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Unusual cardioactive peptide (CCAP) from pericardial organs of the shore crab *Carcinus maenas*

(crustacean neuropeptide/amino acid sequence/cardioactive neurohormone/neurosecretion)

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ABSTRACT **An unusual crustacean cardioactive peptide (CCAP) from the pericardial organs of the shore crab *Carcinus maenas* has been purified to homogeneity by a two-step reversed-phase HPLC procedure. Manual microsequencing using the 4-(*N*,*N*-dimethylamino)azobenzene 4′-isothiocyanate/phenylisothiocyanate double-coupling technique and automated gas-phase sequencing of the oxidized peptide revealed that CCAP is a nonapeptide (M_r 957) of the sequence Pro-Phe-Cys-Asn-Ala-Phe-Thr-Gly-Cys-NH_2. We have confirmed the sequence by chemical synthesis of the C-terminally amidated and nonamidated forms of the peptide. The presence of the amide group was indicated by lack of susceptibility to carboxypeptidase A and Y treatment and was confirmed by the observation that the native CCAP comigrated with the amidated synthetic peptide on HPLC. Native and synthetic CCAP displayed high accelerating activity on a semi-isolated crab heart preparation, whereas the nonamidated synthetic peptide was of much lower potency. The effect of CCAP was both ino- and chronotropic. The two pericardial organs of one animal yielded 30–40 pmol of extractable CCAP. Its sequence does not resemble that of any known neuropeptide. However, a "mirror-image" similarity to vasopressin is conspicuous.**

The crustacean pericardial organs (POs) are conspicuous paired nerve plexuses that are located in lateral positions in the cavity surrounding the heart. They are formed by nerve fibers originating from perikarya in the thoracic ganglionic mass and projecting through openings of the branchiocardiac veins into the pericardium (1, 2). In early morphological and physiological studies, the POs have been recognized as neurohemal structures, which store and release cardioactive substances (3–5). They are, in fact, the largest neurohemal organs in decapod crustaceans. Subsequent studies have identified a variety of cardioexcitatory substances, including the amines serotonin, dopamine, and octopamine (2). There is sufficient evidence to indicate that these amines are released from the POs to act as neurohormones on the heart and, presumably, on other more distant targets as well (2). In addition, the occurrence of cardioexcitatory peptide material has been demonstrated in earlier studies (6), and further work has pointed to the existence of at least two smaller heat-stable peptides (7, 8). Their nature had remained elusive until more recent studies (9–11) demonstrated that one of the peptides was identical with proctolin, which was originally isolated and characterized as a neurotransmitter/modulator from insects (12, 13). There is evidence to indicate that proctolin is released from the POs and acts as a neurohormone in crustaceans (10, 11). This report describes the isolation, sequence analysis, and chemical synthesis of a second cardioexcitatory peptide from the POs of *Carcinus maenas*. The sequence of this peptide, for which we propose the acronym CCAP (for crustacean cardioactive peptide), does not resemble that of any known invertebrate or vertebrate neuropeptide.

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MATERIALS AND METHODS

Animals. Shore crabs, *Carcinus maenas*, from the North Sea were supplied by the Nederlands Instituut voor de Onderzoek van de Zee, Texel Island. They were kept in recirculating and filtered seawater at 12°C and were fed on pelleted cat food.

Heart Bioassay. Solutions to be assayed were applied to a semi-isolated heart preparation from *Carcinus maenas* by superfusion (11). The heart activity was recorded by a force transducer (type K 30, Hugo Sachs Elektronik, March-Hügstetten, F.R.G.) connected to an oscillographic chart recorder (Watanabe, Japan, through Hugo Sachs).

Extraction and Purification. POs were dissected from crabs of both sexes and immediately collected in a chilled Reacti-Vial (Pierce) containing the extraction medium (5% formic acid/1% CF_3COOH/1% NaCl/1 M HCl) (14). The vials were stored at −20°C between dissections until batches of 100–150 POs (from 50–75 animals) had been collected. Extraction was carried out by sonication (Branson sonifier with microtip) followed by stirring for 2 hr. After centrifugation at 10,000 × *g*, the pellet was resuspended in extraction medium and reextracted by sonication, with stirring for 12 hr followed by centrifugation. All steps were carried out at 0–4°C. The combined supernatants were applied to a Sep-Pak C_{18} cartridge (Waters Associates) equilibrated with 0.11% CF_3COOH. The adsorbed material was eluted with 2 ml of 60% CH_3CN/0.11% CF_3COOH. The eluate was concentrated to ≈50 μl in a Speed-Vac concentrator (Savant) and subjected to reversed-phase HPLC with a chromatographic system that consisted of two type 510 pumps, a model 680 solvent programmer, a U6K injector (all Waters Associates), and a Shimadzu (Kyoto, Japan) SPD 2A UV/visible detector. Columns and chromatographic conditions are described in the legends to Figs. 1, 3, and 7.

Amino Acid Analysis and Sequencing. Peptide samples were hydrolyzed *in vacuo*, either with 50 μl of constant boiling HCl (5.7 M, Pierce) containing 0.1% phenol for 24 hr at 110°C or with 25 μl of 4 M methanesulfonic acid containing 0.2% tryptamine (Sigma) for 20 hr at 120°C. Analyses were performed with a Biotronik (Munich, F.R.G.) LC 5000 analyzer with ninhydrin detection. Microsequencing of oxidized or carboxymethylated (CM)-peptides was carried out manually by the 4-*N*,*N*-dimethylaminoazobenzene-4′-isothiocyanate/phenylisothiocyanate double-coupling technique (15). The 4-*N*,*N*-dimethylaminoazobenzene-4′-thiohydantoin-conjugated amino acids were identified by HPLC on an Altex

Abbreviations: CCAP, crustacean cardioactive peptide; CCAP-OH, nonamidated CCAP; PO, pericardial organ; Acm, acetamidomethyl.

(Berkeley, CA) Ultrosphere ODS column (4.6 × 250 mm). Solvents A and B were 35 mM NaOAc (pH 5.0) and CH_3CN, respectively. A gradient from 30–70% solvent B in 1 hr was applied at a flow rate of 0.9 ml/min by using curve 5 on the solvent programmer. Automated sequence analysis was performed on a gas-phase sequencer [Applied Biosystems 470A (Foster City, CA)] directly connected to an on-line phenylthiohydantoin analyzer (Applied Biosystems 120 A). The separation of the phenylthiohydantoin-conjugated amino acids was carried out according to the manufacturer's protocol.

Peptide Synthesis. Solid-phase synthesis was performed on an automated synthesizer (Applied Biosystems 430 A) with *N-t*-butoxycarbonyl (Boc)-amino acids (Bachem) activated by conversion to symmetrical anhydrides. Side chain-protecting groups were *O*-benzyl for threonine and *S*-acetamidomethyl (Acm) for cysteine. For the synthesis of nonamidated CCAP (CCAP-OH), *N-t*-Boc-*S*-Acm-cysteine was coupled to a conventional Merrifield polymer (Fluka) by boiling a suspension of resin and protected amino acid in triethylamine for 60 hr under reflux. The amidated peptide was synthesized on a *p*-methylbenzhydrylamine resin (16). For direct formation of disulfide bonds from Acm-protected cysteines (17), the resin-bound peptides were gently agitated in a solution of 250 mg of I_2 in 20 ml of CH_2Cl_2 for 16 hr at room temperature and then washed with CH_2Cl_2. The unreacted I_2 was reduced in 1 M $Na_2S_2O_3/CH_3OH$, 1:1 (vol/vol), for 30 min at room temperature. The peptide-conjugated resin was then rinsed with CH_2Cl_2 and CH_3OH and dried in a vacuum desiccator. Full deprotection of the peptide and cleavage from the resin were performed by reaction with HF containing 15% (vol/vol) anisole for 1 hr at 0°C (18). After the peptides were washed with diethyl ether, they were extracted with 50% HOAc and purified on a Bio-Gel P4 column (200–400 mesh, 2 × 180 cm; Bio-Rad) equilibrated with 1 M HOAc. Fractions that were eluted in the appropriate molecular weight range were pooled and lyophilized. The purity was checked by HPLC and gas-phase sequencing. Ellmann's reagent (19) was used for the determination of free SH groups.

Cleavage and Derivatization of the Disulfide Bond. Performic acid oxidation of purified peptide was carried out as described by Hirs (20). After the reaction, the sample was diluted 1:9 with H_2O and immediately lyophilized. Carboxymethylation was performed by cleavage with dithiothreitol (without urea or guanidine hydrochloride), and alkylation was performed with iodoacetic acid (21, 22). The sample was acidified with CF_3COOH to pH 2.0, and the CM-peptides were directly analyzed by HPLC (for conditions, see the legend to Fig. 3) and manual sequencing.

Carboxypeptidase Treatment. Samples containing ≈2 μg each of CM-CCAP and CM-somatostatin (Sigma) were prepared. For carboxypeptidase A (Boehringer) treatment, the CM-peptide mixture was dissolved in 50 μl of 20 mM triethanolamine hydrochloride (pH 7.5) containing 200 mM NaCl. Two units of the enzyme (≈50 μg) in 20 μl of the same buffer was added. For treatment with carboxypeptidase Y (Boehringer), an identical sample was dissolved in 50 μl of 100 mM NH_4OAc (pH 5.5), and 4 units (≈200 μg) of enzyme dissolved in 20 μl of 100 mM NH_4OA_c was added. In both cases, incubation was carried out for 3 hr at 25°C. After addition of 100 μl of 5% CF_3COOH, the solutions were directly applied to an HPLC column. For chromatographic conditions, see the legend to Fig. 3.

RESULTS

In the chromatogram obtained by HPLC of a Sep-Pak-prepurified PO extract, two zones with cardioexcitatory activity were readily and reproducibly detected (Fig. 1). One of these can be ascribed to proctolin (11). The second zone,

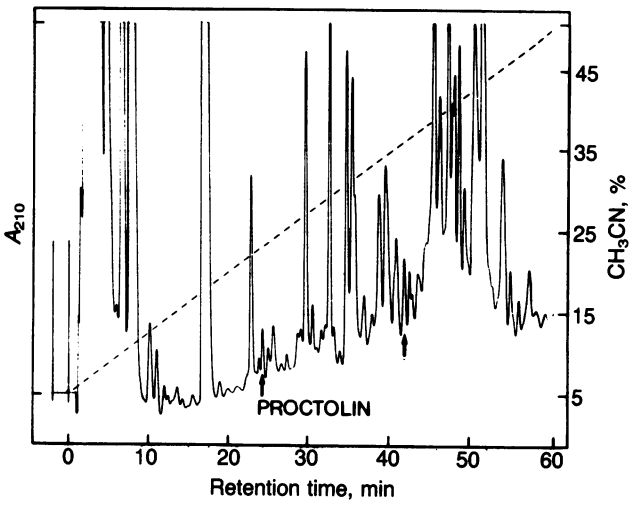

FIG. 1. Reversed-phase HPLC of an extract of 100 POs. The Sep-Pak-purified material was applied to a μBondapak phenyl column (Waters Associates, 0.39 × 30 cm), which was equilibrated with 90% solvent A (0.11% CF_3COOH in H_2O) and 10% solvent B (60% CH_3CN in 0.10% CF_3COOH). Elution was carried out with a linear gradient from 10% to 80% solvent B in 60 min. The flow rate was 0.9 ml/min. The eluate was collected in 0.9-ml fractions. Bioassay of fraction aliquots equivalent to 0.25 PO revealed two cardioactive factors, one of which is proctolin (11), and the other was eluted later as indicated by the arrow (fraction 42).

which was associated with a peak eluted later, was collected. An aliquot of this material, equivalent to ≈0.25 PO equivalents, displayed the biological effect shown in Fig. 2*A*. Rechromatography on a different column with a shallower solvent gradient proved sufficient to separate all of the activity as a distinct, symmetrical peak (Fig. 3). Amino acid analysis of this material yielded the composition shown in Table 1. The results indicated that the cardioactive substance, CCAP, was apparently highly pure, only serine,

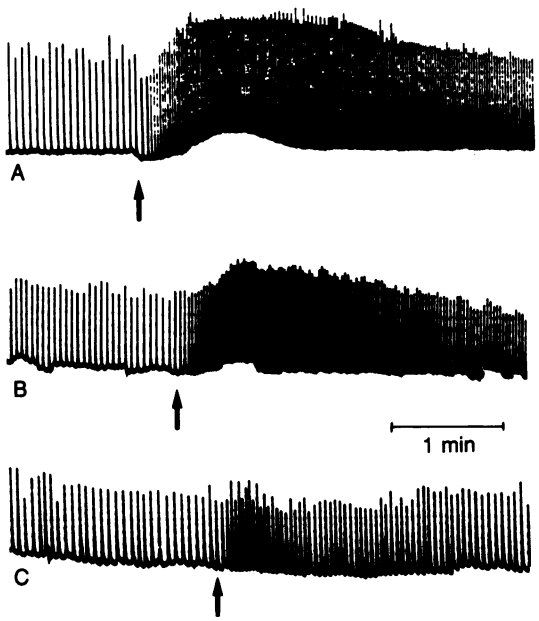

FIG. 2. Representative records of the heart-accelerating effect of CCAP. (*A*) An amount equivalent to 0.25 POs from fraction 42 (Fig. 1) was dried, dissolved in 100 μl of Pantin saline (23), and applied to a semi-isolated crab heart preparation. (*B*) Effect of 100 μl of a 1 nM solution (100 fmol) of synthetic CCAP. (*C*) Effect of CCAP-OH at the same dose. Application of samples is indicated by arrows.

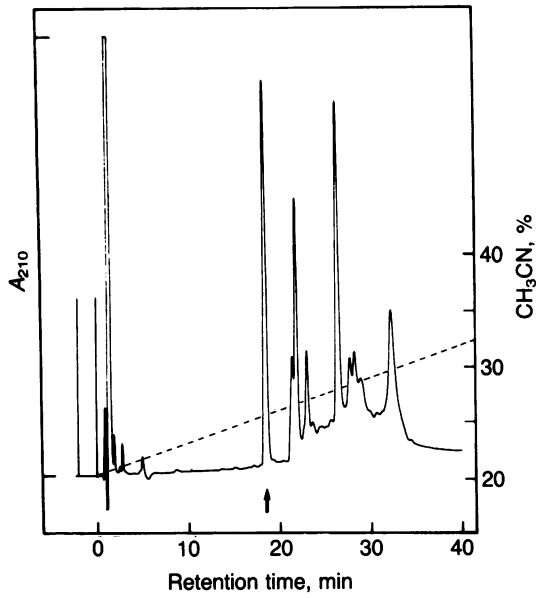

FIG. 3. Rechromatography of the cardioactive material in fraction 42 from the first chromatography (Fig. 1) on a Bakerbond wide-pore C_{18} column (Baker, 0.46 × 25 cm) with the same solvents as described in the legend to Fig. 1. A linear gradient from 30% to 80% solvent B in 90 min was applied. All the cardioactivity was associated with the peak indicated by the arrow.

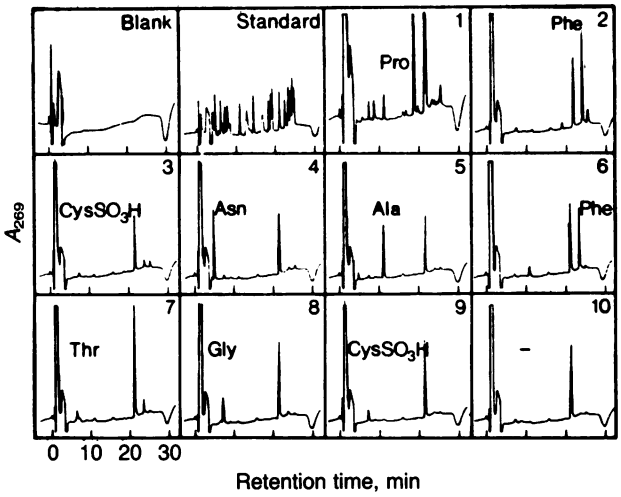

FIG. 4. Identification of phenylthiohydantoin-amino acids from 10 cycles (1–10) of automated Edman degradation of 300 pmol of isolated CCAP oxidized with performic acid. Phenylthiohydantoin-cysteinesulfonic acid comigrates reproducibly at 4 min close to the solvent peak. Included are a blank HPLC trace (blank) and a chromatogram of 12.5 pmol of the phenylthiohydantoin-amino acid standards, with the following order of elution: aspartic acid, asparagine, serine, glutamine, threonine, glycine, glutamic acid, dimethylphenylthiourea, alanine, histidine, tyrosine, arginine, proline, methionine, valine, diphenylthiourea, tryptophane, phenylalanine, isoleucine, lysine, and leucine.

glutamine, and lysine being present as negligible impurities. Manual sequencing of a 500-pmol sample of the performic acid-oxidized CCAP enabled the assignment of residues 1–6 and 8, whereas residues 7 and 9 could not be identified with certainty (data not shown). Complete, unambiguous assignment of all residues was possible by automated gas-phase sequencing of the oxidized peptide (Fig. 4) and also by manual sequencing with CM-CCAP (data not shown). To test whether the C-terminal cysteine residue was free or blocked, we subjected samples of the CM-peptide to carboxypeptidase A and Y digestion. In the reaction mixture, a sample of CM-somatostatin, which has a free C-terminal cysteine residue, was included for comparison. HPLC separation of the mixture before and after incubation with carboxypeptidase showed complete degradation of somatostatin, whereas the CCAP peak remained unaffected, indicating a blocked C-terminal residue (Fig. 5; the data from the carboxypeptidase Y digestion experiment, which were identical, are not shown here). To verify the deduced sequence, we synthesized the nonapeptide, both in its C-terminally free and amidated forms. Due to formation of mixed disulfides, the yield of the correct peptides did not exceed 15% (CCAP-OH) and 20% (CCAP). Both peptides were obtained in a >98% purity by HPLC (data not shown), and identity with the native peptide was ascertained by amino acid analysis (Table 1) and sequencing. The presence of the disulfide bond was indicated by the fact that the *S*-Acm protecting group was unstable in the HF treatment and by the negative result of the test with Ellmann's reagent. Further direct evidence came from laser Raman spectroscopy experiments which showed disulfide stretching bands but no bands corresponding to SH-groups (B. Kisters, personal communication).

Table 1. Amino acid composition of CCAP from three different purifications (batches) and of synthetic CCAP

Amino acid	Composition, mol%				
	Batch 1	Batch 2 oxidized	Batch 3	Synthetic CCAP oxidized	Residues, no.
Asn	13.33	11.82	12.37	12.13	1
Thr	11.27	9.31	9.70	10.33	1
Ser	1.44	0.63	1.51	0.84	—
Gln	1.94	3.51	4.31	0.93	—
Pro	11.26	7.94	11.79	11.96	1
Gly	14.52	12.98	16.98	13.25	1
Ala	13.08	11.58	12.99	13.01	1
$^1/_2$ Cys	7.36	—	7.83	—	
Phe	23.05	23.00	22.47	20.62	2
Lys	0.47	0.31	—	0.35	—
Cya	—	18.33	—	16.57	2
Total					9

Batch 3 was hydrolyzed with methanesulfonic acid; all others were hydrolyzed with HCl. The total amount of peptide in each batch was 0.68 nmol (batch 1), 1.10 nmol (batch 2 oxidized), 0.85 nmol (batch 3), and 1.45 (synthetic CCAP oxidized).

After the synthetic peptides became available, we were able to analyze whether the C-terminal residue of the native substance was amidated. In the standard HPLC run with 0.10% CF_3COOH/CH_3CN, pH 1.5, as solvent system, the synthetic free carboxyl and amidated forms were eluted together as a single peak. However, base-line separation was possible after carboxymethylation of both forms (Fig. 6). Complete separation of the underivatized synthetic forms was achieved by use of a different solvent system at a pH of 3.42 (Fig. 7). In both systems, the retention time of the native CCAP was identical to that of the amidated synthetic peptide. These results together with the sequence analyses are consistent with the following structure for *Carcinus* CCAP:

$$\text{Pro-Phe-}\overline{\text{Cys-Asn-Ala-Phe-Thr-Gly-Cys}}\text{-NH}_2.$$

Bioassays with both synthetic peptides revealed that the amidated form was far more potent than the nonamidated peptide (Fig. 2 *B* and *C*). The increase of the heart rate elicited by an amount of native CCAP corresponding to 0.25 PO equivalents in 100 μl was higher than that caused by superfusion of the semi-isolated heart with a 100-μl sample of a 1 nM

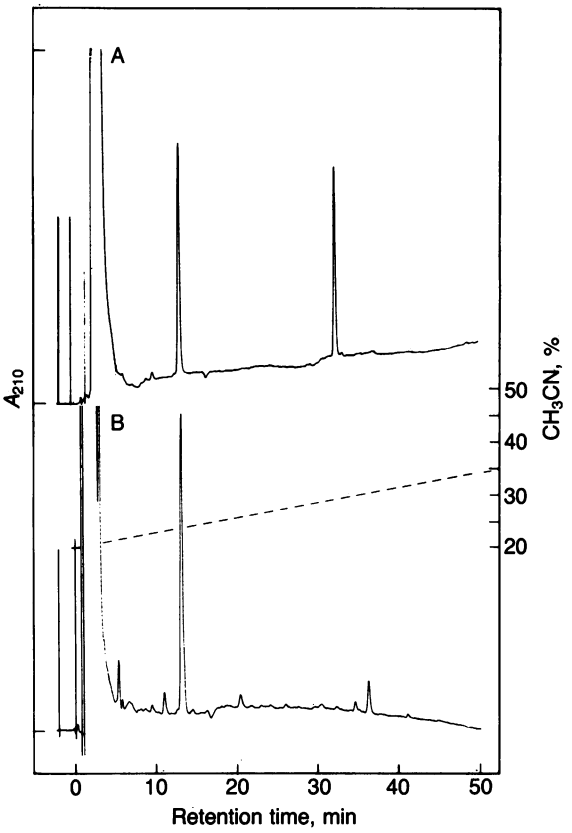

FIG. 5. Incubation of CM-CCAP and CM-somatostatin with carboxypeptidase A. Traces: A, CM-CCAP (retention time, ≈13 min) and CM-somatostatin (35 min) used as a control were separated under the same chromatographic conditions as described in the legend to Fig. 3; B, after carboxypeptidase A treatment for 3 hr at 25°C, CM-CCAP was unchanged, whereas CM-somatostatin was totally degraded.

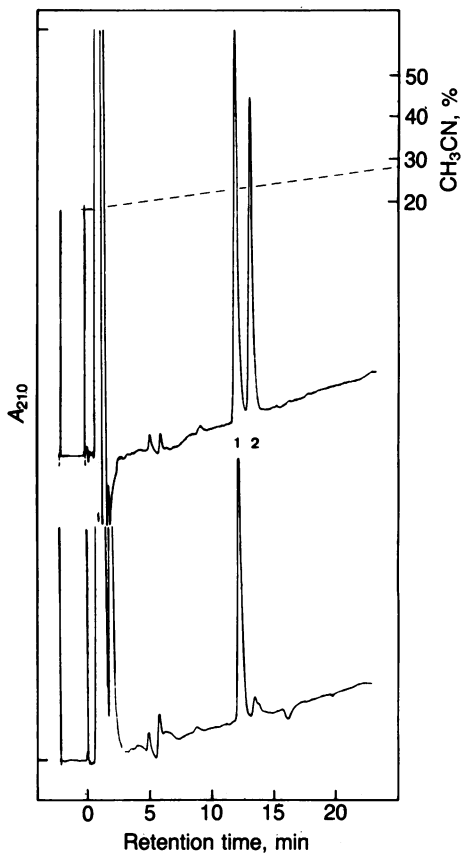

FIG. 6. HPLC separations of synthetic CM-CCAP (peak 1 of upper trace) and CM-CCAP-OH (peak 2 of upper trace) in comparison with the native CM-CCAP (lower trace). The same chromatographic conditions were used as described in the legend to Fig. 3.

solution (100 fmol) of the synthetic amidated peptide (250% vs. 150% increase). This is consistent with our calculations of the content of CCAP in the POs. Based on the amino acid analysis, we estimate that ≈15–20 ng (=pmol) of CCAP was extracted from a single PO, and, thus, 0.25 PO equivalent would amount to 3.7–5 pmol, applied in a 100-μl sample.

The complete structural elucidation reported here required the processing of ≈1200 POs from 600 animals.

DISCUSSION

We report here the isolation and complete structural elucidation of CCAP from the POs of *Carcinus maenas*. It is a C-terminally amidated nonapeptide with a disulfide bridge. Based on the amino acid sequence, CCAP has a M_r of 957. Its amino acid sequence does not resemble that of any known vertebrate and invertebrate neuropeptide. However, a conspicuous inverse similarity to vasopressin is revealed when CCAP in the N→C direction and vasopressin in the C→N direction are aligned as shown below in single-letter amino acid code. The corresponding five residues include four of the six invariant vasopressin residues.

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          (1)              (9)
CCAP      P F C N A F T G C
    G R P - C N Q F Y - C  vasopressin
(9)                      (1)
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Whether this arrangement of residues shared (but in opposite direction) between these neuropeptides is of functional significance could be tested with antibodies.

Earlier work on the POs of the brachyuran *Cancer borealis* (8) has indicated the presence of two cardioactive peptides in the M_r range 700–1500. It may be suggested that proctolin, Arg-Tyr-Leu-Pro-Thr, which has been shown unequivocally to be present in *Carcinus maenas* POs (11) and has heart-

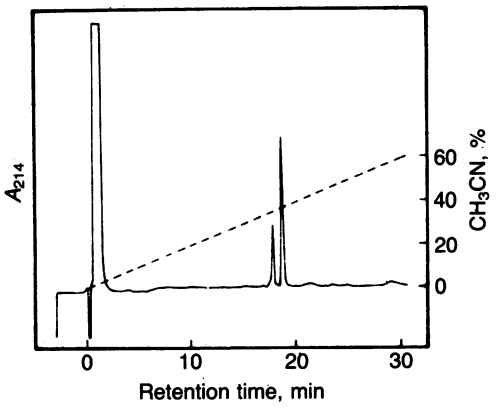

FIG. 7. Reversed-phase HPLC of a mixture of 10 nmol of synthetic CCAP and 5 nmol of synthetic CCAP-OH on a Bakerbond wide pore C_8 column (Baker, 0.46 × 25 cm). Solvent A was 25 mM NH_4HCO_3/0.1% CF_3COOH, pH 3.42; solvent B was 24 mM NH_4HCO_3/0.1% CF_3COOH/60% CH_3CN, pH 3.42. Gradient elution was carried out from 0% solvent B to 100% in 30 min. The flow rate was 1.0 ml/min. The larger peak that was eluted at a retention time of ≈19 min is CCAP (10 nmol). CCAP-OH (5 nmol) was eluted as the lower first peak. The retention time of native CCAP in this system was identical to that of synthetic CCAP.

accelerating activity, is one of them and that CCAP is the second. Inconsistent with this is the observation that all cardioactivity associated with peptide material was destroyed by trypsin treatment (6, 7), although neither proctolin nor CCAP is susceptible to degradation by this enzyme. This discrepancy could be explained in different ways: First, species-specific differences may exist. Second, there may be other, as yet uncharacterized cardioactive peptides that are trypsin sensitive. The third, and in our view most likely explanation, is that a trypsin preparation was used in the early studies that contained chymotrypsin as an impurity. Species specificity, at least as far as different brachyurans are concerned, is, in our opinion, unlikely, as preliminary evidence (based on HPLC retention times and bioassay) indicates that both proctolin and CCAP are present in the POs of *Cancer pagurus* and *Liocarcinus puber* (unpublished data).

To assess whether CCAP, like proctolin (11), can be considered to be a neurohormone that is released from the neurohemal structures in amounts sufficient to produce effective concentrations in the hemolymph, it is important to consider the amounts in the tissue and to compare them with the amounts that proved effective in the bioassay. A sample of 100 μl of a 1 nM solution of synthetic CCAP (total amount, 100 fmol) produced an increase of the heart rate that was ≈150%. We have not established thus far a complete dose–response relationship, but preliminary experiments gave significant responses already after application of 1–10 fmol in 100 μl. Thus, CCAP meets the requirements for hormonal status in that it is active at low concentrations. The yield of extractable CCAP from both POs of an animal was 30–40 pmol. The bioassay indicates that the release of <1% of this amount would produce a concentration in the hemolymph of the pericardial cavity that would significantly affect the heart. Therefore, release of effective amounts would not significantly deplete the stores in the POs. For comparison, the amount of the other cardioactive, neurohormonal peptide, proctolin, is lower—i.e., 5.4 pmol in both POs of an animal (11).

We suggest that CCAP is a neurohormone that is stored in relatively high amounts in the PO, acting upon release into the hemolymph on the heart and, perhaps, also on more peripherally located targets.

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