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Isolation and structure of corazonin, a cardioactive peptide from the American cockroach

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Corazonin, a new cardioaccelerating peptide, has been isolated from the corpora cardiaca of the American cockroach, *Periplaneta americana*, and its structure determined to be Glp-Thr-Phe-Gln-Tyr-Ser-Arg-Gly-Trp-Thr-Asn-amide. The peptide stimulated heart beat at concentrations as low as 0.2 nM, which makes it the most potent insect cardioactive neuropeptide.

Amino acid sequence; Cardioacceleration; Insect; Neuropeptide; (*Periplaneta americana*)

1. INTRODUCTION

The cardiostimulatory effects of corpus cardiacum extracts in insects are well known [1,2]. Two cardioexcitatory peptides were recently isolated from the corpus cardiacum of the American cockroach, *Periplaneta americana* [3–5]. Both are octapeptides related to crustacean red pigment concentrating hormone (RPCH) and locust adipokinetic hormone (AKH), which are called periplanetins CC1 and CC2 [4], or M1 and M2 [5]. The same peptides were also found to be responsible for the hypertrehalosemic effects of corpus cardiacum extracts [4,6]. Here the isolation and structure of a third cardioacceleratory peptide is reported, which has been called corazonin, from corazon, Spanish for heart.

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Abbreviations: AKH, adipokinetic hormone; CC, corpora cardiaca; HFBA, heptafluorobutyric acid; HPLC, high-performance liquid chromatography; HTH, hypertrehalosemic hormone; PITC, phenyl isothiocyanate; PTC, phenylthiocarbamyl; PTH, phenylthiohydantoin; RPCH, red pigment concentrating hormone; TFA, trifluoroacetic acid

2. MATERIALS AND METHODS

2.1. *Experimental animals*

American cockroaches, *Periplaneta americana*, were derived from a laboratory culture kept at 27 ± 2°C. They were fed dog chow and had access to water.

2.2. *Bioassays*

Heart accelerating activity was tested on isolated abdominal hearts from young adult males. Hearts were kept in 1 ml of Baumann and Gersch's saline [3]. After 30 min the rate of heart beat was determined by measuring the time needed for ten beats, these measurements were repeated every 30 s. Only regularly beating hearts were used for the assays, which consisted of exposing the isolated heart to increasing doses of corazonin. Each subsequent dose was only applied after the heart beat had stabilized. This stabilized rate was used for the dose response curve.

2.3. *Preliminary extraction*

For peptide extraction 2- to 6-month-old animals were used. They were decapitated and the corpora cardiaca dissected under 0.9% NaCl and stored in a glass homogenizer on dry ice. Batches of up to 600 pair of corpora cardiaca were homogenized in 1 ml of Bennett's mixture (1% NaCl, 5% formic acid, 1% TFA and 1 M HCl in water [7]) to which 12 μl thiodiglycol had been added. The extract was centrifuged and the pellet reextracted in 0.5 ml of Bennett's mixture. The combined super-

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natants were loaded on a previously activated and equilibrated C-18 Sep-Pak (Waters Associates, MA, USA). The Sep-Pak was washed with 5 ml water and 5 ml of 13% acetonitrile and corazonin was then eluted with 5 ml of 32% acetonitrile (all eluants contained 0.1% TFA). This fraction was concentrated by nitrogen evaporation and purified by HPLC.

2.4. *Peptide HPLC*

The HPLC apparatus consisted of two Waters model 510 pumps, a Waters model M730 data module, a model 680 gradient controller and a model 481 spectrophotometer. Separations were performed on a 15 × 0.46 cm column and a 5 × 0.46 cm precolumn (Tecnocroma, San Cugat des Valles, Spain) filled with 5 μm particles of Spherisorb ODS-II. Solvents were water (A) and 65% acetonitrile (B), which contained either TFA (0.1% and 0.11%) or HFBA (0.13% and 0.145%) as pairing ions. In both cases the column was eluted isocratically for 30 min with 21% B, followed by a linear gradient from 21% to 51% B over 60 min at flow rate of 1 ml/min. Absorption was monitored at 210 nm.

2.5. *Amino acid composition*

The amino acid composition was determined using a Pico-Tag system (Waters Associates, MA, USA), after hydrolysis in HCl vapor for 1 h at 150°C [8].

2.6. *Enzymatic deblocking*

Dry corazonin was solubilized in 10 μl of 0.1 N HCl, 100 μl of Zalut's buffer [9] containing 2.5 units of pyroglutamate aminopeptidase was then added and the mixture incubated for 15 min at 37°C under nitrogen. The reaction was terminated by addition of 250 μl of 0.13% HFBA in water. The deblocked corazonin was isolated by HPLC using HFBA as the pairing ion at a flow rate of 0.8 ml/min. After 20 min isocratic elution of 21% B, a linear gradient to 81% B over 30 min was used.

2.7. *Sequence analysis*

The sequence of despyroglutamyl-corazonin was performed by 'Servicio de sequenciación de Proteinas de la Universidad de Barcelona', using a model 470A protein sequencer of Applied Biosystems Inc. (Foster City, CA, USA), according to the program supplied by the manufacturer. PTH amino acids were analyzed online by a model 120A from the same company.

2.8. *C-terminal analysis*

About 1 nmol of purified corazonin was digested with 1 μg trypsin in 50 μl ammonium bicarbonate buffer at 37°C for 1 h. The C-terminal tetrapeptide was isolated by HPLC as described above, using TFA as the pairing ion. In this case after 20 min isocratic elution of 0% B, a linear gradient to 100% B over 60 min was employed at a flow rate of 1 ml/min; absorption was monitored at 280 nm. The peptide was coupled with PITC as described [8], and after removal of excess reagents the mixture was incubated with concentrated HCl for 5 min at room temperature to cleave the PTC-amino acid residue [10]. Coupling with PITC and cleavage with HCl was performed repeatedly. After the fourth coupling with PITC the reaction sequence was halted. The presence of PTC-Asn-amide in the final reaction mixture was analyzed using HPLC with the following conditions: column (25 × 0.46 cm), containing 10 μm particles of Spherisorb ODS-II (Tecnocroma, San Cugat des Valles, Spain); solvent A: 25 mM sodium acetate, 0.05% triethylamine in water, pH 6.35; solvent B: 60% acetonitrile in water; a linear gradient from 10% to 52% B over 20 min; column temperature: 25°C; flow rate: 0.8 ml/min; detection at 254 nm. The identity of the PTC-Asn-amide peak from corazonin was confirmed by lyophilization and treatment with concentrated HCl for 7 min at room temperature, which converted authentic PTC-Asn-amide to PTH-Asn and some PTC-Asn. Analysis of the latter reaction was performed using the same HPLC conditions as for the PTC-Asn-amide.

3. RESULTS AND DISCUSSION

With the same gradient using successively HFBA and TFA as pairing ions (figs 1 and 2) a complete purification of corazonin was achieved. Amino acid analysis showed the presence of approximately 2 pmol per pair of corpora cardiaca and the following amino acid composition: Glx(2), Asx(1), Phe(1), Tyr(1), Thr(1), Ser(1), Gly(1) and Arg(1). Only alanine was found as a contamination at ~8% of the amount of peptide. The difference in elution time of small peptides using either TFA or HFBA as pairing ions can be accounted for by the number of positively charged groups present in the peptide [11]. Since corazonin contained arginine and the difference in elution time indicated exactly one positively charged group, this suggested the N-terminus of corazonin to be blocked, possibly by pyroglutamate. After digestion with pyroglutamate aminopeptidase a new peak was observed. This peak yielded the following sequence: Thr-Phe-Gln-Tyr-Ser-Arg-Gly-Trp-Thr-Asn, while the next cycle was empty. This sequence, together with

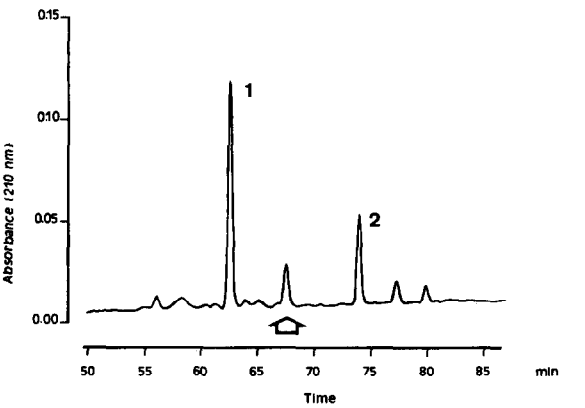

Fig.1. HPLC fractionation of Sep-Pak prepurified corazonin from 80 CC using HFBA as pairing ion. The peak indicated by the arrow contains corazonin. Peaks 1 and 2 contain periplanetins CC1 and CC2, respectively.

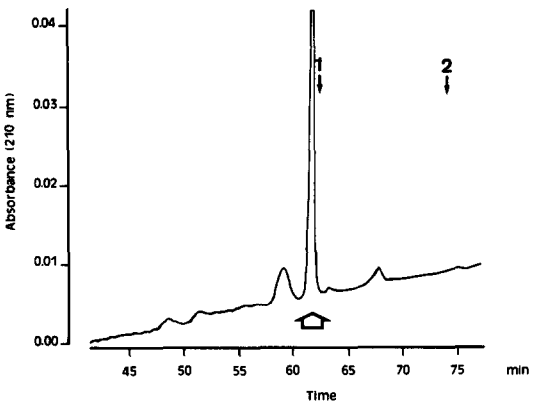

Fig.2. Final purification of corazonin (large arrow) using TFA as pairing ion. The small arrows labelled 1 and 2 indicate the elution position of periplanetins CC1 and CC2 in this system.

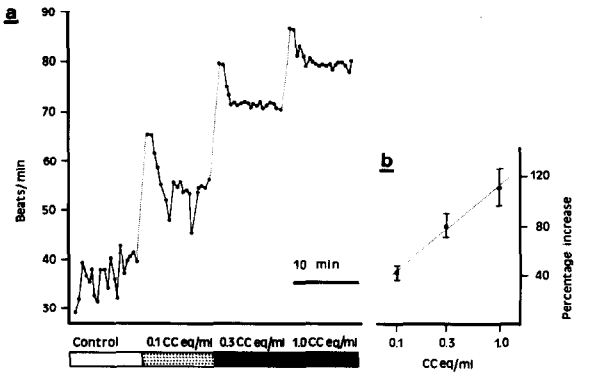

Fig.4. Effect of increasing doses of purified corazonin on heart beat. (a) Effect of corazonin on a single isolated abdominal heart. (b) Dose response curve of seven preparations as shown in a, indicated are means ± SE.

the N-terminal Glp, accounts for all amino acids found in the amino acid analysis and an additional Trp, which is destroyed during hydrolysis. Thus the sequence of corazonin must be: Glp-Thr-Phe-Gln-Tyr-Ser-Arg-Gly-Trp-Asn. The presence of a C-terminal amide group in several recently isolated insect neuropeptides [12] suggested that corazonin might have a blocked C-terminal. In the mixture generated to demonstrate Asn-amide, a peak was found to elute at the position of authentic PTC-Asn-amide. The identity of PTC-Asn-amide was confirmed by treatment with concentrated HCl, which yielded a small peak of PTH-Asn and a trace of PTC-Asn. Thus the complete structure of corazonin is Glp-Thr-Phe-Gln-Tyr-Ser-Arg-Gly-Trp-Asn-amide.

Corazonin has interesting sequence similarities with some members of the AKH/RPCH family. The recently isolated hypertrehalosemic hormone from *Heliothis zea* [13] shows the largest sequence similarity. Corazonin also has a small sequence similarity with leucopyrokinin, a peptide believed to be homologous with periplanetin CC2 [14], another member of the AKH/RPCH family (fig.3). However, some of its characteristics, notably the presence of an arginine, the absence of phenylalanine in position 4, and the presence of 11, rather than 8, 9 or 10 amino acids, seem to exclude it from family membership.

Corazonin was found to have strong cardioacceleratory effects. At concentrations as low as 0.2 nM the heart beat rate was significantly increased (fig.4). This makes it the most potent insect neuropeptide in this respect and about 100 times as potent as the other two cardioacceleratory neuropeptides isolated from this species. Although the amplitude and strength of the heart beat were not measured, they both seemed to be increased by corazonin. Since the corpus cardiacum is a neurohemal organ, it seems reasonable to suggest that corazonin is released into the hemolymph. The release of only one percent of the peptide present in a pair of corpora cardiaca into the hemolymph, which has a volume of about 0.2 ml [1], should lead to a significant increase in the heart beat rate. It seems therefore likely that corazonin is involved in the physiological regulation of the heart beat in the American cockroach.

Heliothis hypertrehalosemic hormone	Glp-Leu-Thr-Phe-Ser-Ser-Gly-Trp-Gly-Asn-NH_2
Corazonin	Glp-Thr-Phe-Gln-Tyr-Ser-Arg-Gly-Trp-Thr-Asn-NH_2
Leucopyrokinin	Glp-Thr-Ser-Phe-Thr-Pro-Arg-Leu-NH_2

Fig.3. Structure of corazonin, leucopyrokinin and *Heliothis* hypertrehalosemic hormone.

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