Amino acid sequence of CAP_{2b}, an insect cardioacceleratory peptide from the tobacco hawkmoth *Manduca sexta*

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Abstract The primary structure of a novel insect neuropeptide, Cardioacceleratory Peptide 2b (CAP_{2b}), from the tobacco hawkmoth *Manduca sexta* has been established using a combination of mass spectroscopy, Edman degradation microsequencing, amino acid analysis, and biological assays. The sequence of CAP_{2b}, pyroGlu-Leu-Tyr-Ala-Phe-Pro-Arg-Val-amide, has a molecular weight of 974.6 and is blocked at both the amino and carboxyl ends. Examination of several national computer protein data bases failed to reveal other peptides or proteins with any sequence homology to CAP_{2b} indicating that this is likely to be a novel insect neuropeptide. This peptide may be a general activator of insect viscera since it causes an increase in heart rate in *Manduca* and in *Drosophila*, and has also been implicated in the regulation of fluid secretion by the Malphigian tubules of *Drosophila*.

Key words: Manduca sexta; Drosophila melanogaster; Insect cardioactive peptide; Cardioacceleratory peptide; Neuropeptide; Insect neurobiology; Invertebrate neurobiology

1. Introduction

Visceral muscle in insects is modulated in vivo by many neurohumoral factors including biogenic amines and a number of neurally derived peptides [1]. One example of the latter are the Cardioacceleratory Peptides (CAPs) [2], a set of neuropeptides originally isolated from the CNS of the tobacco hawkmoth *Manduca sexta*. In *Manduca* they serve at least four different, stage-specific functions. The CAPs act as cardiostimulatory neurohormones twice in adult moths, once immediately after adult emergence to facilitate wing inflation [3–7] and again during flight for metabolic purposes [8]. A subset of the CAPs are also active in earlier developmental stages in *Manduca*, although their primary target is the hindgut rather than the heart. They trigger the initial contractions of the hindgut during embryogenesis [9] and also facilitate clearing of the alimentary canal at the beginning of metamorphosis, which occurs midway through the final larval instar [10]. The CAPs have also been implicated in ovary function in *Manduca* (S.E. Reynolds, personal communication), in the control of fluid secretion by the Malphigian tubules in *Drosophila melanogaster* [11], in the control of heart and gut activity in *Locusta migratoria* [12], and in the regulation of cardiac activity in crustaceans [13].

While much is known about the physiology of the CAPs, progress in obtaining their primary structures have been hampered by the paucity of enough purified material for sequence analyses. Early biochemical studies using low pressure column chromatography fractionated the CAPs into two distinct groups, CAP_1 and CAP_2 [3,4,7]. More recent investigations using reverse phase HPLC has shown that these two groups can be further subdivided into at least two CAP_1s (1a and 1b) and three CAP_2s (2a, 2b, and 2c) [14]. To date only one CAP, CAP_{2a}, has been fully sequenced [14]; its structure is identical to a peptide isolated from crustaceans called Crustacean Cardioactive Peptide (CCAP) [15] and to avoid confusion we will refer to CAP_{2a} as *Manduca* CCAP. In this paper we elucidate the amino acid sequence of another CAP, CAP_{2b}. Using microsequencing techniques we show that the structure of CAP_{2b} is not similar to that of *Manduca* CCAP (CAP_{2a}) and that CAP_{2b} has no sequence homology with any known peptides or proteins.

2. Materials and methods

The protocol we followed to isolate and purify CAP_{2b} to homogeneity is described in detail elsewhere [14]. In brief, the abdominal portion of the ventral nerve cord (ANCs) was removed from 6000 pharate adult *Manduca* and frozen at −80°C for further processing. Following a brief heat treatment to precipitate large proteins (80°C for 5 min), the ANCs were homogenized in 0.5 M acetic acid and microfuged at high speed for 5 min to remove cellular debris and insoluble proteins. The resultant supernatant was applied to a low pressure C-18 cartridge (Sep-pak, Waters) which was then exposed to increasing step-wise concentrations of acetonitrile. The CAPs eluted in the 60% acetonitrile fraction [7] and this fraction was subjected to a six step HPLC protocol. The first three steps were performed by reverse-phase HPLC on an Isco model 2350 two pump system using Aquapore 300 C-18 columns (Brownlee) with an inner diameter of 4.6 mm (steps 1 and 2) and 2.1 mm (step 3). The fourth chromatographic step was performed with a 1.0 mm (I.D.) microbore column using a syringe pump HPLC. The last two chromatographic steps were performed on microcapillary columns (0.53 mm and 0.25 mm I.D., respectively) described elsewhere [16,17]. Each step was spectrophotometrically monitored at 214 nm (steps 1–3), 220 nm (step 4), and 200 nm (steps 5, 6). CAP containing fractions were confirmed at steps 1–5 using an isolated pharate adult heart bioassay [3,4]. Following the fifth HPLC step, CAP_{2b} was analyzed by secondary ion mass spectrometry (SIMS) using a TSQ-700 triple quadrupole instrument (Finnigan-MAT, San Jose, CA) equipped with a cesium ion gun (Phrasor Scientific Inc., Duarte, CA) to determine monoisotopic mass. The ion source acceleration potential was 8 keV and the conversion

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Abbreviations: CAPs, cardioacceleratory peptides; CCAP, crustacean cardioactive peptide; CAP_{2a}, cardioacceleratory peptide 2a; CAP_{2b}, cardioacceleratory peptide 2b; HPLC, high performance liquid chromatography, ANCs, the abdominal portion of the ventral nerve cord.

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dynode was set to −12 keV. Scans were acquired every 3 seconds in a mass per charge range from 400 to 2000. An aliquot of the sample was applied directly after HPLC onto the sample stage which had been previously covered with 3-mercapto-1,2-propanediol (thioglycerol) as matrix. Following the 6th HPLC step, CAP2b was mass analyzed on-line using a TSQ-700 triple quadrupole instrument (Finnigan-MAT, San Jose, CA) equipped with an electrospray ion source operating at atmospheric pressure (LC-MS) [17]. The electrospray needle was operated at a voltage differential of 3–4 keV and a sheath flow of 2 μl/min of methoxyethanol was used. The same instrument was used to perform tandem mass spectrometry for primary sequence determination (LC-MS/MS). The collision offset was set to −20 keV and the collision cell was filled with argon to a pressure of 3 milliTorr. Scans were continually acquired every 3 s and spectra were generated by averaging the scans containing the peak.

3. Results

The first HPLC run splits the heart accelerating bioactivity from the *Manduca* CNS into two peaks, CAP_1 and CAP_2 [7]. The CAP_1-containing fractions were set aside at this point and all subsequent experiments focussed on the CAP_2 peak. The second HPLC step fractionates CAP_2 into three bioactive peaks, CAP_{2a}, CAP_{2b}, and CAP_{2c} [14]. Four additional HPLC steps were required to purify CAP_{2b} to homogeneity. From an initial starting sample of 6000 ANCs, we obtained about 100 picomoles of pure CAP_{2b}.

Two attempts were made to obtain sequence information on the purified CAP_{2b} with standard Edman techniques. Both attempts were unsuccessful at cycle one suggesting that CAP_{2b} might be blocked at the N-terminus. CAP_{2b} was further analyzed by SIMS, which yielded a protonated molecule with a monoisotopic mass of 975.6 (Fig. 1). MS/MS analysis produced a daughter ion spectrum which, when analyzed, gave a proposed sequence of pyroGlu-(Leu/Ile)-Tyr-Ala-Phe-Pro-Arg-Val-amide. Attempts were made to both acetylate and methylate the peptide. However, no change in the value of the protonated molecular ion was observed in either instance. This is consistent with a blocked amino terminus and an amide on the carboxy terminus. The ambiguity of the amino acid designation

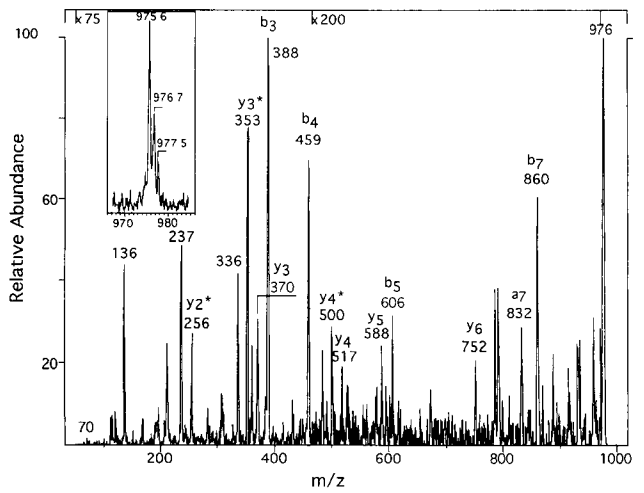

Fig. 1. Monoisotopic mass (MH+) determination (inset) of native CAP_{2b} and collision-activated dissociation spectrum of m/z 976 ion. The collision energy was 20 eV and the argon pressure in the collision cell was 3×10^{-3} Torr. The fragment ions are labelled using the Biemann nomenclature [20]. Ion series members that have a additional loss of ammonia are denoted with an asterisk.

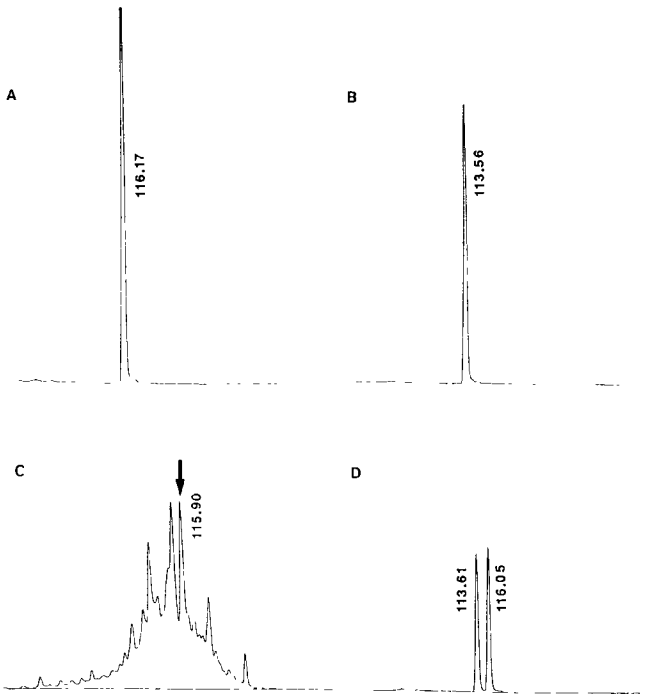

Fig. 2. HPLC elution profiles of native CAP_{2b} and the Leu- and Ile-containing synthetic peptides when chromatographed on the same HPLC column under identical conditions. (A) Leu synthetic peptide (elution time, 116.17 min). (B) Ile synthetic peptide (elution time, 113.56 min). (C) Native CAP_{2b}, (arrow; elution time, 115.90 min). (D) Elution profile of the two synthetic peptides chromatographed together (elution times, 113.61 min for the Ile form and 116.05 min for the Leu form).

at the second position (Leu vs. Ile) could not be resolved using mass spectroscopy since both residues have the same molecular mass. The amount of material purified to homogenity was not sufficient for amino acid analysis.

As a more direct approach towards resolving which sequence was correct, both peptides were synthesized to compare their chromatographic and cardioactive properties to that of native CAP_{2b}. Microbore HPLC runs (step 4) were conducted on the two synthetic peptides to compare their elution times to that of the native peptide. The native and the two synthetic peptides were chromatographed either individually or in pairs following the protocol used in step 4 of the purification protocol [14]. When chromatographed separately, the native peptide eluted at 115.9 min, while the Ile and Leu versions of the synthetic peptide eluted at 113.4 min and 116.1 min, respectively (Fig. 2a–c). Two distinct peaks were obtained when the two synthetic peptides were mixed together (Fig. 2d). These data indicate that the sequence of the native peptide is likely to contain a Leu residue at position 2.

Another approach to resolving the Leu/Ile ambiguity was to bioassay equivalent doses of all three peptides, the two synthetics and the native, on the isolated *Manduca* heart. All three peptides were separately chromatographed on the microbore HPLC following the same procedures used in the previous set of experiments. HPLC injection samples were adjusted to ensure similar peak size for all three peptides. The appropriate fractions from each run were collected, lyophilized, resus-

pended in *Manduca* saline [8], and applied on the isolated heart bioassay at equivalent concentrations. To guard against interassay variability, each bioassay preparation was exposed to all three peptides. The Leu-synthetic and native CAP2b each produced comparable cardioacceleratory responses at comparable OD units, whereas an equimolar application of the Ile form elicited a much smaller cardioexcitatory effect (Fig. 3).

Fig. 4 depicts the dose–response relationship of the three peptides when tested on the isolated heart bioassay. Because individual heart preparations varied in their CAP sensitivity, each individual heart bioassay was exposed to a full range of concentrations for each peptide. The shape of the dose response curves for the two synthetic peptides were very similar, however, the heart was more sensitive to the Leu peptide than the Ile form at all concentrations. Threshold (i.e. the lowest bioactive concentration) for the Leu-peptide was less than 10^{-9} M whereas threshold for the Ile-peptide was slightly greater than 10^{-8} M. Although the 5 native CAPs, the 2 CAP_1s and the 3 CAP_2s, each elicit a dose-dependent increase in heart rate when assayed on the *Manduca* heart bioassay [14], their effects are not identical. The cardioexcitation produced by *Manduca* CCAP is fairly transient in comparison to that caused by CAP_{2b} (Fig. 5). The CAP_{2b} response is characterized by a relatively slow time-to-peak and an even slower decline to baseline. The temporal characteristics of the cardioexcitation produced by both synthetic peptides was similar to that of native CAP_{2b} (Fig. 5), with both peptides triggering an increase in heart rate that slowly reached maximal and which returned to basal over a period of many minutes. Thus, the temporal response characteristics of the Leu-containing synthetic peptide is consistent with the hypothesis that the native CAP_{2b} contains a Leu residue.

4. Discussion

Several lines of evidence presented in this paper support the conclusion that the amino acid sequence of CAP_{2b} is pyroGlu-

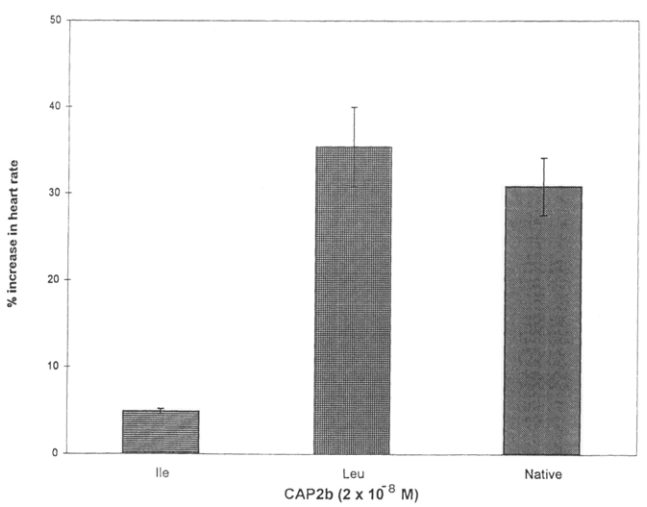

Fig. 3. Response of the isolated *Manduca* heart to equimolar concentrations (2×10^{-8} M) of native CAP_{2b} and the Leu- and Ile-containing synthetic peptides. Concentrations were determined by chromatographing each peptide on microbore HPLC and assaying samples from equivalent sized peaks on the isolated *Manduca* heart bioassay. Each histogram represents the mean ± S.E.M. of at least 5 separate samples.

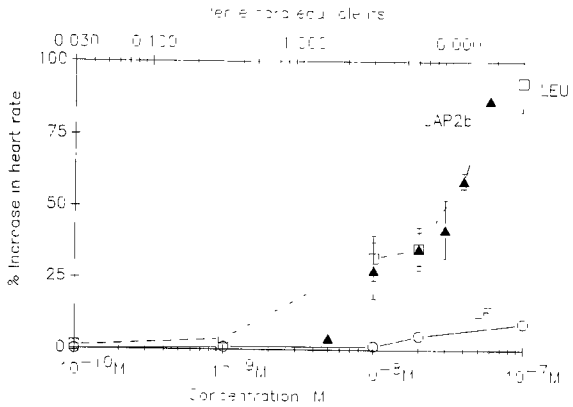

Fig. 4. Dose–response relationship of native CAP_{2b} and the Leu- and Ile-containing synthetic peptides on the isolated *Manduca* heart bioassay. Native CAP_{2b} is measured in terms of nerve cord equivalents (NCE) where one NCE is equal to the total CAP bioactivity extracted from a single pharate adult nerve cord. Each point represents the mean ± S.E.M. of at least 5 individual samples except for one data point (native CAP_{2b}, 18 nerve cord equivalents) for which $n = 1$. Data points without error bars indicate that the S.E.M. is smaller than the size of the data symbol.

Leu-Tyr-Ala-Phe-Pro-Arg-Val-amide. Mass spectroscopic analysis indicates that CAP_{2b} has a molecular weight of 974.6, is amidated at the C-terminus, and blocked at the N-terminus, the latter which is consistent with the lack of success at sequencing using standard Edman techniques. The MS data also indicates that CAP_{2b} has eight residues, all of which could be unambiguously assigned with the exception of the residue at position 2. The MS data was unable to distinguish between a Leu and a Ile residue at position 2, however this ambiguity was resolved by comparing elution times of the Leu and Ile forms of the peptide with native CAP_{2b} using HPLC (Fig. 2). These data, combined with the amino acid composition results, clearly showed that Leu was the appropriate assignment for the residue at position 2. The proposed sequence was further confirmed by the results from physiological assays, that showed that equivalent concentrations of native CAP_{2b} and the Leu-containing synthetic peptide produced identical responses when applied onto the isolated *Manduca* heart (Fig. 3). Moreover MS sequence analysis of the native and the Leu-containing synthetic peptide generated identical daughter ion spectra. The primary structure proposed here for CAP_{2b} appears to be unique, based upon a search of national protein data bases (Swiss-Pro and PIR using the BLAST program) which failed to find any peptide or protein which contained this sequence.

These results force a re-evaluation of some of the initial assumptions about the CAPs. The CAPs were originally identified and named on the basis of their cardioexcitatory bioactivity [3–6,14], and although several studies have shown that the CAPs cause an increase in heart rate in a variety of insects and other arthropods [12], recent investigations have demonstrated that their effects are not limited to the heart. For example, some of the CAP_2s have potent physiological and pharmacological effects on gut activity of several insects including moths [2] and locusts [12]. CCAP has also been shown to affect the activity of the insect ovary (S.E. Reynolds, personal communication). Others have proposed that the CAP_1s may have an allatotropin-like stimulatory effect on the release of juvenile

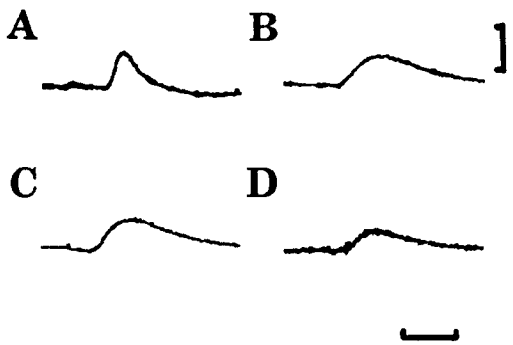

Fig. 5. Time course of the response of the isolated *Manduca* heart to CCAP (A), native CAP_{2b} (B), and the two synthetic peptides (C, Leu; D, Ile). Scale bars: 2 min, 20% increase in heart rate.

hormone by the insect corpora allata [18], although biochemical data does not support this conclusion (Tublitz, unpublished observations). Thus, it is likely that a number of the CAPs perform a variety of functions in insects in addition to affecting the heart.

This multiplicity of function also applies to CAP_{2b}. Although CAP_{2b} modulates heart rate in *Manduca* [14] and *Drosophila melanogaster* [11], it also is a potent stimulator of fluid secretion in adult *Drosophila* Malphigian tubules, the insect equivalent of the vertebrate kidney [11,19]. CAP_{2b}-like bioactivity in flies has been localized to a set of midline mesodermal cells that exhibit a number of neuronal-like properties. One intriguing property is the presence of an axonal-like process in the neurosecretory transverse nerve, suggesting a secretory function for these cells [11]. CAP_{2b} acts on the Malphigian tubules through the cGMP second messenger system [19] and preliminary evidence suggests that CAP_{2b}-triggered rise in cGMP may be occurring through the nitric oxide pathway (Davies et al., unpublished observations). Taken together these data support the hypothesis that CAP_{2b}, like the other CAPs, acts on tissues other than the heart. Thus, the CAPs should not be considered as exclusively cardioregulatory.

The results presented here also indicate that the CAPs, although grouped together on the basis of functional similarities, may not be structurally related to each other. The only other CAP that has been sequenced to date, CCAP (CAP_{2a}), is a cyclical nonapeptide (Pro-Phe-Cys-Asn-Ala-Phe-Thr-Gly-Cys-amide) with no sequence homology to the CAP_{2b} structure presented here. Answers to questions on the structural relationships among the 5 CAPs must await sequence analyses of the remaining CAPs.

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References

[1] Raabe, M. (1989) Recent Developments in Insect Neurohormones, Plenum Press, NY, pp. 235–266.
[2] Tublitz, N.J., Broadie, K.S., Loi, P.K. and Sylwester, A.W. (1991) Trends Neurosci. 14, 254–259.
[3] Tublitz, N.J. and Truman, J.W. (1985) J. Exp. Biol. 114, 365–379.
[4] Tublitz, N.J. and Truman, J.W. (1985) J. Exp. Biol. 114, 381–395.
[5] Tublitz, N.J. and Truman, J.W. (1985) Science 228, 1013–1015.
[6] Tublitz, N.J. and Truman, J.W. (1985) J. Exp. Biol. 116, 395–410.
[7] Tublitz, N J. and Evans, P.D. (1986) J. Neurosci. 6, 2451–2456.
[8] Tublitz, N.J. (1989) J. Exp. Biol. 142, 31–48.
[9] Broadie, K.S., Sylwester, A.W., Bate, C.M. and Tublitz, N.J. (1990) Development 108, 59–71.
[10] Tublitz, N.J., Allen, A.T., Cheung, C.C., Edwards, K.K., Kimble, D.P., Loi, P.K. and Sylwester, A.W. (1992) J. Exp. Biol. 165, 241–264.
[11] Tublitz, N.J., Bate, M., Davies, S.H., Dow, J.A.T. and Maddrell, S.H.P. (1994) Soc. Neurosci. Abstr. 20, 533.
[12] Stangier, J. (1991) in: Comparative Aspects of Neuropeptide Function (Florey, E. and Stefano, G.B. eds.), Manchester University Press, UK, pp. 201–210.
[13] Stangier, J., Hilbich, C. and Keller, R. (1989) J. Comp. Physiol. B. 159, 5–11.
[14] Cheung, C.C., Loi, P.K., Sylwester, A.W., Lee, T.D. and Tublitz, N.J. (1992) FEBS Lett. 313, 165–168.
[15] Stangier, J., Hilbich, C., Beyreuther, K. and Keller, R. (1987) Proc. Natl. Acad. Sci. USA 84, 575–579.
[16] Swiderek, K.M., Chen, S., Feistner, G.J., Shively, J.E. and Lee, T.D. (1992) In: Techniques in Protein Chemistry III (R.H. Angeletti ed.), pp. 457–465, Academic Press, NY.
[17] Swiderek, K.M., Lee, T.D. and Shively, J.E. (1994) In: Methods of Enzymology (B.L. Karger and W.S. Hancock eds.) Academic Press, NY, in press.
[18] Veenstra, J.A., Lehman, H.K. and Davis, N.T. (1994) J. Exp. Biol. 188, 347–354.
[19] Davies, S.A., Maddrell, S.H.P., Dow, J.A.T., O'Donnell, M.J., Huesmann, G.R. and Tublitz, N.J. (1995) Am. J. Physiol. in press
[20] Biemann, K. (1988) Biomed. Environ. Mass Spectrom. 16, 99–111.