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Primary structure of a cardioactive neuropeptide from the tobacco hawkmoth, *Manduca sexta*

Clement C. Cheung[a], Poh Kheng Loi[a], Andy W. Sylwester[a,*], Terry D. Lee[b] and Nathan J. Tublitz[a]

[a]*Institute of Neuroscience, University of Oregon, Eugene, OR 97403, USA* and [b]*Division of Immunology, Beckman Research Institute of the City of Hope, Duarte, CA 91010, USA*

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The amino acid sequence of the first of a family of insect cardioregulatory peptides from the tobacco hawkmoth, *Manduca sexta*, has been determined using a combination of Edman degradation microsequencing and mass spectroscopy. This peptide contains 9 amino acid residues and an observed mass for the monoisotopic protonated molecule of 956.4 Da. There are two cysteines at positions 3 and 9 forming a disulfide bridge and the carboxyl-terminus is amidated. The structure of this peptide, Pro-Phe-Cys-Asn-Ala-Phe-Thr-Gly-Cys-NH_2, is identical to a peptide recently isolated from crabs called crustacean cardioactive peptide (CCAP) and we propose that this peptide be named *Manduca* CCAP.

Cardioregulation; Insect neuropeptide; Invertebrate neuropeptide

1. INTRODUCTION

The insect heart is modulated in vivo by several neurohumoral factors, including the biogenic amine octopamine, as well as by a number of neurally derived peptides [1]. Included in this latter group are the cardioacceleratory peptides (CAPs), a family of peptides which have been studied extensively in the tobacco hawkmoth, *Manduca sexta* [2]. Early work isolated a pair of CAPs, known as CAP_1 and CAP_2, from the *Manduca* central nervous system [3]. Physiological studies have determined that the CAPs act as cardiostimulatory neurohormones twice in adult *Manduca*, once immediately after adult emergence to facilitate wing inflation [4,5] and again during flight episodes for increased blood circulation between abdomen and thorax [6]. The CAPs are also functionally active in earlier developmental stages, for which the hindgut is the primary target. One of the CAPs, CAP_2, is released during the latter stages of embryogenesis to stimulate the initial contractions of the hindgut [7]. CAP_2 is also released in caterpillars at the beginning of metamorphosis to trigger clearing of the alimentary canal [8]. The CAPs thus serve a variety of stage-specific functions throughout this organism's life cycle.

Despite the extensive physiological work on the *Manduca* CAPs, their primary structures have remained unresolved, in large part due to the paucity of available material to analyze. These difficulties have been compounded by the recent report that there are at least 2 distinct CAP_1s and 3 CAP_2s [9]. This paper reports on the elucidation of the amino acid sequence of the first of the CAP_2s using microsequencing techniques.

Correspondence address: N.J. Tublitz, Institute of Neuroscience, University of Oregon, Eugene, OR 97403, USA. Fax: (1) (503) 346-4548

Abbreviations: CAPs, cardioacceleratory peptides; CAP_1, cardioacceleratory peptide 1; CAP_2, cardioacceleratory peptide 2; CCAP, crustacean cardioactive peptide; HPLC, high performance liquid chromatography; HFBA, heptafluorobutyric acid; TFA, trifluoroacetic acid.

2. MATERIALS AND METHODS

The abdominal portion of the ventral nerve cord (ANC) from over 6,000 pharate adult moths were dissected and stored at −20°C. Frozen ANCs were heat-treated at 80°C for 5 min and extracted in 0.5 M acetic acid. Following an initial low pressure separation through a C-18 cartridge (Sep-pak, Waters), CAP bioactivity was purified to homogeneity using a 6-step HPLC procedure whose details are described in Table I. The first four HPLC steps were performed by reverse-phase HPLC using an Isco model 2350 system and standard bore (4.6 mm and 2.1 mm) columns [10]. The final two chromatographic steps used microbore (1.0 mm and 0.5 mm) columns and a syringe pump HPLC. All chromatographic steps used variations of a standard acetonitrile gradient in dilute TFA or HFBA [10]. Each step was spectrophotometrically monitored at 200 (step 6) or 215 nm (steps 1–5) and CAP-containing fractions confirmed using an isolated pharate adult heart bioassay [3,5,8]. The peptide peak from the fifth purification step was subjected to amino acid sequencing on an Applied Biosystems 670A sequencer equipped with a PTH on-line analyzer. Following a sixth chromatography step, the same peak was analyzed further on a Finnigan MAT TSQ-700 triple sector quadruple mass spectrometer using a 10 kV Cs ion primary beam and thioglycerol as the sample matrix.

3. RESULTS

Isolation and purification of the CAPs first required that the CAP_1s were fully separated from the CAP_2s, and this was accomplished by the initial HPLC run (Table I). The second chromatography step fractionated the combined CAP_2 activities into three distinct

Table I

Purification procedure for isolation of *Manduca* CCAP/CAP_{2a}

Step	Column	Column size	Flow rate	ΔGradient A→B	Counter ion	λ (nm)
1	Spheri-5 ODS	220 × 4.6 mm	1 ml min^{-1}	2% min^{-1}	TFA	215
2	Aquapore 300	220 × 4.6 mm	1 ml · min^{-1}	0.5% min^{-1}	HFBA	215
3	Aquapore 300	220 × 4.6 mm	1 ml · min^{-1}	0.1% · min^{-1}	TFA	215
4	Aquapore 300	220 × 2.1 mm	250 μl · min^{-1}	0.25% min^{-1}	TFA	215
5	Aquapore 300	250 × 1.0 mm	50 μl min^{-1}	0.1% min^{-1}	TFA	215
6	Vydac C-18	300 × 0.5 mm	20 μl · min^{-1}	2% min^{-1}	TFA	200

Columns used in steps 1–5 were manufactured by the Brownlee Corporation. All runs were performed at room temperature. TFA, trifluoroacetic acid; HFBA, heptafluorobutyric acid

bioactive peaks, which we have called CAP_{2a}, CAP_{2b}, and CAP_{2c}. This paper focusses exclusively on the analysis of CAP_{2a}. Four additional HPLC steps, including a final run using a 0.5 mm i.d. microbore column at very low flow rates, were required to purify CAP_{2a} to homogeneity. Peptide purity was confirmed using Edman degradation, which produced a single residue signal in each cycle. From an initial starting sample of 6,000 ANCs we obtained about 150 pmol of pure CAP_{2a}.

The Edman degradation results from a starting sample of approximately 80 pmol are shown in Table II. With the exception of cycle 3, strong signals were obtained in each cycle. The low yield in cycle 3 compared that from other cycles suggested that the assignment of Tyr at residue 3 might be open to other interpretations. One likely possibility was that the Tyr signal of cycle 3 was the result of a partially oxidized Cys residue that co-chromatographed with Tyr. This hypothesis was investigated by analyzing the same sample with mass spectrometry, which yielded a mass of 956.4 for the protonated molecule (Fig. 1). This is the value that would be calculated if the sequence from the Edman degradation was modified to include a Cys at positions 3 and 9 joined by a disulfide bond and with an amide at the C-terminus. MS/MS analysis of the molecular ion gave a daughter ion spectrum consistent with this structure (data not shown). Fragmentation ions from the parent peptide were relatively weak, as would be expected for a cyclized molecule. Nonetheless, there was sufficient sequence information to confirm the proposed structure.

To test the validity of the MS/MS data, we had the proposed sequence synthesized by American Peptide Co., Sunnyvale, CA. The synthetic peptide co-eluted precisely with native CAP_{2a} on each of the first 5 HPLC purification steps. For technical reasons, the final microbore step was not tested. When assayed on an isolated pharate adult *Manduca* heart, native and synthetic peptides produced identical dose-dependent cardioexcitatory responses (Fig. 2). Both synthetic and native peptides were biologically inactivated upon reduction with performic acid (data not shown). Based on these results, we conclude that the primary sequence of CAP_{2a} is Pro-Phe-Cys-Asn-Ala-Phe-Thr-Gly-Cys-NH_2.

4. DISCUSSION

The data presented here firmly establishes the amino acid sequence of one of the CAPs, CAP_{2a}, using Edman degradation and mass spectrometry procedures. The mass of the protonated molecule (m/z 956.4) for this nine-residue peptide is in the range of values determined previously by gel filtration on Sephadex G-15 (ca. 500 Da) [3,4]. This nonapeptide exhibits several distinctive properties for a neuropeptide: (i) although water soluble it is very hydrophobic, a characteristic which explains the long retention times on all reverse-phase columns, and (ii) it is cyclized, with the two Cys residues forming a disulfide bridge in the active conformation. This latter conclusion is supported by previous reports which showed that CAP_2 lost much of its bioactivity when exposed to reducing agents such as performic acid [10,11]. These studies also demonstrated that the C-terminal amide was required for biological activity.

A search of the protein data base at the National Center for Biotechnology Information at the National Library of Medicine revealed only one other peptide

Table II

Amino acid sequence of *Manduca* CCAP/CAP_{2a} using Edman degradation

Residue no.	Identity	Yield (pmol)
1	Pro	76.8
2	Phe	78.7
3	(Tyr)	5.8
4	Asn	32.6
5	Ala	25.8
6	Phe	26.6
7	Thr	20.9
8	Gly	13.8
9	–	–
10	–	–
11	–	–

Cycles 9–11 yielded no detectible signal.

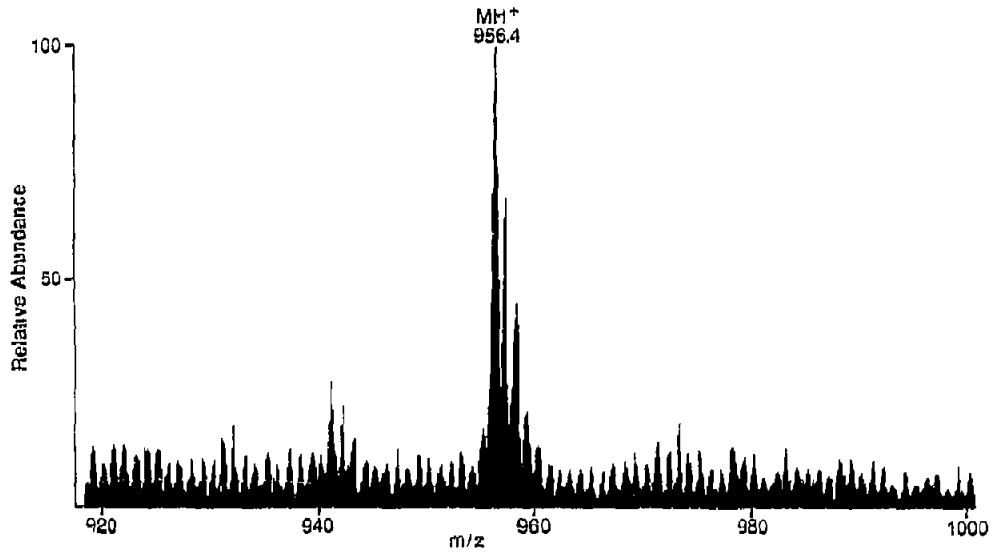

Fig. 1 Molecular ion region of the positive ion mass spectrum of CAP_{2a}/CCAP

with a significant degree of homology (more than 2 residues) to CAP_{2a}. This was crustacean cardioactive peptide (CCAP), an invertebrate peptide originally isolated from the pericardial organs of the shore crab, *Carcinus maenas* [12], whose structure is 100% homologous, including the C-terminus amide, with CAP_{2a}. CCAP appears to be widely distributed among the crustacea, with CCAP-like bioactivity isolated from other crabs, such as the edible crab *Cancer pagurus*, and the crayfish, *Orconectes limosus* [13]. Although its function is unknown, CCAP is a potent cardioexcitor in all these crustacea, producing both inotropic and chronotropic effects when applied to semi-isolated hearts [13]. CCAP has also been isolated in an insect, *Locusta migratoria*, but in that species it is the hindgut rather than the heart that is most sensitive to CCAP application [14]. Thus, there is a striking degree of overlap between CCAP's pharmacological profile in crustaceans and locusts, and the physiology of the CAPs in *Manduca* since the CAPs have been shown to have physiologically significant actions on both the hindgut and heart [2,6]. Because CAP_{2a} and CCAP share identical structures and targets, we propose that CAP_{2a} be renamed as *Manduca* CCAP.

The present elucidation of the primary structure of *Manduca* CCAP should provide a springboard for future molecular studies on its physiology and mode of action in regulating insect behavior.

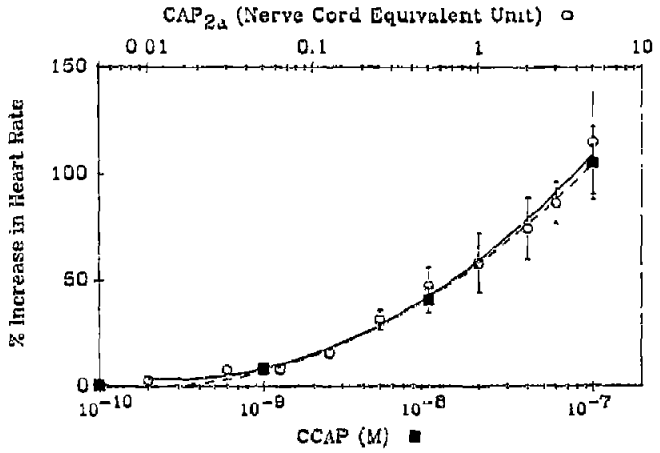

Fig. 2. The effects of native CAP_{2a} and synthetic CCAP on the isolated *Manduca* heart bioassay. Each point represents the mean ± S E M of at least 8 different assays with the exception of the 5 nerve cord equivalent CAP_{2a} data point which is the average of 5 replicate assays. (○) Native CAP_{2a}; (■) synthetic CCAP.

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