3, and canine CRSP-3, in cDNA cloning by the cross-hybridization technique using porcine CRSP-2 and CRSP-3 cDNA as probes. The cDNA sequence identity between these putative peptides and porcine CRSP-2/CRSP-3 may not be as high as that detected by cross-hybridization, even under the low stringent hybridization and washing conditions. In fact, mature canine CRSP-2 has low amino acid sequence identity with that of porcine CRSP-2 (47%), although the amino acid sequences of CRSP-1s determined to date have higher identity in the four species (>60%) (Fig. 1C). Despite the low amino acid identity between canine and porcine CRSP-2, they have common structural and biological features. Fig. 2 and our previous study for porcine CRSP-2 [2] showed that the deduced precursors of canine and porcine CRSP-2s do not have typical PC cleavage sites. As were the cases of porcine CRSP-2 and CRSP-3, synthetic canine CRSP-2 did not stimulate either the CT receptor or CL receptor, even in the presence of RAMPs (Figs. 3 and 4). As the deduction of a mature form of canine CRSP-2 may not be correct, further analyses are required to identify the endogenous molecular form of this peptide in the brain and thyroid gland, along with other peptides identified by cDNA cloning.

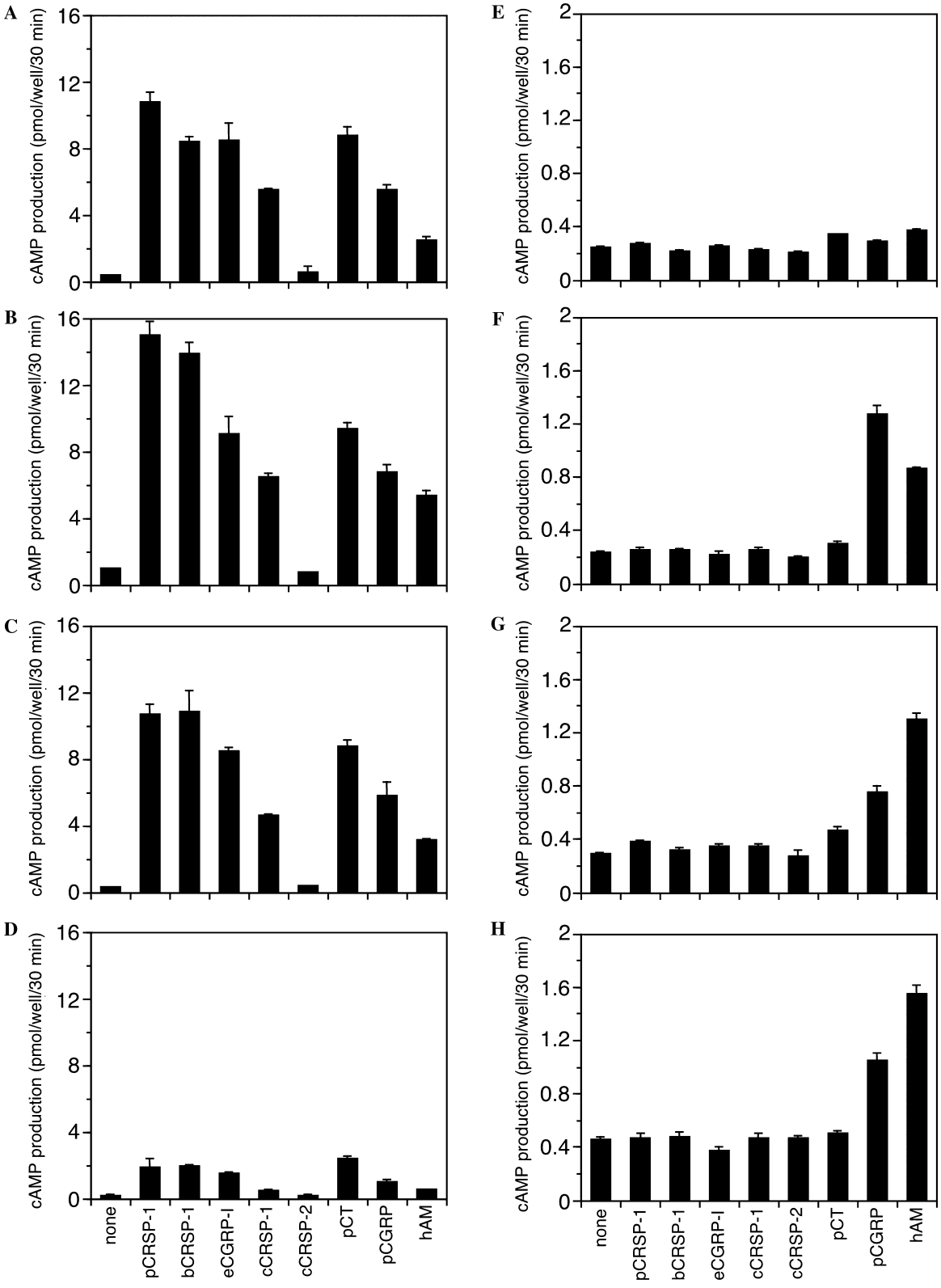

Fig. 4. Effect of CRSPs and its related peptides on the CT or CL receptor in the absence or presence of RAMPs. COS-7 cells were co-transfected with pcDNA inserted with CT receptor cDNA and pcDNA inserted with none (A), RAMP1 (B), RAMP2 (C) or RAMP3 (D) cDNAs, or pcDNA inserted with a CL receptor and pcDNA inserted with none (E), RAMP1 (F), RAMP2 (G) or RAMP3 (H) cDNAs. The cells were stimulated with the incubation medium or a medium containing 100 nM of porcine CRSP-1 (pCRSP-1), bovine CRSP-1 (bCRSP-1), equine CGRP-I (eCGRP-I), canine CRSP-1 (cCRSP-1), canine CRSP-2 (cCRSP-2), porcine CT (pCT), porcine CGRP (pCGRP) or human AM (hAM). Each point represents the mean ± SEM of three separate determinations.

In conclusion, our study demonstrated that CRSP-1 is present in the pig, cow, dog, and horse, and elicits its biological activity via the CT receptor. CRSP-1s are now recognized to be common candidate ligands for a brain CT receptor in mammals and constitute a new class of the biologically active peptide, suggesting the existence of CRSP-1 in humans and rodents. The presence of canine CRSP-2 along with porcine CRSP-2 and CRSP-3, which do not stimulate the known receptors for the CGRP superfamily, indicates the presence of a still unidentified mechanism and diverse effects in the CGRP superfamily.

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