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博　士　論　文

Crustacean cardioactive peptide (CCAP) in the midgut and the nervous systems of the cockroach, *Periplaneta americana*

「ワモンゴキブリ中腸及び神経系における
甲殻類心筋活性ペプチド（CCAP）について」

平成 17 年 1 月

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Table of contents

ABBEVIATIONS.....2

CHAPTER Ⅰ. GENERAL INTRODUCTION 3

CHAPTER Ⅱ. Characterization of Crustacean Cardioactive Peptide (CCAP) as a Novel Insect Midgut Factor: Isolation, Localization, and Stimulation of α-Amylase Activity and Gut Contraction 8

Ⅱ-1. INTRODUCTION 8

Ⅱ-2. MATERIALS AND METHODS 11

Ⅱ-3. RESULTS 20

Ⅱ-4. DISCUSSION 30

CHAPTER Ⅲ. Nutrients-induced α-amylase and protease activity is regulated by crustacean cardioactive peptide in the cockroach midgut 35

Ⅲ-1. INTRODUCTION 35

Ⅲ-2. MATERIALS AND METHODS 37

Ⅲ-3. RESULTS 42

Ⅲ-4. DISCUSSION 49

SUMMARY AND PERSPECTIVE..... 52

REFERENCES 55

LIST OF PUBLICATIONS 63

ACKNOWLEDGEMENTS 64

Abbreviations

bp	base pair
CCAP	crustacean cardioactive peptide
cDNA	complementary DNA
DEPC	diethypyrocarbonate
DIG	digoxigenin
DNA	deoxyribonucleic acid
EC_{50}	50% effective concentration
HPLC	high performance liquid chromatography
mRNA	messenger RNA
ODS	octadecylsilyl
PCR	polymerase chain reaction
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
RT	reverse transcriptase
SSC	standard saline citrate
TFA	trifluoroacetic acid
Tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet

Chapter I

General Introduction

Neuropeptides are key regulators of diverse physiological processes, including growth, reproduction, response to stresses, maintenance of energy balance, and feeding (Strand 1999). Neuropeptides were found to exert neurohormonal and neurotransmitter effects in the central and peripheral nervous system. Recently, an increasing number of findings have shown that neuropeptides, in addition to such traditional biological roles, also act as regulators of non-neuronal cells such as endocrine cells. In vertebrates, cholecystokinin, orexin, neuropeptide Y and ghrelin, which are produced and secreted by the gastrointestinal endocrine cells as well as neurons, participate in coordinated regulations of feeding and energy expenditure response through neural and/or humoral input (Murphy and Bloom, 2004).

Feeding is a critical behavior for supply of nutrients with all organisms, which is necessary for energy production, maintenance of biochemical processes, growth and development. Thus, understanding of control mechanism of feeding is very important in biology and medicine. In half of the past century, scientists revealed the role of neuropeptides in feeding behavior including hunger, satiety, digestion, metabolism and excretion. Peptidergic regulations of feeding behavior of vertebrates have been well

investigated, whereas those of invertebrates have not been well elucidated. Therefore, characterization of invertebrate neuropeptides involved in feeding and comparison of vertebrate and invertebrate counterparts would provide profound understanding of general and species-specific peptidergic regulation mechanisms of all living creatures.

Insects, the predominantly largest invertebrate species, are widely diverse in food and feeding habits, and have correspondingly high diversity in the gut structure and function. The evolutionary success of insects has been driven by their ability to occupy a variety of ecological niches and utilize many different types of food sources. Food sources of insects can be roughly divided into broad categories of solid foods or liquid foods and plants or animals. The most likely prototypic feeding behavior of insects was believed to be omnivorous, followed by divergence toward more specialized phytophagous or carnivorous properties (Dow, 1986).

One of the representative omnivorous insects is a cockroach, which is featured by their ability to ingest almost all organic materials. They have succeeded in adapting their lives to man-made habitats, while the origin of cockroaches date back 250 million years to the Carboniferous period. The fossil records show that the shape or body organization of cockroaches have changed very little since ancient era, as compared with their present-day descendants (Cornwell, 1968). The exceptional omnivorous character of cockroaches in the insect kingdom suggests that the cockroach's digestive tract has the ability to digest

many kinds of foods. For instance, American cockroach, *Periplaneta americana* feeds on dead and injured individuals of its own species, bread, cheese, vegetables, and cardboard (Bignell 1981). The shape of cockroach gut is neither as short and broad as those of solid feeders, nor as convoluted as those of liquid feeders. Figure Ⅰ-1 shows the gross morphology of the cockroach alimentary canal.

The midgut, which is responsible for secretion of digestive enzymes, digestion of food, and absorption of nutrients, has a single layer of columnar cells producing digestive enzymes such as amylase, invertase, maltase, and lactase, proteases and lipase (Cornwell, 1968). The cockroach midgut is much simpler in structure than mammalian guts. The epithelium is a flat layer without villi or crypt formation and is composed of three cell types: columnar, endocrine, and regenerative cells. The regenerative cells, which differentiate to columnar cells and endocrine cells, form relatively large nests or nidi at the base of the epithelium. The majority of epithelial cells are tall and uniform columnar cells. The endocrine cells reside solely among columnar cells and midgut nidi.

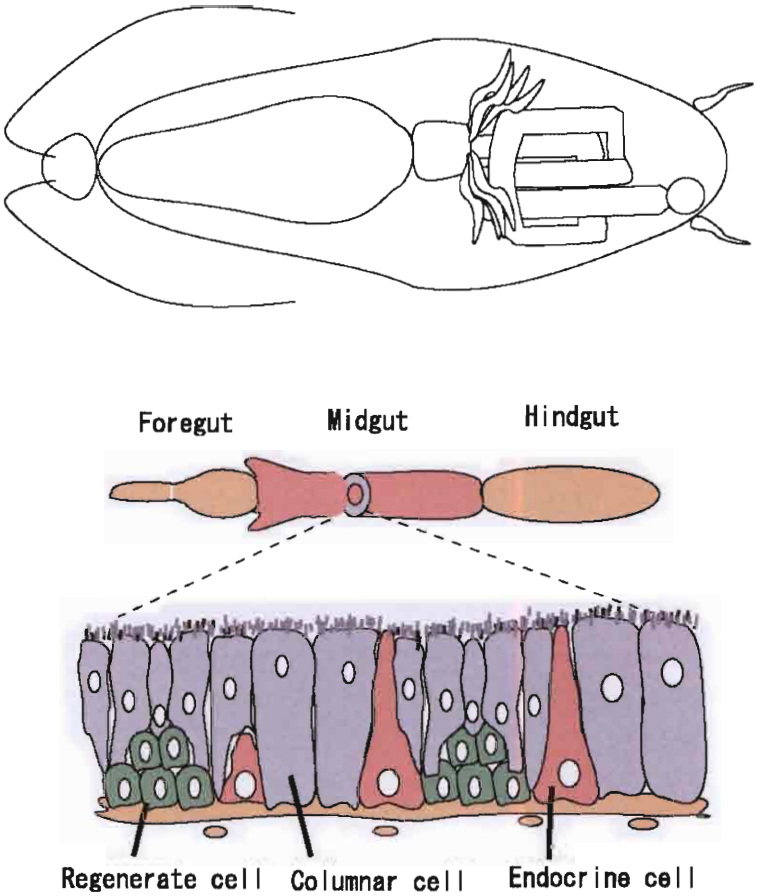


Fig. Ⅰ-1. The gross morphology of the cockroach alimentary canal (Upper figure) and midgut cell structure (lower figure).

In early studies, secretory granules were observed in insect midgut endocrine cells residing in the epithelial layer (Nishiitsutsuji-Uwo and Endo 1981), and several mammalian neuropeptide-like immunoreactivities were detected in the endocrine cells (Montuenga *et al.* 1989, Schols *et al.* 1987). Insect midgut endocrine cells were also found

to express several genes of insect neuropeptides such as allatostatin (Reichwald *et al.* 1994), leucomyosuppressin (Fuse *et al.* 1998), and neuropeptide F (Brown *et al.* 1999). These findings support the notion that neuropeptides produced by the midgut endocrine cells play pivotal roles in food digestion as gut regulatory factors in a non-neuronal fashion. Compared with the neuronal peptidergic systems, however, the non-neuronal regulatory mechanism in gut neuropeptide expression and secretion has yet to be fully elucidated. Furthermore, detailed physiological roles of gut neuropeptides and their functional relationship with neuronal elements on the midgut are still unclear.

Consequently, we focused on the functions of an arthropod neuroepeptide, crustacean cardioactive peptide (CCAP) in feeding behavior in the midgut and nervous system of the cockroach, *Periplaneta americana,* by following approaches: (1) isolation of CCAP from the midgut and characterization of its cDNA sequence, (2) localization of the mRNA and peptide product, (3) determination of the physiological functions in feeding and inducible expression and secretion of CCAP. This study revealed the unprecedented multifunctional roles of the neuropeptide in the midgut and novel non-neuronal regulatory mechanism in feeding behavior, leading to the presumption that the cockroach midgut is an excellent model for an integrated analysis of neuropeptide functions in feeding behavior.

Chapter Ⅱ

Characterization of Crustacean Cardioactive Peptide (CCAP) as a Novel Insect Midgut Factor: Isolation, Localization, and Stimulation of α-Amylase Activity and Gut Contraction

Ⅱ-1. Introduction

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 (Strand 1999).

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 (Cornwell 1968). 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 (Lehane 1998). Thus, non-neural regulatory mechanisms are expected to regulate digestion and absorption

in insect midgut (Lehane *et al.* 1995). Apart from the visceral nervous system, the insect midgut epithelium contains secretory cells called endocrine cells or midgut paraneurons (Nishiitsutsuji-Uwo *et al.* 1985). Ultrastructural studies have detected exocytosis of secretory granules from insect midgut endocrine cells which have morphological similarity to vertebrate gut endocrine cells (Nishiitsutsuji-Uwo and Endo 1981). Immunohistochemical observations using antibodies against mammalian neuropeptides also support the presence of diverse neuropeptide-like compounds in midgut endocrine cells (Montuenga *et al.* 1989, Schols *et al.*1987). To date, gene expression of insect neuropeptides such as allatostatin, leucomyosuppressin, and neuropeptide F have been detected in the midgut endocrine cells of insects (Reichwald *et al.*1994, Fuse *et al.* 1998, Brown *et al.* 1999), 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. I have been studying the functions of neuropeptides in the cockroach midgut. In this article, I 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.

II-2. Materials and Methods

Animals

The American cockroach, *Periplaneta americana,* and the cricket, *Teleogryllus 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 phosphate-buffered saline (PBS; 0.01M 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 ℃. The pooled tissues, which weighed 34.3 g, were boiled for 10 min in 5 volumes 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 × g for 20 min at 4 ℃. 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 μm membrane filter (Chromatodisk 4N, Kurabo, Tokyo), applied to a reversed-phase HPLC (RP-HPLC) column (Capcell Pak C18, 10 × 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 ℃. 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 (Fujisawa *et al. 1993*). Myostimulatory fractions were pooled, reduced in volume using a centrifugal-vacuum evaporator, and applied to a cation-exchange column (TSKgel SP-5PW, 4.6 × 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 ℃. 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, USA), 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 reverse transcription polymerase chain reactions (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 dNTP on a thermal cycler (model GeneAmp PCR system 9600; PE-Biosystems, Foster City, CA, USA). First-strand cDNA was synthesized with the oligo(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 ℃ for 30 sec, 40 ℃ for 30 sec and 72 ℃ for 3 min, followed by 35 cycles each consisting of 94 ℃ for 15 sec, 50 ℃ for 30 sec, and 72 ℃ 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)ACIGGITG(T/C)GG-3' , corresponding to the sequences NAFTGCG, where the last G was regarded as a C-terminal amidation signal. PCR was done using five cycles of 94 ℃ for 30 sec, 37 ℃ for 1 min, and 72 ℃ for 2 min, followed by 15 cycles of 94 ℃ for 30 sec, 45 ℃ for 30 sec, and 72 ℃ for 2 min, and a final extension at 72 ℃ 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, San Diego, CA, USA) 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 nt 595-615 (5'-GGCTCCAGGGCTTCGTGATC-3'), followed by dA-tailing of the cDNA with dATP and terminal transferase (Roche Diagnostics). The tailed cDNA was amplified with the oligo(dT)-anchor primer (Roche Diagnostics) and gene-specific primer 1 (5'-CTCCTGGAGTTGAGATGTGG-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'-CTGCAAATCTCTTCCTCCGG-3', complementary to nucleotides 508-527). Both first-round and second-round PCR steps were performed for 30 cycles of 30 sec at 94 ℃, 30 sec at 55 ℃ and 1.5 min at 72 ℃. 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 analysed 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 μl reaction volume with 2 μg of total RNA, using oligo(dT) primer and reverse transcriptase (Takara). Three microliters of the reverse transcription 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 ℃ 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 μm thickness were made and treated as previously described (Satake *et al. 1999*). Whole-mount *in situ* hybridization for the ingluvial ganglion was performed as previously described (Fujisawa *et al. 1999*). To prepare a sense or antisense probe, 53-mer oligonucleotides complementary or identical to CCAP precursor cDNA located between nucleotides 245 and 298 were tailed at the 3'-terminus by DIG-11-dUTP 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 ℃ in Bouin fluid, and embedded in paraffin from which 7-μm 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 (Sattelle *et al.* 2000) using the anti-CCAP antiserum diluted to 1:1000. Immunoreactivity was visualized by the avidin-biotin peroxidase method with a diaminobenzidine (DAB) as a chromogen (Vectastain ABC Elite kit, Vecta Lab. Inc, CA, USA). The ingluvial ganglion was dissected from animal, fixed in 4% paraformaldehyde/PBS at 4℃ overnight, and further processed for whole-mount immunohistochemistry as previously described (Fujisawa *et al.* 1999). The tissue was incubated with the anti-CCAP antiserum diluted to 1:300 and immunoreactivity was visualized by the avidin-biotin peroxidase method with DAB.

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

Measurement of α-amylase activity from the midgut

Cockroach midguts were dissected in low glucose saline (154 mM NaCl, 13 mM KCl, 10

mM $CaCl_2$, 3 mM $MgCl_2$, 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. α-amylase and maltase activities released into the supernatant was quantified using the α-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 $CaCl_2$ and 11 mM D(+)-glucose in 10 mM HEPES (pH 7.0), and treated as previously described (Fujisawa *et al.* 1993). 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 ± SE. Dose-response study on the midgut α-amylase and maltase activities were analyzed by the one-way ANOVA with Dunnett error protection. Differences were accepted as significant when $P < 0.05$.

II-3. Results

HPLC purification

The chromatogram of the first-round RP-HPLC purification is shown in Fig. II-1A. Fractions #26 and #27, showing myostimulatory activities on the cricket foregut (Fig. II-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. II-1C) and subjected to an amino acid sequence analysis, revealing that the primary structure of the peptide was PFCNAFTGC, which is identical to CCAP (Stangier *et al.* 1987).

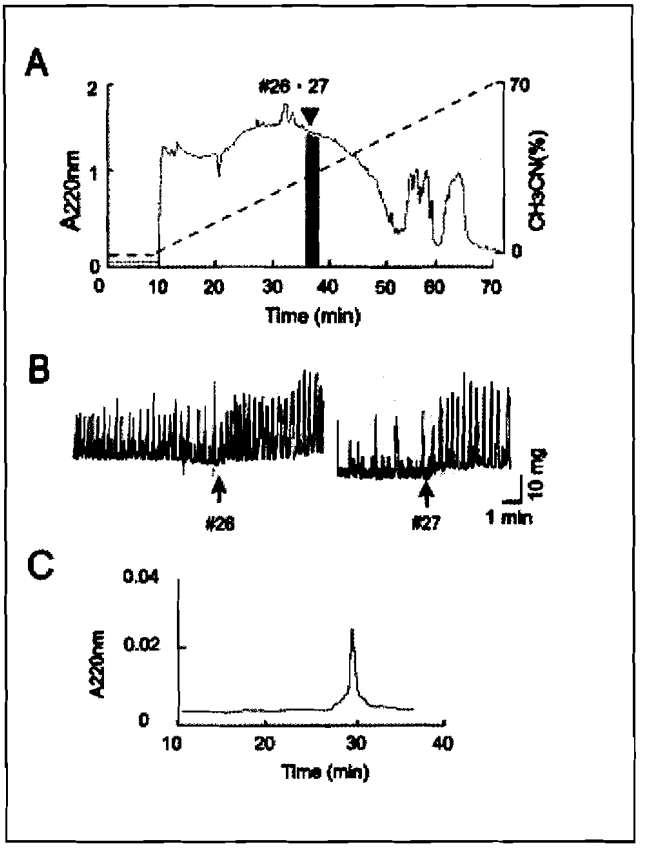


Fig. II-1. Purification of CCAP from the midgut of the cockroach using a bioassay. **A**, First was a separation step using RP-HPLC. The column (Capcell Pak C18; 10 × 250 mm) was developed by 0-70% acetonitrile and 0.1% TFA. A shaded bar indicates the bioactive fractions from which CCAP was purified. **B**, Fractions #26 and #27 in (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 reversed-phase HPLC. The column (TSKgel ODS-80TM; 4.6 × 150 mm) was eluted isocratically with 21 % acetonitrile and 0.1% TFA.

Characterization of a cDNA encoding CCAP precursor

Since this neuropeptide has been isolated from the central nervous system of arthropods (Dircksen 1998), I 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.

I attempted to clone the CCAP cDNA from the cockroach ventral nerve cord, given that high CCAP gene expression was detected in the ventral nerve cord of the moth, *Manduca sexta* (Loi *et al.* 2001). I 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 Figure 2.2A, 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

925, 1013, 1028 and 1107. 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 precursors (Fig. II-2B), although the biological significance of such conserved amino acid residues remains unknown.

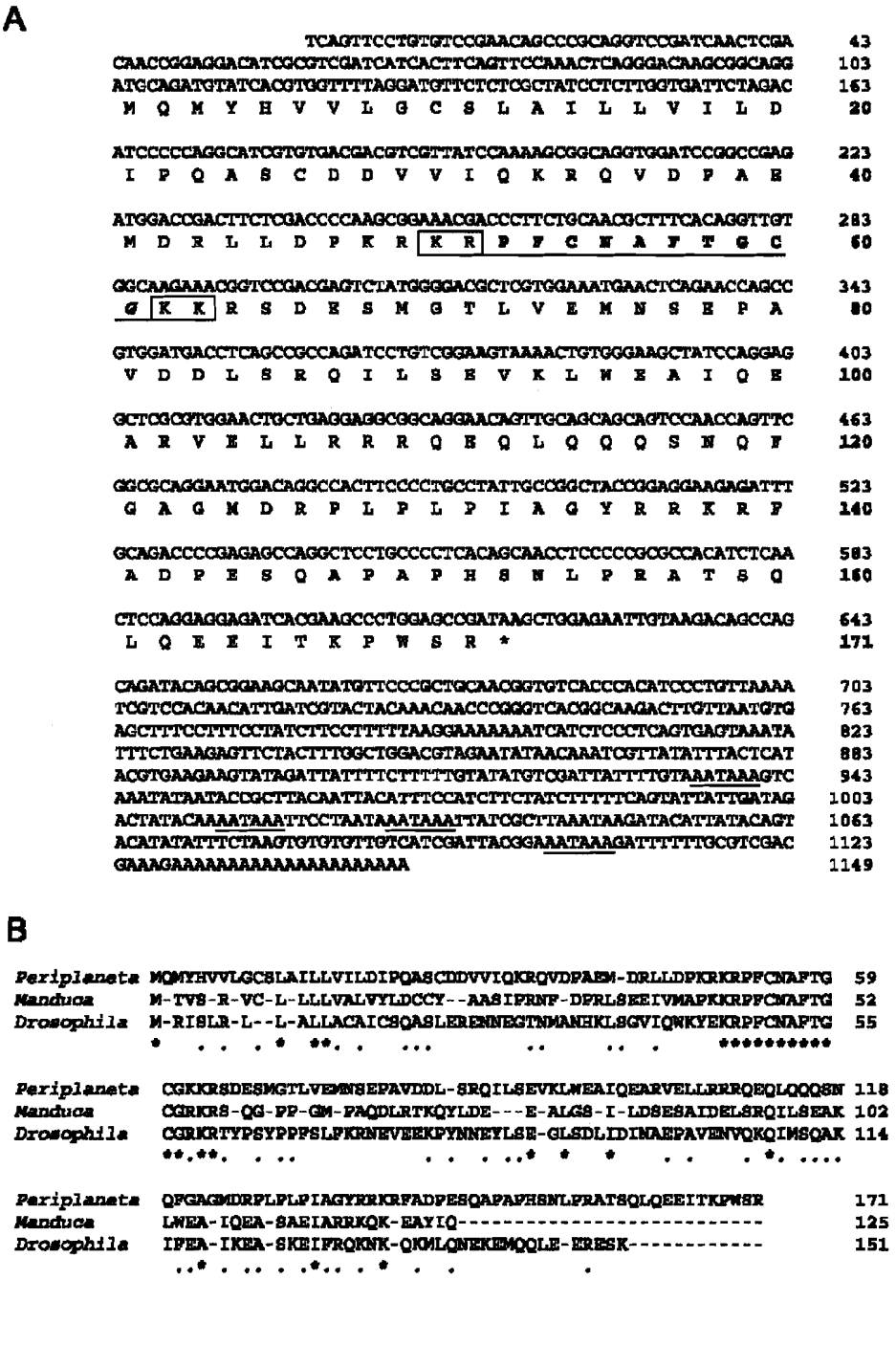


Fig. Ⅱ-2. Nucleotide sequence and deduced amino acid sequences of cockroach CCAP cDNA. **A,** The sequence of CCAP is indicated in boldface type and underlined. The neuropeptide has a C-terminal Gly residue (boldfaced italics), which is a consensus residue for amidation. The dibasic cleavage site is boxed. Four polyadenylation signals are underlined. **B,** Comparison of amino acid sequences of CCAP precursors. The CCAP precursors are aligned with those of the cockroach (*Periplaneta*), the tobacco hawkmoth (*Manduca*) and the fruit fly (*Drosophila*). Amino acid sequences are available from the DDBJ/EMBL/GenBank databases (accession no. AF451838, the moth) and FlyBase (FBgn0039007, the fruit fly). Asterisks and dots denote identical amino acid residues among all or two precursors, respectively. Gaps marked by hyphens were inserted to optimize homology.

Expression of the CCAP precursor gene

Subsequently, RT-PCR for the CCAP mRNA was performed to investigate the tissue distribution of CCAP gene expression. A single band for the 516 bp RT-PCR product between nt 126 and 641 was detected exclusively in the ventral nerve cord and the midgut but not in the brain, foregut, hindgut and Malpighian tubules (Fig. Ⅱ-3). These results led to the conclusion that CCAP was synthesized in the midgut as well as in the nerve cord.

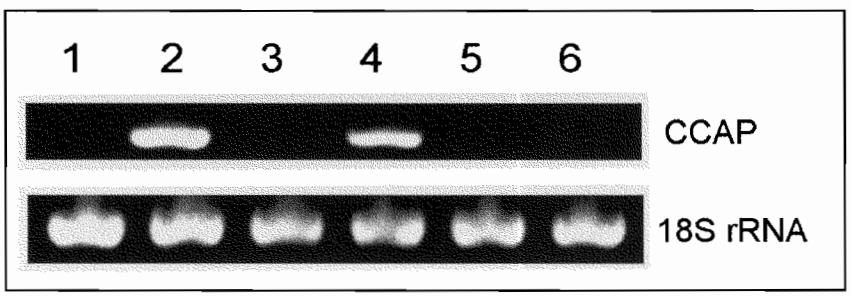


Fig. Ⅱ-3. Expression analysis of CCAP precursor in the cockroach. Total RNA (2 μg) was prepared from the brain (lane1), ventral nerve cord (lane 2), foregut (lane3), midgut (lane4), hindgut (lane6), and Malpighian tubules (lane7), and subjected to RT-PCR using specific primers for the CCAP precursor. 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-μm serial sections of the tissue using an antisense DIG-labeled 53-mer oligonucleotide probe. As shown in Fig. Ⅱ-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. Ⅱ-4B), which is in complete agreement with the results obtained by *in situ* hybridization (Fig. Ⅱ-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. Ⅱ-.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. Ⅱ-4D), while 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. Ⅱ-5A and 5B). 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.

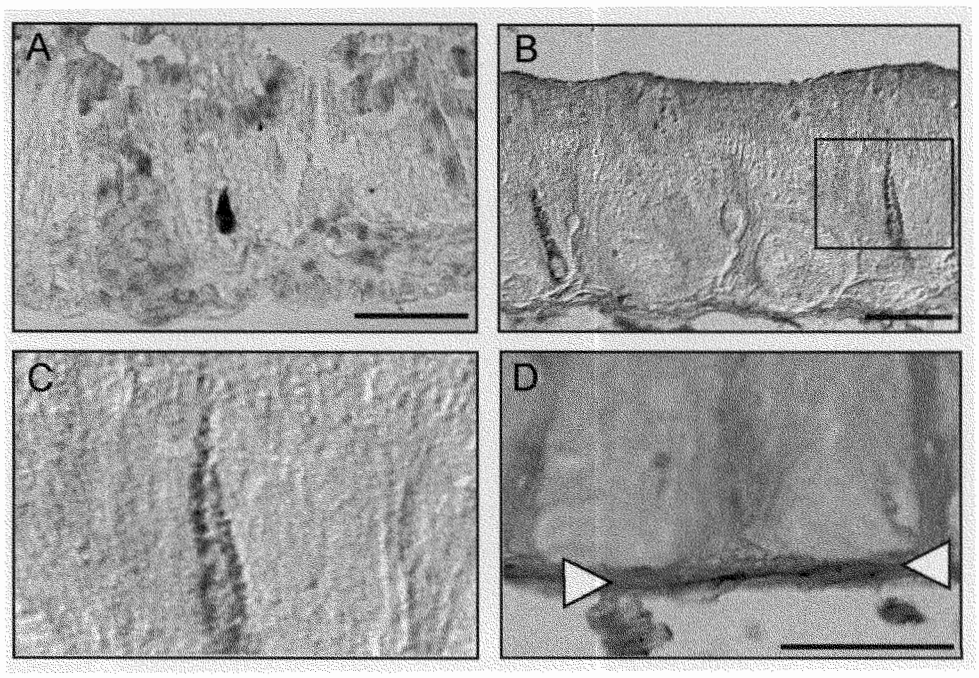


Fig. Ⅱ-4. Localization of the CCAP gene product in the midgut. All positive cells or nerve fibers were equally detected in three separate experiments. **A,** *In situ* hybridization of an antisense DIG-labeled 53-mer oligonucleotide probe to fixed 7-μm sections of the cockroach midgut detected the presence 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 μm.

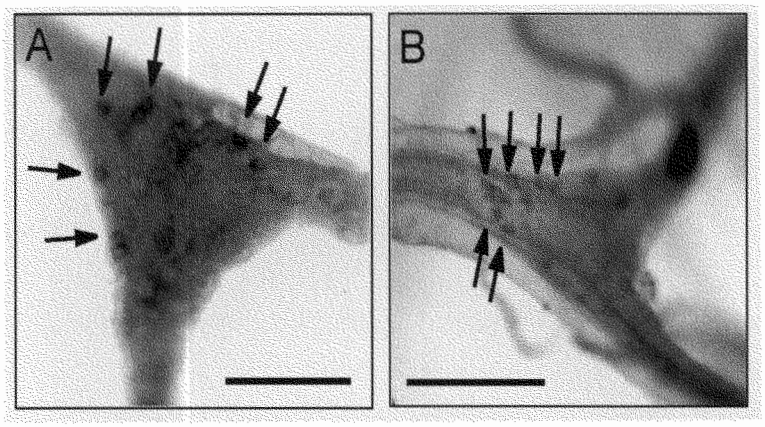


Fig. II -5. Localization of the CCAP gene product in the ingluvial ganglion. All positive neurons were equally detected in three separate experiments. **A,** Whole-mount *In situ* hybridization was performed on the ingluvial ganglion using the DIG-labeled antisense probe. Arrows indicate positively stained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

Stimulatory effect of CCAP on α-amylase activity in the midgut

Subsequently, I 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 α-amylase (Fig. II-6A), whereas only the low glucose saline showed no effects (data not shown). Furthermore, CCAP failed to affect activities of maltase activity (Fig. II-6B). These results provided evidence that CCAP is responsible for digestion of carbohydrates, at least starch.

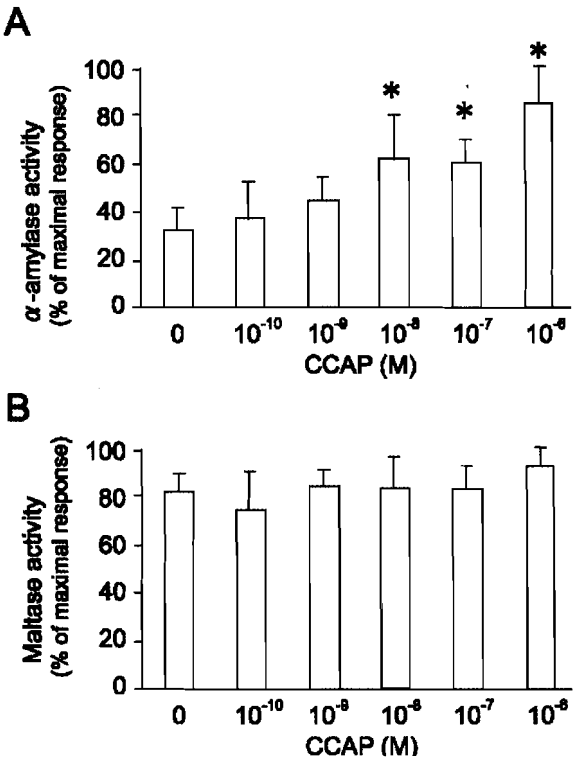


Fig. II-6. Stimulatory effect of CCAP on the midgut α-amylase (A) and maltase (B) activities. Midguts were incubated for 30 min with various concentrations of CCAP. Each point represents the mean ± SE of 3 preparations. *, $P < 0.05$, compared with α-amylase activity in the absence of CCAP. No significant difference in maltase activity in the presence or absence of CCAP was observed.

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. Ⅱ-7A-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 x 10^{-8} M, 1.1 x 10^{-8} M and 3 x 10^{-8} M, respectively. These results clearly show that CCAP at physiological concentrations stimulates contraction of each part of the alimentary tract.

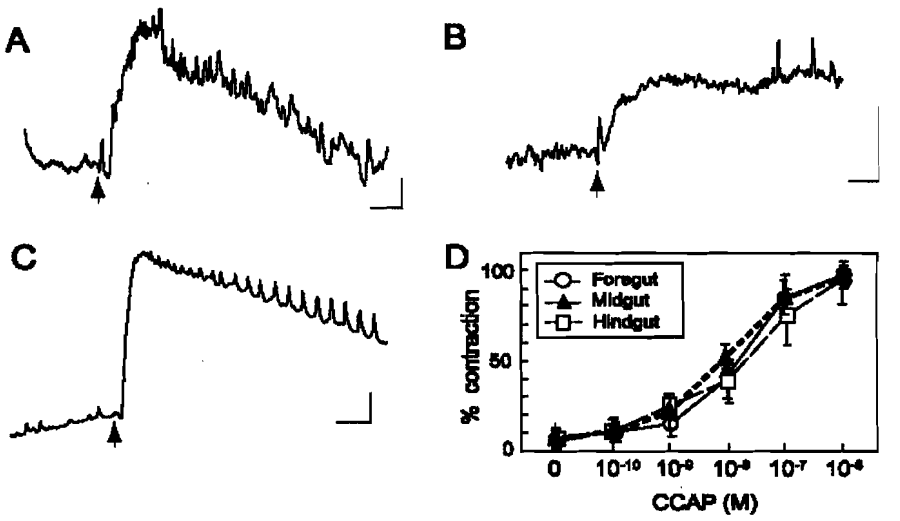


Fig. Ⅱ-7. Myostimulatory effects of CCAP on various parts of the alimentary tract of adult *P. americana*. 10^{-7} M CCAP increased the amplitude of the contractions of the foregut (A), midgut (B), and hindgut (C). The peptide was applied at the time indicated by the arrows. Vertical and horizontal bars in each scale represent 15 mg and 1 min, respectively. Dose-response curves showing the effects of varying concentrations of CCAP on foregut, midgut, 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 x 10^{-8} M, 1.1 x 10^{-8} M and 3 x 10^{-8} M, respectively. Each point represents the mean ± SE of 3 preparations.

Discussion

CCAP was originally isolated as a cardioaccelerator in the pericardial organs of the shore crab, *Carcinus maenas* (Stangier *et al.* 1987), and later identified in the nervous system of diverse insects (Dircksen 1998). Furthermore, the CCAP gene was cloned from the central nervous system of *M. sexta* (Loi *et al.* 2001) and *D. melanogaster* (Park *et al.* 2003). CCAP was also found to exhibit myomodulatory effects in various organs including the heart, the leg muscle, the oviduct, and the hindgut of insects (Dircksen 1987, Lehman *et al.* 1993), and to stimulate the *in vitro* release of adipokinetic hormone (AKH) by glandular cells of locust corpora cardiaca (Veelaert *et al.* 1997). 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 (Gammie and Truman 1997), and CCAP-knockout *D. melanogaster* abolished the pupal ecdysis, indicating that CCAP is requisite for initiation of the ecdysis into pupa (Park *et al.* 2003). These findings have suggested multiple and vital roles for CCAP as a neuronal factor in insects.

In the present study, I showed the isolation of CCAP from the midgut of *P. americana,* localization of the CCAP mRNA and the peptide, stimulation of α-amylase activity from midgut, and contractile effects on the alimentary tract. RT-PCR analysis (Fig. II-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. II-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. II-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 (Lehane 1998). Since the midgut epithelial cells are not subject to neuronal regulation (Lehane 1998), 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 (Lehane *et al.* 1995). Notably, the CCAP-expressing cells are closely associated with columnar cells (Figs. 2.4A-C), and CCAP was shown to increase the activity of the midgut α-amylase (Fig. II-.6A). These data are in good agreement with the fact that α-amylase-containing columnar cells were localized in the midgut epithelium of *P. americana* (Lima *et al.* 2003)

and that high α-amylase activity in the midgut was also observed in the cockroaches, *D. punctata* (Fuse *et al.* 1999) and *Nauphoeta cinerea* (Elpidina *et al.* 2001). 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 α-amylase produced and secreted by the columnar cells, although the possibility cannot be excluded that CCAP may regulates activities of other digestive enzymes than carbohydrate-degrading enzymes.

CCAP was also shown to elicit a prominent contraction of all gut muscles (Fig. II-.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. II -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. II-4D). The ingluvial ganglion, which is a major component of the stomatogastric nervous system, is known to innervate the foregut and anterior midgut (Miyoshi and Endo 1998), and CCAP production was in fact detected in the ingluvial ganglion (Fig. II-5A, 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. II-4B). These histochemical observations 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. II-.3). The cockroach abdominal ganglia of the ventral cord innervate the dorsal and ventral muscles, the dorsal heart, and the hindgut (Cornwell 1968). The immunohistochemical and physiological studies on a variety of insects showed that CCAP from the ventral cord controls the heart beat and hindgut contraction (Lehman *et al.* 1993, Stangier *et al.* 1989, Dircksen *et al.* 1991, Dulcis and Levine 2001), 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. II-7C 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.

I for the first time showed the expression of both the CCAP gene and peptide in the insect midgut endocrine cells (Figs 2.3 and 2.4). The CCAP gene expression has yet to be investigated in the midgut of other insects including *D. melanogaster* (Park *et al.* 2003) and *M. sexta* (Loi *et al.* 2001). Similarly, the gene expression of other myomodulatory neuropeptides such as leucomyosuppressin (Fuse *et al.* 1998) and tachykinin-related

peptides (Siviter *et al.* 2000) 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 α-amylase activity (Fig. II-6) and contraction of the midgut (Fig. II-7B and D) suggest the presence of a CCAP receptor in this tissue. Recently, identification of a CCAP receptor from *Drosophila* was reported (Cazzamali *et al.* 2003). 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, I 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.

Chapter Ⅲ

Nutrients-induced α -Amylase and Protease activity is Regulated by Crustacean Cardioactive Peptide (CCAP) in the Cockroach Midgut

Ⅲ-1. Introduction

Feeding behavior is critical for all organisms' lives. Most insects have either herbivorous or carnivorous property, whereas cockroaches are known to feed on a variety of organic sources. The exceptional omnivorous characteristic of cockroaches in the insect kingdom suggests that the cockroach midgut has the ability to recognize and digest many kinds of foods. The primary tissue for digestion of nutrients in insects is the midgut, which produces diverse digestive enzymes such as amylase, invertase, maltase, and lactase, proteases and lipase (Cornwell, 1968). The midgut epithelium responsible for digestion and absorption of nutrients is not innervated by sensory neurons (Lehane 1998), indicating the non-neuronal sensory mechanism functions in digesting processes. In early studies, secretory granules were observed in insect midgut endocrine cells residing in the epithelial layer (Nishiitsutsuji-Uwo *et al.* 1981), and several mammalian neuropeptide-like immunoreactivities were detected in the endocrine cells (Montuenga *et al.* 1989). Insect

midgut endocrine cells were also found to express several genes of insect neuropeptides such as allatostatin (Reichwald *et al.* 1994), leucomyosuppressin (Fuse *et al.* 1998), and neuropeptide F (Brown *et al.* 1999). These findings support the notion that neuropeptides produced by the midgut endocrine cells play pivotal roles in food digestion as gut regulatory factors in a non-neuronal regulatory fashion. Furthermore, the omnivorous property of cockroaches indicates the multiple gut-peptidergic regulations for digestion.

In our previous study, I identified a neuropeptide crustacean cardioactive peptide (CCAP) as a novel gut factor in insect. The expression of the CCAP gene in the endocrine cells of the midgut was detected by *in situ* hybridization, and immunohistochemical analysis showed that CCAP was distributed around the lateral surfaces of the endocrine cells. Furthermore, elevation of α-amylase activity was also observed upon addition of CCAP to the midgut.

In this report, I show the CCAP-regulatory digestion cascade where nutrients-inducing α-amylase and protease activities are mediated by CCAP.

Ⅲ-2. *Materials and methods*

Animal

The adult male American cockroach, *Periplaneta americana,* reared in mass and fed with water and artificial diet (MF, Oriental Yeast Corp., Tokyo, Japan) at 25 ℃ were used for experiments. They were kept at 25 ℃ under a 12:12h light:dark cycle. Animals were anesthetized by cooling under crushed ice before its dissection. To eliminate food in the gut completely, the cockroaches were kept individually in clear plastic cups containing a tube filled with water for 3 weeks without food. They were fed with 100% starch, casein, talc and none, respectively, after 3-week starvation. The midgut from each cockroach was collected for subsequent analysis at 1h, 2h, 3h, 4h, and 5h after re-feeding the experimental diets.

Immunohistochemistry

The midgut was dissected from *P. americana,* adult males, fixed in Bouin fluid at 4 ℃ overnight, and embedded in paraffin from which 7-μm sections were cut. Immunohistochemistry was performed as previously described in Chapter Ⅱ. Primary antibodies used were rabbit anti-CCAP (used at 1:1,000; Genemed Synthesis, Inc, CA,

USA). Immunoreactivity was visualized by the avidin-biotin peroxidase method with a diaminobenzidine (DAB) as a chromogen (Vectastain ABC Elite kit, Vector Lab. Inc, CA, USA).

Controls included (i) 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 (ii) deletion of the primary antiserum. No specific immunostaining was observed in either of the controls.

Morphometric analysis

The immunohistochemical reaction was quantified using the point counting method. Four sections of the midgut from each experimental animal were randomly selected from 50 sections. Points falling on the endocrine cells were counted and divided by the total number of points on the midgut epithelium. Data were reported as the amount of endocrine cells per 100 points grid. All morphometric parameters determined for all groups were compared by one-way ANOVA and the level of significance was set at 5% in all analyses.

Competitive ELISA

Isolated midguts from the re-fed cockroaches were homogenized individually in TBS after removing food particles and peritrophic membrane, and then each homogenate was centrifuged. The supernatants were used for samples. Three samples were tested at each time point. The antiserum against CCAP and a synthetic CCAP conjugate were used in a competitive ELISA to quantify CCAP in the samples. A CCAP-BSA conjugate was prepared by coupling CCAP to BSA with dimethyl suberimidate, DMS. For the analysis, the coating plates were prepared with CCAP-BSA (0.6 μg/ml per well) in 0.05 M sodium carbonate–bicarbonate buffer (pH 9.0) for 3h and blocked with 2% skimmed milk for 1 h. Standard peptides (0.01–100 nmol/well) or the midgut supernatant samples were added respectively in a volume of 50 μl/well. Subsequently 50 μl of the diluted antiserum against CCAP (1:20,000 final concentration in TBST with 2% skimmed milk) were added in the wells. Plates were incubated overnight at 4 °C. Plates were washed three times with TBST after incubation, and incubated with a secondary antibody solution (goat serum anti-rabbit IgG labeled with alkaline phosphatase, 1:2000 final concentration in TBST with 2% skimmed milk) for 1 h. 100 μl of the substrate (p-nitrophenylphosphate disodium salt hexahydrate, NPP) was added to each well and incubated for 1h. The reaction was stopped with 50 μl of 4 M NaOH. Finally, the results were measured at 405 nm using an

automated microplate reader (Tosoh, Tokyo, Japan).

Digestive enzyme assay: α-amylase and protease

The α-amylase activities of the midgut were measured as described previously using the α-amylase measuring kit (Kikkoman Corp. Chiba, Japan). The protease activities of the midgut were measured by digestion of azocasein (Elpidina *et al.* 2001). The cockroach's midguts were dissected and cut to remove lumen contents in 50mM Tris-HCl (pH 7.4). Each tissue was homogenized in fresh 50mM Tris-HCl and centrifuged at 4,000× g for 10 min at 4 °C. Aliquots were taken from the supernatant to measure proteases activities. Proteases activities were quantified by incubating 0.3ml supernatant with 0.3ml of 0.5% azocasein solution in Tris-HCl (pH 7.4). After 15min of incubation at 37 °C, the reaction was stopped by adding 0.9ml of 30% trichloroacetic acid. The incubation mixture was centrifuged at 4,000× g at 4 °C for 15min and the supernatant was read at 335nm with a UV-Spectrophotometer.

CCAP effect on the enzyme activities

The midguts prepared as described above were incubated in the 50mM Tris-HCl (pH 7.4) in the presence or absence of CCAP at room temperature for 20 min. α-amylase

activities and protease activities released into the supernatant and the tissues were measured.

Statistical analysis

Results are shown as the mean ± SE. Data were analyzed by the one-way ANOVA with Dunnett error protection. Differences were accepted as significant when $P < 0.05$.

Ⅲ-3. Results

Localization of enzymes activity in the midgut

To investigate the regions responsible for starch and protein digestion, distribution of two of the digestive enzymes, α-amylase and protease, was determined. The midgut was divided into three regions, anterior, middle and posterior for enzyme distribution studies. Fig. Ⅲ-1AB shows that both α-amylase and protease enzyme activities in the anterior region were 2 times stronger than middle and posterior region. These results showed that the anterior part of the midgut plays a major role in protein and starch digestion.

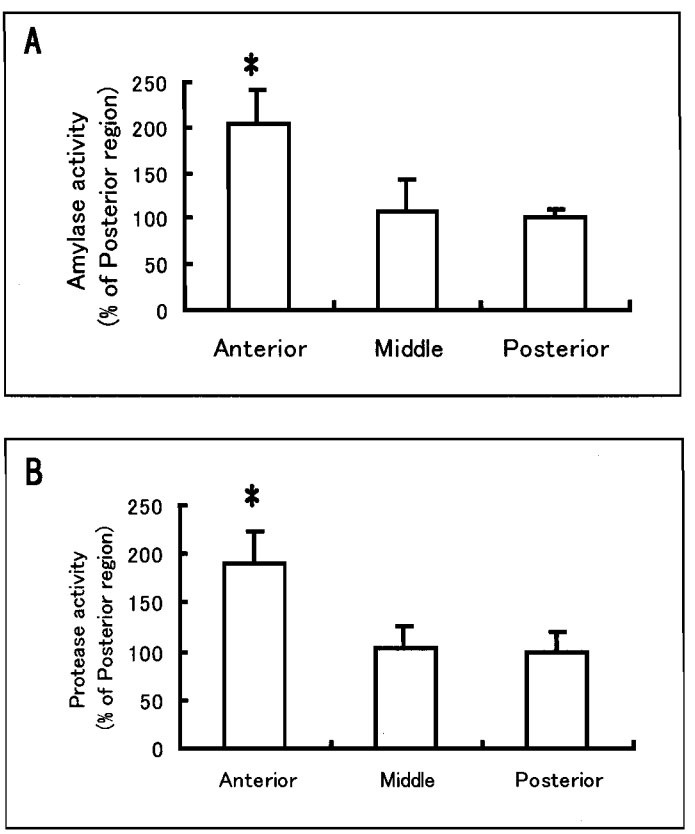


Fig. Ⅲ-1. The distribution of α-amylase (A) and protease (B) activity in the midgut of *P. americana* from fed adults. Each point represents the mean ± SE of three preparations. *, $P<0.05$, compared with the enzyme activity of posterior midgut.

Localization of CCAP-positive endocrine cell in the midgut epithelium

Previous study showed that CCAP stimulated α-amylase activity in the midgut, suggesting direct up-regulation of α-amylase activity with CCAP. This result allowed us to presume that more CCAP-producing cells would localize in the anterior region than in any other parts. Immunohistochemical analysis using the midgut paraffin section of *ad libitum* individuals demonstrated that a large number of endocrine CCAP-immunoreactive cells were observed in the anterior midgut. Fig. Ⅲ-2A shows the represented CCAP-endocrine cells in the anterior region. Fig. Ⅲ-2B indicates the number of CCAP-endocrine cells per unit area: more numerous CCAP-positive endocrine cells were detected in the anterior part than in the middle and posterior midgut. This analysis revealed that CCAP-positive endocrine cells were situated in the digestive enzymes-containing area in the anterior midgut.

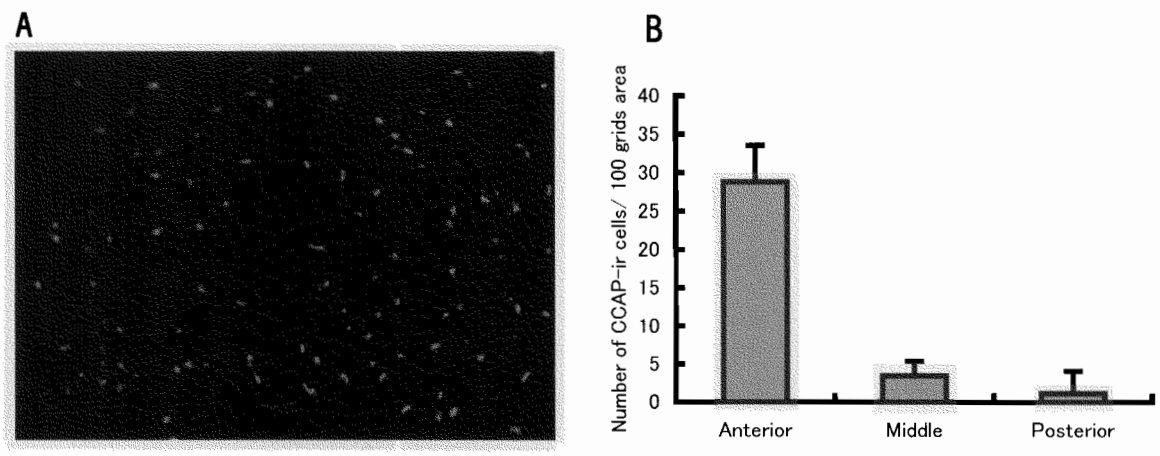


Fig. Ⅲ-2. CCAP-endocrine cells in the anterior midgut region (A). The distribution of number of CCAP-immunoreactive cells in the anterior, middle, and posterior midgut of adult *P. americana* (B). Each point represents the mean ± SE of three preparations. *, $P<0.05$, compared with the number of CCAP-immunoreactive cells of the posterior.

Stimulatory effect of CCAP on digestive enzyme activity in the midgut

Subsequently, I examined whether CCAP modulates protease activity in the midgut. Intriguingly, incubation of the dissected midgut with CCAP at more than 10^{-8} M led to an increase in the activity of protease (Fig. Ⅲ-3), whereas only the buffer showed no effects (data not shown). This result provided evidence that CCAP is responsible for digestion of proteins as well as starch. Fig. Ⅲ-4A and 4B show the digestive enzyme activities released into the buffer over a 4h incubation period. Three hours incubation of the midgut treated with CCAP exhibited approximately 8-fold higher the α-amylase activity than the midgut untreated with CCAP (Fig. Ⅲ-4A). In addition, Fig. Ⅲ-4A demonstrated the time-dependent α-amylase activity; the activity remained continuously elevated up to 3h, and then reduced. Likewise, the released protease activity of the CCAP-treated midgut was 3-fold higher than that of untreated one (Fig. Ⅲ-4B). Taken together, these results clearly showed that CCAP up-regulated both the α-amylase and protease activities released from the midgut.

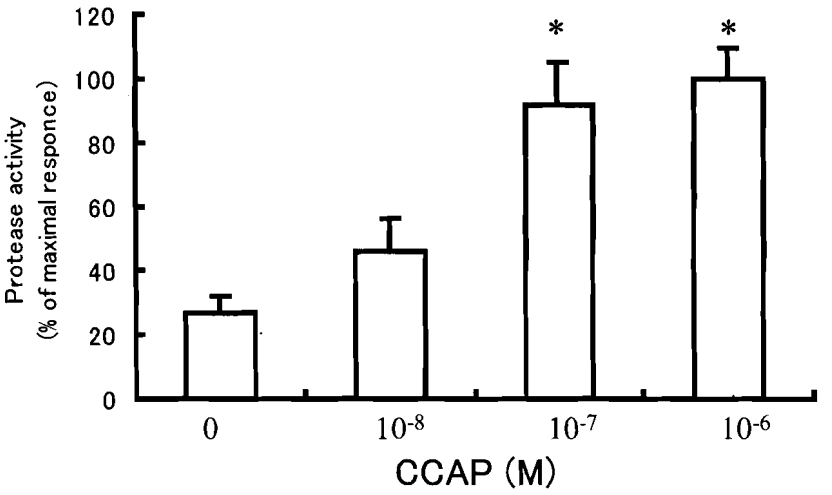


Fig. Ⅲ-3. Dose-response for protease activity in the midgut tissue of *P. americana*, from fed adults as a result of *in vitro* treatment with CCAP. Each point represents the mean ± SE of three preparations. *, $P<0.05$, compared with protease activity in the absence of CCAP.

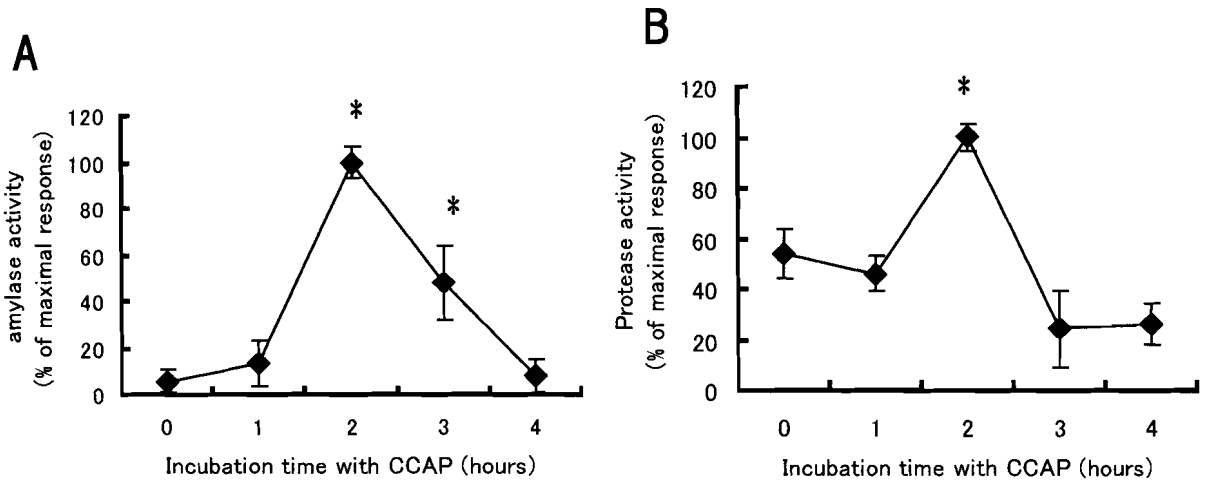


Fig. Ⅲ-4. Time course of change in midgut α-amylase activity in the incubated buffer (A) and midgut protease activity in the incubated buffer (B) following treatment *in vitro* with 10^{-7} M CCAP. Each point represents the mean ± SE of three preparations. *, $P<0.05$, compared with enzyme activity in the point at the incubation time 0 hour.

Effect of feeding on CCAP content in the midgut

The above results suggested that CCAP released from the midgut endocrine cells stimulates digestive enzyme activities in response to food contents. We initially examined whether the CCAP-endocrine cells number and CCAP amount were dependent on nutrients.

Quantitative analysis of stained cells indicated that there was an increase in the number of CCAP-expressing cells after re-feeding starch (Fig. Ⅲ-5A) and casein (Fig. Ⅲ-5C), but not after re-feeding talc (Fig. Ⅲ-5E). The tissue section collected after 3 hr post-re-feeding starch showed twice increase in the number of CCAP-positive endocrine cells than those unfed and after 1 hr post-re-feeding, and the sections prepared from the midgut after 5h post-re-feeding showed decrease in number of positive cells to the basal level. Similar data was obtained from the quantitative analysis of the midgut section prepared by the cockroaches with casein (Fig. Ⅲ-5C). Competitive ELISA on the midgut homogenate of re-feeding starch and casein individuals also confirmed that CCAP amount in the midgut of cockroaches after 2h and 3h post-re-feeding increased 1.5-fold more than in the unfed midgut, and then decreased after 4h post-re-feeding (Fig. Ⅲ-5B and D). The midgut homogenate of re-feeding talc did not change CCAP amount during 4h post-re-feeding (Fig. Ⅲ-5F). It is noteworthy that these nutrients-inducing elevation of

the CCAP amount was in good agreement with the up-regulation of the α-amylase and protease activities by CCAP (Fig. Ⅲ-4.), given that both of them reached the maximum level after at 3-hr incubation after treatment with nutrients or CCAP. Consequently, we concluded that both starch and proteins induce the synthesis of CCAP in the midgut.

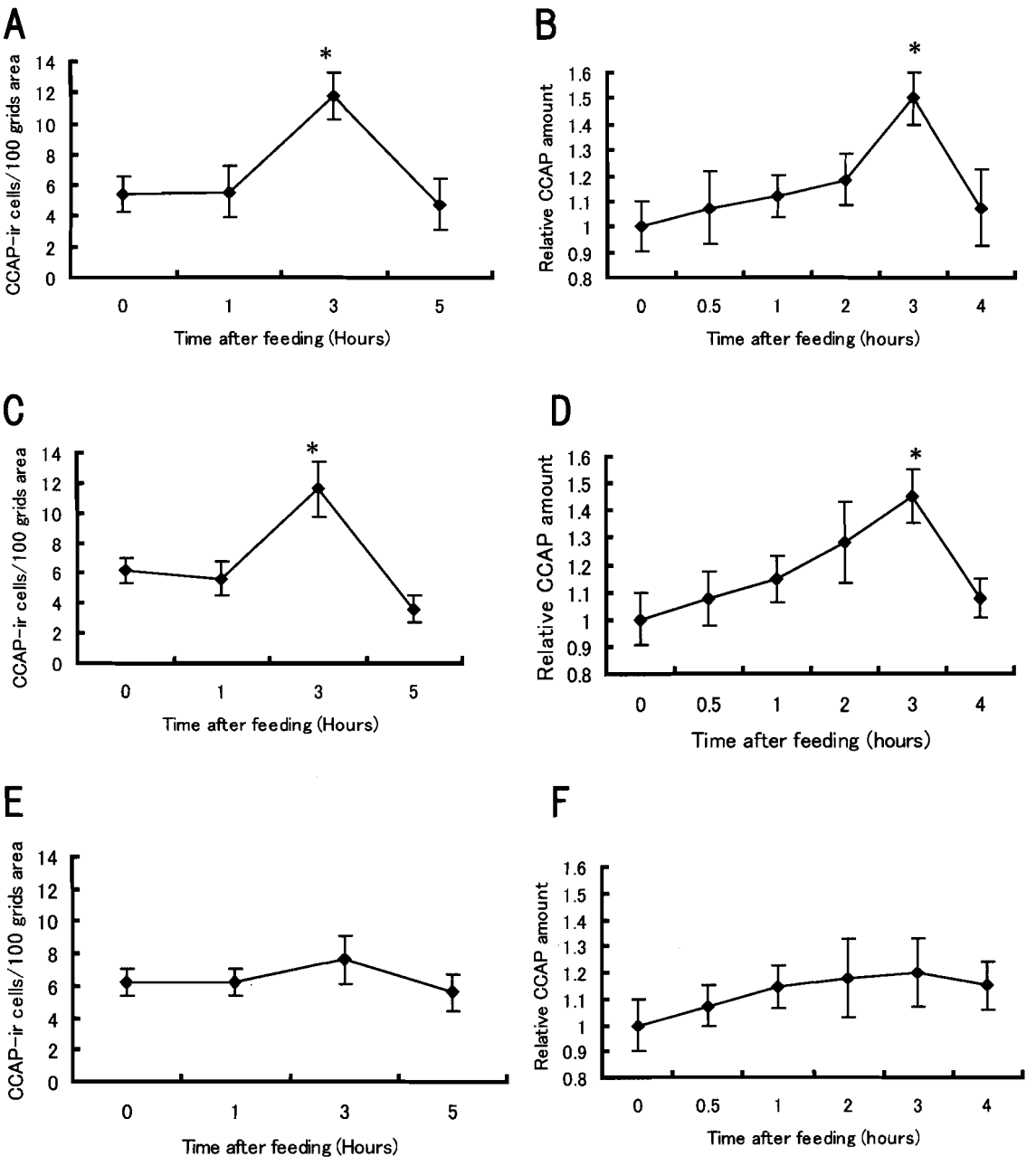


Fig. Ⅲ-5. Percentage of CCAP immunopositive cells (mean ± SE) in the midgut epithelium of which fed starch (A), casein (C), and talc(E). Competitive ELISA detected relative amount of the midgut epithelium of which fed starch (B), casein (D), and talc(F). Each point represents the mean ± SE of three preparations. *, $P<0.05$, compared with CCAP-cell number or CCAP amount in the point at the time 0 hour.

Ⅲ-4. *Discussion*

The digestive system of cockroach is a coiled tube composed of the foregut, the midgut and the hindgut from mouth to anus. When foods move thorough the alimentary canal from mouth to anus, almost digestive processes and absorption of nutrients are initiated in the midgut (Cornwell 1968). The midgut plays a critical role in absorption and digestion. The midgut is composed of a single layered epithelium resting on a continuous basal membrane surrounded by circular and longitudinal muscles. The major cells in the midgut are columnar cells, responsible for processing of the diet. Endocrine cells and regenerative cells are scattered among the population of columnar cells, throughout the epithelium. The nervous systems innervate only the midgut musculature, indicating that the digestive and absorptive cells in the epithelial layer are not controlled by any neuronal cells (Lehane *et al* 1996). Thus, endocrine cells are expected to regulate the digestion and absorption in insect midguts.

As described in Chapter Ⅱ, neuropeptide CCAP was found to be produced in the cockroach midgut endocrine cells as a stimulator of amylase activity; CCAP mRNA was detected in the endocrine cells, and that administration of CCAP to the midgut resulted in elevation of α-amylase activity.

In this chapter, I show the functional correlation of CCAP with digestion of nutrients.

Predominant α-amylase and protease activities were detected in the anterior region of the midgut (Fig. Ⅲ-1). Immunohistochemical analysis demonstrated that a large number of CCAP-endocrine cells were also localized in the anterior midgut (Fig. Ⅲ-2). Notably, CCAP was shown to stimulate not only amylase but also protease activity in the midgut in a time-dependent manner (Fig. Ⅲ-3). These results provided evidence that CCAP is responsible for digestion of carbohydrate and proteins. Furthermore, a quantitative analysis of CCAP-immunoreactive cells and ELISA for CCAP induced by nutrients (Fig. Ⅲ-5) revealed the time-dependent increase in CCAP production in the endocrine cells, which is consistent with induction of α-amylase and protease activities by CCAP (Fig. Ⅲ -4). In addition, CCAP is continuously elevated up to 3hr after feeding (Fig. Ⅲ-5). Taken together, these results led to the conclusion that the nutrients such as starch and protein stimulates the release of CCAP, which in turn up-regulates the α-amylase and protease activities. Furthermore, long-term (3h) continuous elevation of both CCAP-positive endocrine cells (Fig. Ⅲ-5A, C) and CCAP amounts (Fig. Ⅲ-5B, D) strongly suggest that the *de novo* synthesis of CCAP is also enhanced by the pre-existing CCAP, nutrients and/or other secondary inducible factors. Investigation of such regulatory systems is currently in progress.

The CCAP-immunoreactivity was observed at the lateral margins of the midgut

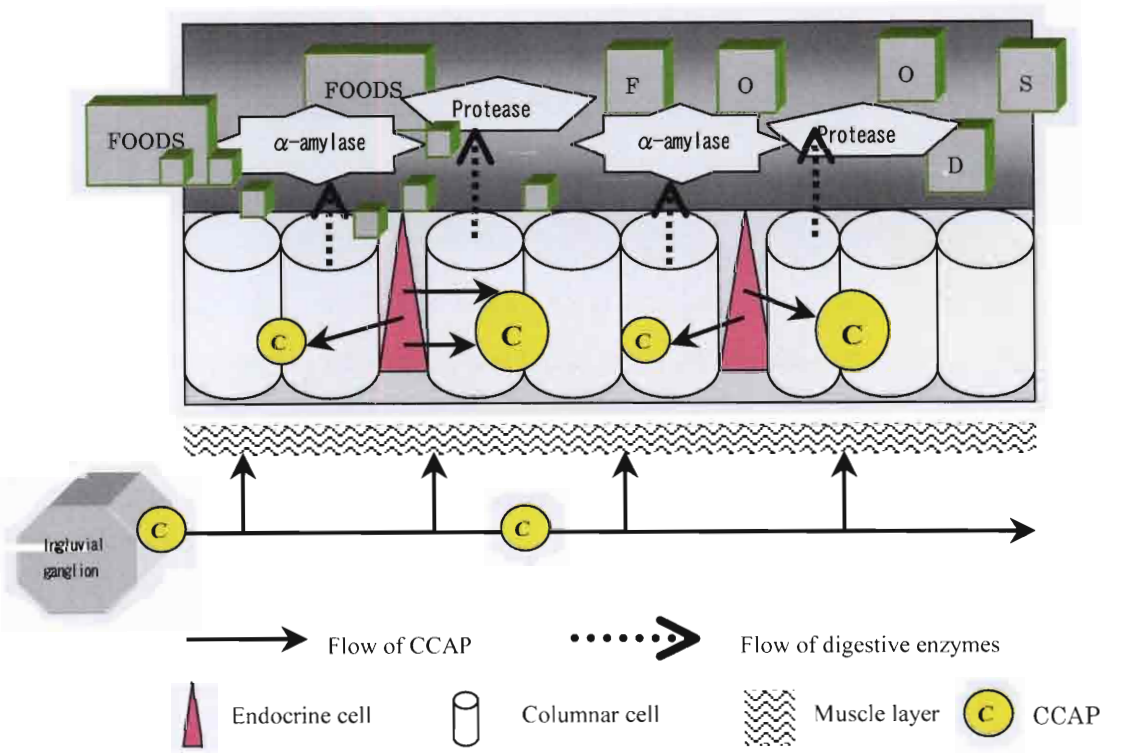
endocrine cells (Sakai *et al*, 2004). In addition, microscopic observation of the midgut implies that the CCAP-immunoreactivity likely spreads from the CCAP-positive endocrine cells into gaps among adjacent cells. These findings, combined with the up-regulation of α-amylase and protease activities by CCAP, support the notion that CCAP released from the midgut endocrine cells participates in the regulation of digestion in a paracrine manner. In keeping with this, also of interest is the localization of CCAP receptor expressing cells. The stimulatory effects of CCAP on the midgut digestive enzyme activities suggest the presence of a CCAP receptor in the columnar cells which produce the digestive enzymes. CCAP receptor was cloned from *Drosophila*, although the affinity to CCAP is relatively low (Cazzamali *et al,* 2003). Localization of an authentic CCAP receptor in the cockroach midgut is expected to provide a crucial clue to the understanding of the mechanism and physiological roles of CCAP in the digestive system. Identification of a CCAP receptor from the cockroach is currently in progress. In summary, we present the regulation of nutrients-inducing α-amylase and protease activities by CCAP, revealing a novel non-neural gut peptidergic digestive system.

Summary and Perspective

In the present study, I showed the isolation of CCAP from the midgut of *P. americana,* characterization of cDNA sequence, localization of the CCAP mRNA and the peptide, stimulation of α-amylase and protease activity from the midgut, and contractile effects on the alimentary tract by CCAP administration, and the functional correlation of CCAP expression and release with digestion of nutrients. This study has revealed novel biological roles of CCAP, regulations of feeding through neural and humoral input.

The experiments in Chapter 2 led to the conclusion that CCAP released from the midgut endocrine cells is involved in food digestion and/or absorption. CCAP was shown to be expressed in the ingluvial ganglion and ventral nerve cord as well as in the midgut endocrine cells, suggesting that CCAP also plays an essential role in gut movement through the nervous system in the cockroach. This notion is compatible with the result showing prominent gut contraction by CCAP. Therefore, it is presumed that CCAP participates in the regulation of feeding behavior by concerted functions as a non-neuronal substance for induction of food digestion by the midgut epithelium and a myostimulatory neuropeptide for each of the gut tissues. This invertebrates peptidergic control of feeding is reminiscent of the brain-gut axis hormone system in vertebrates.

Physiological experiments in Chapter 2 and 3 provide evidence that the nutrients such as starch and protein stimulates the release of CCAP, which in turn up-regulates the α-amylase and protease activities. The result indicates that a nutrient rise both digestive enzyme activities in the lumen through stimulating the release of CCAP. This mechanism, which allows the midgut digests diverse nutrients in food with digestive enzymes cocktail, is suitable to the omnivorous insect. Furthermore, long-term continuous elevation of both CCAP-positive endocrine cells and CCAP amounts strongly suggest that the *de novo* synthesis of CCAP is enhanced by the nutrients and/or other secondary inducible factors.



Present study strongly suggest that CCAP produced in the midgut endocrine cells 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. Localization of an authentic CCAP receptor in the cockroach midgut is expected to provide a crucial clue to the understanding of the paracrine mechanism and physiological roles of CCAP in the digestive system. Adding this, molecular regulatory mechanisms of the neuropeptide synthesis and secretion of the endocrine cells have yet been well elucidated. Further investigation is required to examine whether CCAP synthesis is directly induced by nutrients or other secondary inducible factors, and whether the neuropeptide processing enzymes, such as precursor convertases and peptidyl-hydroxyglycine-amidating lyase, are also newly synthesized to produce a mature peptide. Also of interest is the regulation of the digestive enzymes, given that it is unclear whether CCAP promotes *de novo* synthesis of digestive enzymes or only enzyme secretion.

In summary, CCAP was shown to have multifunctional roles in the regulation of gut tissues as both a neuronal and gut peptidergic factor, which regulates nutrients-inducing α-amylase and protease activities.

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List of publications

(1) Sakai T, Satake H, Minakata H, and Takeda M. Characterization of crustacean cardioactive peptide as a novel insect midgut factor: Isolation, localization, and stimulation of α-amylase activity and gut contraction. *Endocrinology* 2004, 145(12): 5671–5678

(2) Sakai T, Satake H, and Takeda M: Nutrients-induced α-amylase and protease activity is regulated by crustacean cardioactive peptide (CCAP) in the insect midgut.

in preparation

Related paper

(1) Takeda M, Sakai T, Fujisawa Y, Narita M, Iwabuchi K, Loeb MJ: Cockroach midgut peptides that regulate cell proliferation, differentiation, and death in vitro. *In Vitro* Cell Developmental Biology of Animal. 2001, Jun; 37(6): 343-347

(2) Slama K, Sakai T and Takeda M: Effect of corazonin and CCAP on heartbeat in the adult American cockroach (*Periplaneta americana*).

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