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# Characterization of Crustacean Cardioactive Peptide as a Novel Insect Midgut Factor: Isolation, Localization, and Stimulation of $\alpha$ -Amylase Activity and Gut Contraction

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The midgut, which plays a major role in the feeding behavior of insects, is believed to be controlled by various factors including neuropeptides. In the present study, we identified a neuropeptide crustacean cardioactive peptide (CCAP) as a novel gut factor in insects. CCAP was isolated from the midgut of the cockroach, *Periplaneta americana*. RT-PCR analysis detected the expression of the cockroach CCAP mRNA in the ventral nerve cord and the midgut, revealing the production of CCAP in the midgut as well as the central nervous system. Moreover, we observed expression of the CCAP gene in the endocrine cells of the midgut by *in situ* hybridization, and immunohistochemical analysis showed that CCAP was distributed around the lateral surfaces of the endocrine cells. Elevation of  $\alpha$ -amylase activity was observed upon addition of

CCAP to the midgut. These results suggest that CCAP is involved in digestion of carbohydrate in a paracrine manner. *In situ* hybridization and immunohistochemistry also revealed CCAP expression in the cell bodies of the ingluvial ganglion, which innervates the midgut muscle layer but not the epithelium, indicating that CCAP is produced in the ingluvial ganglion and then transported to the muscle layer through nerve fibers. Furthermore, CCAP was found to stimulate the contraction of the foregut, midgut, and hindgut in a dose-dependent manner. Taken together, our data indicate the multifunctionality of CCAP in the regulation of gut tissues as both a neuropeptide and a novel midgut factor. (*Endocrinology* 145: 5671–5678, 2004)

FEEDING BEHAVIOR IS regulated not only by the central nervous system but also by peripheral tissues, including positive and negative sensory feedback, local hormonal regulation, gastric and intestinal digestion, and the effect of nutrients. In mammals, several neuropeptides such as cholecystokinin, orexin, and neuropeptide Y are involved in the regulation of feeding behavior. These neuropeptides are produced in gut endocrine cells as well as in the nervous system (1).

In insects, the midgut is the primary tissue for digestion and absorption of nutrients. The stomatogastric nervous system, which consists of a nerve trunk along the dorsal surface of the foregut, and the proctodeal nervous system, which originates from the terminal ganglion of the ventral nerve cord, innervate the midgut (2). These nervous systems innervate only the midgut musculature, indicating that the digestive and absorptive cells in the midgut epithelium are not regulated by nerve tissues (3). Thus, nonneural regulatory mechanisms are expected to regulate digestion and absorption in insect midgut (4). Apart from the visceral nervous system, the insect midgut epithelium contains secretory cells called endocrine cells or midgut paraneurons (5). Ultrastructural studies have detected exocytosis of secretory granules

from insect midgut endocrine cells that have morphological similarity to vertebrate gut endocrine cells (6). Immunohistochemical observations using antibodies against mammalian neuropeptides also support the presence of diverse neuropeptide-like compounds in midgut endocrine cells (7, 8). To date, gene expression of insect neuropeptides such as allatostatin, leucomyosuppressin, and neuropeptide F have been detected in the midgut endocrine cells of insects (9–11), which is consistent with the physiological role of midgut endocrine cells as a major source of gut neuropeptides. However, molecular and/or functional aspects of midgut neuropeptides have not been well characterized.

The exceptional omnivorous property of cockroaches in the insect kingdom allows us to presume that the cockroach midgut is conferred with not only a highly advanced digestive system, but also multiple gut-peptidergic regulatory mechanisms. We have been studying the functions of neuropeptides in the cockroach midgut. In this article, we describe the bioactivity of an arthropod neuropeptide, crustacean cardioactive peptide (CCAP), the CCAP cDNA sequence, and the tissue-distribution of CCAP peptide and mRNA, providing evidence that CCAP serves as a novel multifunctional midgut factor as well as a neuropeptide.

Abbreviations: CCAP, Crustacean cardioactive peptide; DIG, digoxigenin; dT, deoxythymidine; RACE, rapid amplification of cDNA ends; RP-HPLC, reversed phase-HPLC; TFA, trifluoroacetic acid.

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## Materials and Methods

### Animals

The American cockroach, *Periplaneta americana*, and the cricket, *Telegryllus occipitalis*, reared in mass and fed with water and artificial diet (MF, Oriental Yeast Corp., Tokyo, Japan) under constant light at 25 C,

were used as the tissue source for peptide isolation and functional assays of peptide bioactivity. Animals were anesthetized by cooling under crushed ice.

### Extraction of peptides from the midgut

The midguts of *P. americana* were dissected from 1650 adults and placed immediately into PBS [0.01 M phosphate buffer (pH 7.4), 0.9% NaCl] after luminal food stuffs had been removed. The isolated organs were immediately frozen on dry ice and stored at  $-80^{\circ}\text{C}$ . The pooled tissues, which weighed 34.3 g, were boiled for 10 min in 5 vol of water. After cooling, acetic acid was added to a final concentration of 4%. The tissues were homogenized at ice-cold temperature using a Polytron, and then centrifuged at  $15,000 \times g$  for 20 min at  $4^{\circ}\text{C}$ . The supernatant was chromatographed using C18 cartridges (Mega Bond Elut C18, Varian, Harbor, CA). After washing each cartridge with 0.1% trifluoroacetic acid (TFA), the retained material was eluted with 60% acetonitrile in 0.1% TFA. The eluate was concentrated to a small volume in a centrifugal vacuum-evaporator (CE 1, Hitachi Koki Co., Ltd., Tokyo, Japan).

### HPLC purification and sequencing of peptide

The concentrated material was filtered through a  $0.45\text{-}\mu\text{m}$  membrane filter (Chromatodisk 4N, Kurabo, Tokyo, Japan), applied to a reversed-phase HPLC (RP-HPLC) column (Capcell Pak C18,  $10 \times 250$  mm, Shiseido, Tokyo, Japan), and eluted with a 70-min linear gradient of 0–70% acetonitrile in 0.1% TFA at a flow rate of 1.0 ml/min at  $40^{\circ}\text{C}$ . Column effluent was monitored spectrophotometrically at 220 nm. Forty fractions of 2 ml each were collected and 1/1000 of each fraction was assayed for myomodulatory activity using the foregut of the cricket as previously described (12). Myostimulatory fractions were pooled, reduced in volume using a centrifugal-vacuum evaporator, and applied to a cation-exchange column (TSKgel SP-5PW,  $4.6 \times 150$  mm, Tosoh, Tokyo, Japan). A 0–0.6 M gradient of NaCl in 10 mM phosphate buffer (pH 6.7) was applied to the top of the column over 90 min at a flow rate of 0.5 ml/min at  $40^{\circ}\text{C}$ . Positive fractions were purified by alternating RP-HPLC separations and the assay to produce a single UV-absorbance peak. The amino acid sequence of the purified peptide was determined by an automatic peptide sequencer (PSQ-1, Shimadzu, Kyoto, Japan).

### Total RNA preparation

Frozen ventral nerve cords of the cockroaches (1 g) were pulverized by grinding under liquid nitrogen. Total RNA was extracted from the ground tissue using RNeasy mini kit (QIAGEN, Valencia, CA), according to the manufacturer's instructions.

### Amplification of the partial fragment of CCAP cDNA

All nucleotides were ordered from QIAGEN Japan (Tokyo, Japan) and Kiko-tech (Osaka, Japan), and all RT-PCRs and rapid amplifications of cDNA ends (RACE) were performed using *Taq* Ex polymerase (Takara, Kyoto, Japan) or *rTaq* DNA polymerase (Toyobo, Osaka, Japan), and 0.2 mM deoxynucleotide triphosphate on a thermal cycler (model GeneAmp PCR system 9600; PE Biosystems, Foster City, CA). First-strand cDNA was synthesized with the oligo[deoxythymidine (dT)]-anchor primer supplied in the 5'/3' RACE kit (Roche Diagnostics, Basel, Switzerland) and amplified with the anchor primer (Roche Diagnostics) and the first degenerate primers 5'-CCITT(T/C)TG(T/C)AA(T/C)GCITT(T/C)AC-3' (I represents an inosine residue), corresponding to the amino acid sequence PFCNAFT. These PCR experiments were performed using five cycles each consisting of  $94^{\circ}\text{C}$  for 30 sec,  $40^{\circ}\text{C}$  for 30 sec, and  $72^{\circ}\text{C}$  for 3 min, followed by 35 cycles each consisting of  $94^{\circ}\text{C}$  for 15 sec,  $50^{\circ}\text{C}$  for 30 sec, and  $72^{\circ}\text{C}$  for 3 min. The first-round PCR products were reamplified using the anchor primer and the degenerate primers 5'-AA(T/C)GCITT(T/C)ACIGGTTG(T/C)GG-3', corresponding to the sequences NAFTGCG, where the last G was regarded as a C-terminal amidation signal. PCR was performed using five cycles of  $94^{\circ}\text{C}$  for 30 sec,  $37^{\circ}\text{C}$  for 1 min, and  $72^{\circ}\text{C}$  for 2 min, followed by 15 cycles of  $94^{\circ}\text{C}$  for 30 sec,  $45^{\circ}\text{C}$  for 30 sec, and  $72^{\circ}\text{C}$  for 2 min, and a final extension at  $72^{\circ}\text{C}$  for 10 min. The resulting PCR product was purified using the Qiaquick Gel Extraction kit (QIAGEN) and subcloned into the pCR II-TOPO vector using a TA cloning kit (Invitrogen Life

Technologies, San Diego, CA) according to the manufacturer's instructions. The DNA inserts of the positive clones were amplified by PCR with universal M13 primers.

### Determination of the 5'-end sequence of CCAP precursor cDNA

Template cDNA was synthesized with an oligonucleotide primer complementary to nucleotides 595–615 (5'-GGCTCCAGGGCTTCGTGATC-3'), followed by deoxy-ATP-tailing of the cDNA with deoxy-ATP and terminal transferase (Roche Diagnostics). The tailed cDNA was amplified with the oligo(dT)-anchor primer (Roche Diagnostics) and gene-specific primer 1 (5'-CTCTGGAGTTGAGATGTGG-3', complementary to nucleotides 572–592). This was followed by further amplification of the first-round PCR products with the anchor primer (Roche Molecular Biochemicals) and gene-specific primer 2 (5'-CTGCAAATC-TCTTCCTCCGG-3', complementary to nucleotides 508–527). Both first-round and second-round PCR steps were performed for 30 cycles of 30 sec at  $94^{\circ}\text{C}$ , 30 sec at  $55^{\circ}\text{C}$ , and 1.5 min at  $72^{\circ}\text{C}$ . The second-round PCR products were subcloned and the inserts were amplified as described above.

### DNA sequencing

All nucleotide sequences were determined with Big-Dye sequencing kits (PE Biosystems) and an ABI PRISM TM 310 Genetic analyzer (PE Biosystems), and then analyzed with DNASIS-MAC software (Hitachi Software Engineering, Kanagawa, Japan). Universal M13 primers or gene-specific primers were used to sequence both strands.

### RT-PCR

RT-PCR was performed on tissue samples of the brain, ventral nerve cord, foregut, midgut, hindgut, and Malpighian tubules of the cockroach. Total RNA was extracted from the tissues as described above and then spectrophotometrically quantified. First strand cDNA was synthesized in a  $20\text{-}\mu\text{l}$  reaction volume with  $2\text{ }\mu\text{g}$  of total RNA, using oligo(dT) primer and reverse transcriptase (Takara). Three microliters of the RT product was used for PCR. Amplification of CCAP precursor transcripts was conducted using specific primers for CCAP (forward primer, 5'-TAGGATGTTCTCTCGCTATCC-3'; reverse primer, 5'-GGCTGTCTTACAATTCTCCAG-3'). Then, PCR products were separated on 1.5% agarose gel and stained with ethidium bromide.

### In situ hybridization

The midgut was dissected from a male cockroach and incubated in 4% paraformaldehyde/PBS at  $4^{\circ}\text{C}$  overnight. After washes with PBS to remove the fixative, the fixed midgut was dehydrated in ethanol and xylene and embedded in paraffin. Serial sections with  $7\text{-}\mu\text{m}$  thickness were made and treated as previously described (13). Whole-mount *in situ* hybridization for the ingluvial ganglion was performed as previously described (14). To prepare a sense or antisense probe, 53-oligonucleotides complementary or identical to CCAP precursor cDNA located between nucleotides 245 and 298 were tailed at the 3' terminus by digoxigenin (DIG)-11-deoxyuridine triphosphate using a DIG oligonucleotide tailing kit (Roche Diagnostics). Hybridization and detection were carried out according to the DIG SYSTEM protocol (Roche Diagnostics).

### Immunohistochemistry

The midgut was dissected from *P. americana*, adult males, fixed overnight at  $4^{\circ}\text{C}$  in Bouin fluid, and embedded in paraffin from which  $7\text{-}\mu\text{m}$  sections were cut. Anti-CCAP antiserum was ordered from Genemed Synthesis Inc. (South San Francisco, CA), and no cross-reactivity to prohormonal forms such as the CCAP flanked by a Gly C-terminal amidation signal and/or dibasic endoproteolytic sites at either terminus was confirmed by the manufacturer. Immunohistochemical staining was performed as previously described (15) using the anti-CCAP antiserum diluted to 1:1000. Immunoreactivity was visualized by the avidin-biotin

peroxidase method with a diaminobenzidine as a chromogen (Vectastain ABC Elite kit, Vector Laboratories, Inc., Burlingame, CA). The ingluvial ganglion was dissected from animal, fixed in 4% paraformaldehyde/PBS at 4°C overnight, and further processed for whole-mount immunohistochemistry as previously described (14). The tissue was incubated with the anti-CCAP antiserum diluted to 1:300 and immunoreactivity was visualized by the avidin-biotin peroxidase method with diaminobenzidine.

Controls included 1) preabsorption of the antiserum with synthetic CCAP (Bachem AG, Bubendorf, Switzerland) at a final concentration of  $10^{-5}$  M for overnight at 4°C and 2) deletion of the primary antiserum. No specific immunostaining was observed in either of the controls.

### Measurement of $\alpha$ -amylase activity from the midgut

Cockroach midguts were dissected in low glucose saline [154 mM NaCl, 13 mM KCl, 10 mM  $\text{CaCl}_2$ , 3 mM  $\text{MgCl}_2$ , 0.01% D (+)-glucose, 1% Ficoll Type 400, and 10 mM HEPES (pH 7.0)] containing a protease inhibitor cocktail (Complete, EDTA-free, Roche Diagnostics). Isolated midguts were opened to remove food particles and peritrophic membrane. The tissues were incubated in the low glucose saline at room temperature for 30 min in the presence or absence of CCAP.  $\alpha$ -Amylase and maltase activities released into the supernatant was quantified using the  $\alpha$ -amylase measuring kit (Kikkoman Corp., Chiba, Japan) and Wako glucose test kit (Wako Pure Chemical Industries, Osaka, Japan), respectively.

### Gut contraction assay

The foregut, midgut, and hindgut of *P. americana* were used for bioassays to confirm the effect of CCAP on the cockroach itself. After dissection, these tissue sections were mounted in a chamber containing insect saline of the following composition: 154 mM NaCl, 13 mM KCl, 1 mM  $\text{CaCl}_2$  and 11 mM D(+)-glucose in 10 mM HEPES (pH 7.0), and treated as previously described (12). Stimulatory effect on the contraction of the tissue sections were monitored at different concentrations of CCAP and plotted in percent of maximum contraction for each tissue.

### Statistical analysis

Results are shown as the mean  $\pm$  SE. Dose-response study on the midgut  $\alpha$ -amylase and maltase activities were analyzed by the one-way ANOVA with Dunnett error protection. Differences were accepted as significant when  $P < 0.05$ .

## Results

### HPLC purification

The chromatogram of the first-round RP-HPLC purification is shown in Fig. 1A. Fractions 26 and 27, showing myostimulatory activities on the cricket foregut (Fig. 1B), were pooled and purified on the cation-exchange column. The bioactive fraction was further purified by four more RP-HPLC steps. Finally, the fraction with a single UV-absorbance peak was obtained (Fig. 1C) and subjected to an amino acid sequence analysis, revealing that the primary structure of the peptide was PFCNAFTGC, which is identical to CCAP (16).

### Characterization of a cDNA encoding CCAP precursor

Because this neuropeptide has been isolated from the central nervous system of arthropods (17), we speculated that the midgut CCAP was derived from the stomatogastric nervous system innervating the midgut muscles. To clarify the origin of the peptide isolated, we examined the expression of the CCAP gene in the midgut.

We attempted to clone the CCAP cDNA from the cockroach ventral nerve cord, given that high CCAP gene ex-

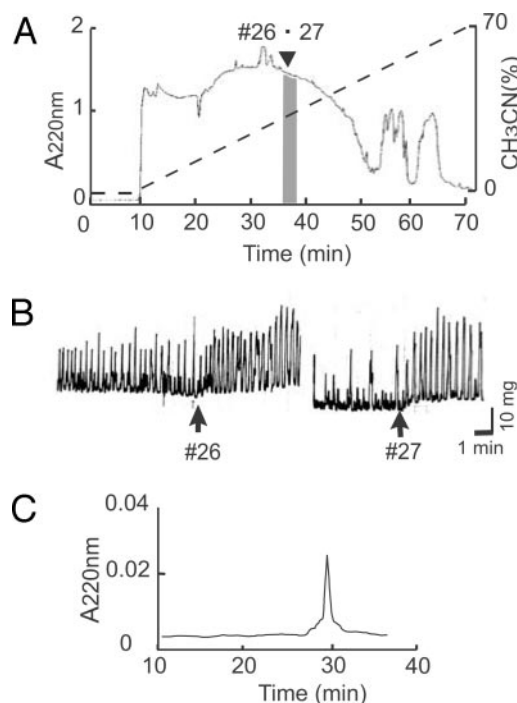


FIG. 1. Purification of CCAP from the midgut of the cockroach using a bioassay. A, First was a separation step using RP-HPLC. The column (Capcell Pak C18;  $10 \times 250$  mm) was developed by 0–70% acetonitrile and 0.1% TFA. A shaded bar indicates the bioactive fractions from which CCAP was purified. B, Fractions 26 and 27 in panel A stimulated contractions of the cricket foregut. The fractions were applied to the tissue at the time indicated by the arrows. C, The final step used RP-HPLC. The column (TSKgel ODS-80TM;  $4.6 \times 150$  mm) was eluted isocratically with 21% acetonitrile and 0.1% TFA.

pression was detected in the ventral nerve cord of the moth, *Manduca sexta* (18). We initially performed an RT-PCR experiment with degenerate primers corresponding to the partial CCAP sequence PFCNAFT and the anchor primer, followed by reamplification of the first-round PCR products with degenerate primers corresponding to the partial CCAP sequence CNAFTGCG and the same anchor primer. Furthermore, 5' RACE with specific primers for the clone was employed to elucidate the full-length sequence of the CCAP cDNA. A representative sequence was submitted to the DDBJ/EMBL/GenBank databases (AB126034). As shown in Fig. 2A, the CCAP precursor cDNA was composed of 1149 nucleotides containing a short 5' untranslated sequence of 103 bp, a single open reading frame of 513 bp, and a 3' untranslated sequence of 533 bp. The open reading frame region began with two putative start codons at positions 104 and 110 and terminated with a TAA stop codon at position 617. Four polyadenylation signals (AATAAA) were found in the 3' untranslated region at positions 935, 1013, 1028, and 1102. A single CCAP sequence, flanked by a glycine C-terminal amidation signal and dibasic endoproteolytic sites at both termini, was shown to be encoded at residue 50–63 in the precursor. In addition, amino acid sequence comparisons of the cockroach CCAP precursor with those of *M. sexta* and *Drosophila melanogaster* revealed that one CCAP sequence and several amino acid residues are conserved among all



FIG. 3. Expression analysis of CCAP precursor in the cockroach. Total RNA (2  $\mu$ g) was prepared from the brain (lane 1), ventral nerve cord (lane 2), foregut (lane 3), midgut (lane 4), hindgut (lane 6), and Malpighian tubules (lane 7), and subjected to RT-PCR using specific primers for the CCAP precursor. The expected position of PCR products (516 bp) is indicated at the *right margin*. The amount of 18S rRNA was used as a control for monitoring PCR demonstration. Results are representative of three independent experiments.

### Distribution of CCAP in the midgut endocrine cell and the nervous system

The localization of CCAP mRNA was directly observed by *in situ* hybridization to 7- $\mu$ m serial sections of the tissue using an antisense DIG-labeled 53-oligomer oligonucleotide probe. As shown in Fig. 4A, several midgut endocrine cells were positively stained. Such positive staining was only detected when the antisense probe was used but was not observed with the sense probe (data not shown). Furthermore, no transcript was detected in the musculature of the midgut. In addition, these CCAP-expressing cells were found to reside solely among columnar cells and midgut nidi, *i.e.* clusters of regenerative epithelial cells. These results provide indisputable evidence that the CCAP gene is expressed in the endocrine cells of the midgut epithelium. Immunoreactivity against CCAP was also detected in the endocrine cells (Fig. 4B), which is in complete agreement with the results obtained by *in situ* hybridization (Fig. 4A). CCAP-immunopositive cells were also found to reside solely among columnar cells and nidi of regenerative cells, and some endocrine cells were extended toward the luminal side. These immunohistochemical studies confirmed the specific production of CCAP in the endocrine cells located in the midgut epithelium. Intriguingly, CCAP immunoreactivity was distributed around the apical site and the lateral surface of the cells (Fig. 4C), but rarely near the circular muscle cells. Such intracellular localization of the immunoreactivity suggested paracrine secretion of CCAP to adjacent midgut epithelial cells rather than to the muscle tissues.

CCAP immunoreactivity was also observed in the nerve fibers within the circular muscles (Fig. 4D), whereas no positive signal was obtained by *in situ* hybridization in the muscle tissues including the nerve fibers. Instead, the ingluvial ganglion cells in the stomatogastric nervous system, which projects nerve fibers to the midgut muscles, showed positive signals by *in situ* hybridization and immunohistochemistry

(Fig. 5, A and B). Taken together, these results indicate that CCAP is not produced in the muscle tissue, but transported from the ingluvial ganglion to the midgut muscle through nerve fibers.

### Stimulatory effect of CCAP on $\alpha$ -amylase activity in the midgut

Subsequently, we examined the effect of CCAP on activities of digestive enzymes. Intriguingly, incubation of the dissected midgut with CCAP at more than  $10^{-8}$  M led to an increase in the activity of  $\alpha$ -amylase (Fig. 6A), whereas only the low glucose saline showed no effects (data not shown). Furthermore, CCAP failed to affect activities of maltase activity (Fig. 6B). These results provided evidence that CCAP is responsible for digestion of carbohydrates, at least starch.

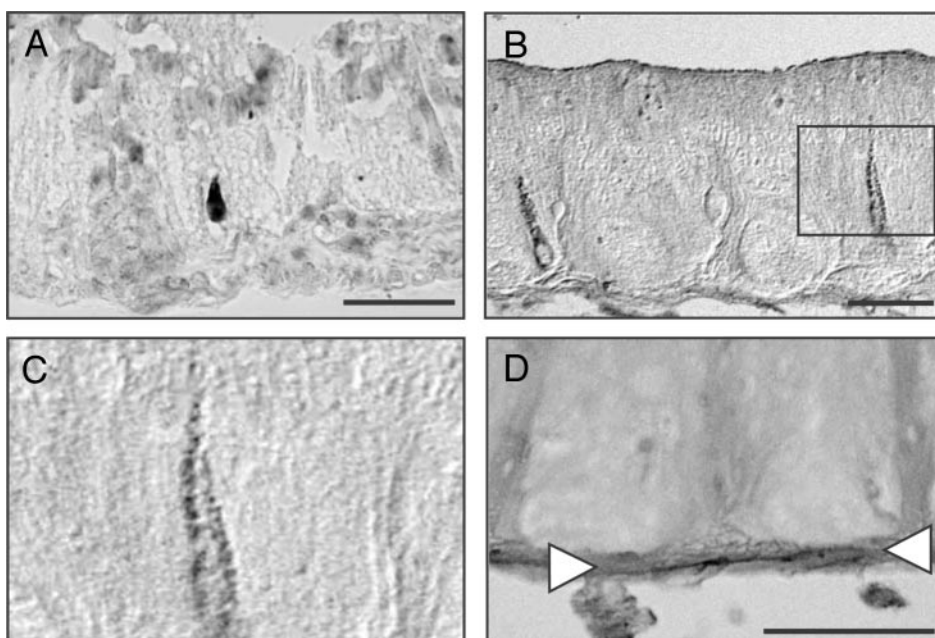
### Myotropic activity of CCAP on the cockroach alimentary tract

The effect of CCAP on the alimentary tract of the cockroach was evaluated using a gut contraction assay. All the preparations of cockroach (foregut, midgut, and hindgut) formed stable spike sequences without CCAP. Administration of CCAP resulted in an increase in the amplitude of contractions of all the gut tissues in a dose-dependent manner with a threshold of  $10^{-9}$  M (Fig. 7, A–D), whereas no change was observed by addition of the insect saline to any guts (data not shown).  $EC_{50}$  values on the foregut, midgut and hindgut were calculated to be  $2.4 \times 10^{-8}$  M,  $1.1 \times 10^{-8}$  M, and  $3 \times 10^{-8}$  M, respectively. These results clearly show that CCAP at physiological concentrations stimulates contraction of each part of the alimentary tract.

### Discussion

CCAP was originally isolated as a cardioaccelerator in the pericardial organs of the shore crab, *Carcinus maenas* (16), and

FIG. 4. Localization of the CCAP gene product in the midgut. All positive cells or nerve fibers were equally detected in three separate experiments. A, *In situ* hybridization of an antisense DIG-labeled 53-oligomer oligonucleotide probe to fixed 7- $\mu$ m sections of the cockroach midgut detected the presence of CCAP mRNA in the cytoplasm of endocrine cells located in the epithelium. B, CCAP-immunoreactive cells were distributed in the endocrine cells of the midgut epithelium. C, The region outlined in panel B is shown at higher magnification. D, CCAP immunoreactivity was observed in nerve fibers within the circular muscle of the midgut. White arrowheads indicate CCAP-immunoreactive fibers. Scale bars, 60  $\mu$ m.





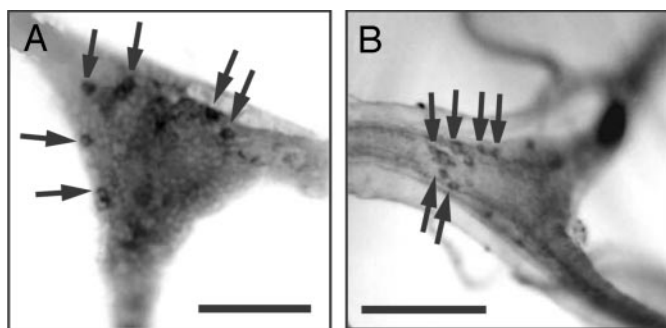


FIG. 5. Localization of the CCAP gene product in the ingluvial ganglion. All positive neurons were equally detected in three separate experiments. A, Whole-mount *In situ* hybridization was performed on the ingluvial ganglion using the DIG-labeled antisense probe. Arrows indicate positively stained neurons. B, CCAP-immunopositive cells were present in the ingluvial ganglion. Arrows indicate positively stained neurons. Scale bars, 100  $\mu$ m.

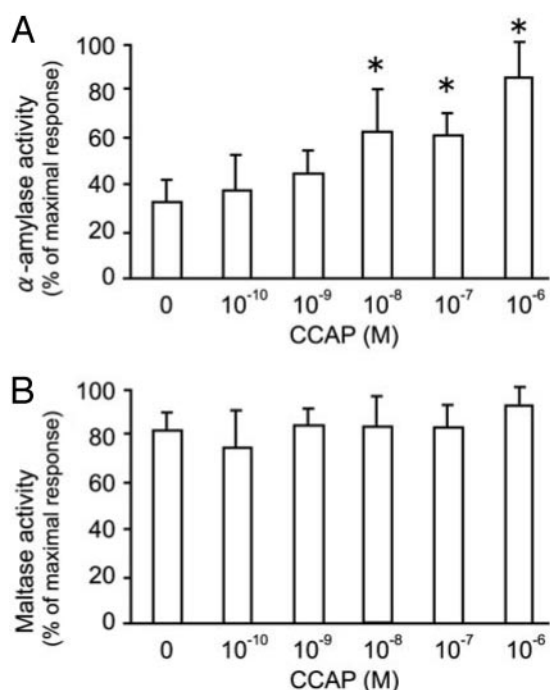


FIG. 6. Stimulatory effect of CCAP on the midgut  $\alpha$ -amylase (A) and maltase (B) activities. Midguts were incubated for 30 min with various concentrations of CCAP. Each point represents the mean  $\pm$  SE of three preparations. \*,  $P < 0.05$ , compared with  $\alpha$ -amylase activity in the absence of CCAP. No significant difference in maltase activity in the presence or absence of CCAP was observed.

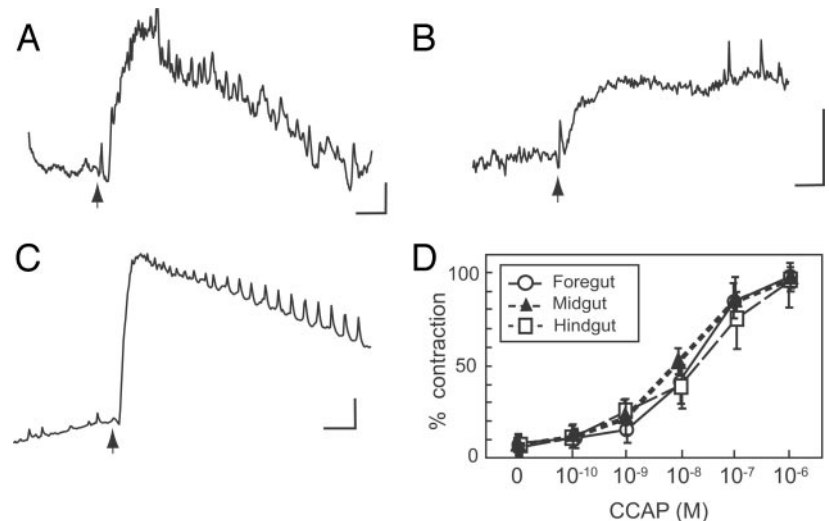
later identified in the nervous system of diverse insects (17). Furthermore, the CCAP gene was cloned from the central nervous system of *M. sexta* (18) and *D. melanogaster* (19). CCAP was also found to exhibit myomodulatory effects in various organs including the heart, the leg muscle, the oviduct, and the hindgut of insects (17, 20), and to stimulate the *in vitro* release of adipokinetic hormone by glandular cells of locust corpora cardiaca (21). Recently, the function of CCAP in ecdysis was also demonstrated; in *M. sexta*, CCAP plays a key role in the initiation of the ecdysis motor program (22), and CCAP-knockout *D. melanogaster* abolished the pupal ecdysis, indicating that CCAP is requisite for initiation of the

ecdysis into pupa (19). These findings have suggested multiple and vital roles for CCAP as a neuronal factor in insects.

In the present study, we showed the isolation of CCAP from the midgut of *P. americana*, localization of the CCAP mRNA and the peptide, stimulation of  $\alpha$ -amylase activity from midgut, and contractile effects on the alimentary tract. RT-PCR analysis (Fig. 3) clearly demonstrates expression of the CCAP gene in the midgut as well as in the ventral cord, providing evidence that CCAP serves as both a neuropeptide and a gut peptide. Furthermore, *in situ* hybridization revealed that the CCAP gene was expressed exclusively in several endocrine cells within the midgut epithelium (Fig. 4A), suggesting a biological role for CCAP as a secretory substance from midgut endocrine cells. A striking feature is that abundant CCAP immunoreactivity was detected at the lateral margins rather than the center of the endocrine cells (Fig. 4C). In addition, a previous ultrastructural study showed exocytosis of secretory granules on the lateral and the basal surface of the midgut endocrine cell of *P. americana* (6). In combination, these findings strongly suggest that the CCAP produced in the midgut is released to adjacent cells as a paracrine factor and participates in the regulation of some midgut epithelial cells such as columnar cells and/or regenerative cells. In the insect midgut, columnar cells are predominantly responsible for synthesis and secretion of digestive enzymes (3). Because the midgut epithelial cells are not subject to neuronal regulation (3), synthesis and secretion of digestive enzymes and/or absorption of nutrients are expected to be stimulated not only by digests in the lumen but also by the midgut endocrine cells (4). Notably, the CCAP-expressing cells are closely associated with columnar cells (Figs. 4, A–C), and CCAP was shown to increase the activity of the midgut  $\alpha$ -amylase (Fig. 6A). These data are in good agreement with the fact that  $\alpha$ -amylase-containing columnar cells were localized in the midgut epithelium of *P. americana* (23) and that high  $\alpha$ -amylase activity in the midgut was also observed in the cockroaches, *D. punctata* (24) and *Nauphoeta cinerea* (25). Taken together, these findings led to the conclusion that CCAP released from the midgut endocrine cells is involved in food digestion and/or absorption via paracrine regulation of the activity of  $\alpha$ -amylase produced and secreted by the columnar cells, although the possibility cannot be excluded that CCAP may regulate activities of other digestive enzymes than carbohydrate-degrading enzymes.

CCAP was also shown to elicit a prominent contraction of all gut muscles (Fig. 7), suggesting that CCAP controls gut movement in the cockroach. However, an *in situ* hybridization signal was absent throughout the midgut circular muscle region (Fig. 4A), revealing that the midgut muscle region is not responsible for the synthesis of CCAP. On the other hand, CCAP-immunoreactivity was observed in nerve fibers running through the midgut circular muscle (Fig. 4D). The ingluvial ganglion, which is a major component of the stomatogastric nervous system, is known to innervate the foregut and anterior midgut (26), and CCAP production was in fact detected in the ingluvial ganglion (Fig. 5, A and B). Moreover, the midgut epithelial CCAP is not likely to be released in the circular muscle layers, given that CCAP-immunoreactive endocrine cells are rarely observed near the circular muscle layers (Fig. 4B). These histochemical obser-

FIG. 7. Myostimulatory effects of CCAP on various parts of the alimentary tract of adult *P. americana*.  $10^{-7}$  M CCAP increased the amplitude of the contractions of the foregut (A), midgut (B), and hindgut (C). The peptide was applied at the time indicated by the arrows. Vertical and horizontal bars in each scale represent 15 mg and 1 min, respectively. Dose-response curves showing the effects of varying concentrations of CCAP on foregut, midgut, and hindgut muscle contractions (D). The maximum contraction caused by  $10^{-6}$  M CCAP was taken as 100%.  $EC_{50}$  values on the foregut, midgut, and hindgut are  $2.4 \times 10^{-8}$  M,  $1.1 \times 10^{-8}$  M, and  $3 \times 10^{-8}$  M, respectively. Each point represents the mean  $\pm$  SE of three preparations.



vations indicate that CCAP present in the midgut circular muscle region is transported there from the ingluvial ganglion and then is involved in regulation of foregut and midgut contraction. In addition, CCAP mRNA was detected in the ventral cord (Fig. 3). The cockroach abdominal ganglia of the ventral cord innervate the dorsal and ventral muscles, the dorsal heart, and the hindgut (2). The immunohistochemical and physiological studies on a variety of insects showed that CCAP from the ventral cord controls the heart beat and hindgut contraction (20, 27–30), suggesting the involvement of nerve cord CCAP in the control of hindgut movement and/or the heart beat in the cockroach. This notion is compatible with the result showing cockroach hindgut contraction by CCAP (Fig. 7, C and D). Therefore, it is presumed that CCAP participates in the regulation of feeding behavior by concerted functions as a paracrine substance for induction of carbohydrate digestion by the midgut epithelium and a myostimulatory neuropeptide for each of the gut tissues and/or hearts. Such functional relationships are now being examined.

We for the first time showed the expression of both the CCAP gene and peptide in the insect midgut endocrine cells (Figs. 3 and 4). The CCAP gene expression has yet to be investigated in the midgut of other insects including *D. melanogaster* (19) and *M. sexta* (18). Similarly, the gene expression of other myomodulatory neuropeptides such as leucomyosuppressin (10) and tachykinin-related peptides (31) in the midgut has so far been investigated in only a single insect species. Further studies are required to establish common or species-specific biological roles for these neuropeptides as midgut factors.

Also of interest is the structure and function of a CCAP receptor. The stimulatory effects of CCAP on the midgut  $\alpha$ -amylase activity (Fig. 6) and contraction of the midgut (Fig. 7, B and D) suggest the presence of a CCAP receptor in this tissue. Recently, identification of a CCAP receptor from *Drosophila* was reported (32). However, the low binding affinity of the receptor with CCAP implied that other receptors serve as authentic CCAP receptors. Therefore, molecular and functional characterization of the cockroach CCAP receptors is expected to contribute to the further investigation of the biological roles and modes of action for CCAP in the insect

kingdom. Identification of CCAP receptor from the cockroach is currently in progress.

In conclusion, we have shown that an arthropod neuropeptide, CCAP, has multifunctional roles in the regulation of gut tissues as both a neuropeptide and a novel midgut factor.

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