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ISOLATION AND IDENTIFICATION OF C-TYPE NATRIURETIC PEPTIDE IN CHICKEN BRAIN

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SUMMARY: C-type natriuretic peptide (CNP) has recently been identified in porcine brain as a third member of the mammalian natriuretic peptide family (1). Using a radioimmunoassay system for porcine CNP, we found a significant concentration of immunoreactive (ir-) CNP in chicken brain, from which a new peptide was isolated. By microsequence analysis, the amino acid sequence of the peptide was determined to be Gly-Leu-Ser-Arg-Ser-Cys-Phe-Gly-Val-Lys-Leu-Asp-Arg-Ile-Gly-Ser-Met-Ser-Gly-Leu-Gly-Cys. Based on its high homology to porcine CNP, the peptide was designated chicken C-type natriuretic peptide. Chicken CNP also elicits pharmacological effects highly similar to porcine CNP, suggesting that CNP functions as a neuropeptide in the chicken central nervous system.

In addition to atrial natriuretic peptide (ANP, A-type natriuretic peptide) and brain natriuretic peptide (BNP, B-type natriuretic peptide) (2, 3), we have very recently identified in porcine brain a novel peptide designated C-type natriuretic peptide (CNP) in porcine brain (1). Porcine CNP has an amino acid sequence highly homologous to ANP and BNP, within a 17-residue ring structure formed by a disulfide linkage, and exerts a pharmacological spectrum similar to that of ANP and BNP. However, CNP completely lacks the C-terminal extension from the ring structure, which is commonly found in ANP and BNP. Porcine CNP stimulates guanylate cyclase activity several times more potently than ANP in cultured vascular smooth muscle cells (4). Such structural and pharmacological features indicate that CNP is a new member of the mammalian natriuretic peptide family. Thus, three highly similar but distinct peptides, ANP, BNP and CNP, are shown to constitute a natriuretic peptide family in mammals.

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Abbreviations: CNP, C-type natriuretic peptide; ANP, atrial natriuretic peptide (A-type natriuretic peptide); BNP, brain natriuretic peptide (B-type natriuretic peptide); HPLC, high performance liquid chromatography; RIA, radioimmunoassay; ir, immunoreactive; TFA, trifluoroacetic acid; PTH, phenylthiohydantoin; IgG, immunoglobulin G; RCM, reduced and S-carboxymethylated.

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In a preliminary study using a radioimmunoassay (RIA) system for porcine CNP, we found CNP-like immunoreactivity not only in mammals but also in other vertebrates, including chicken and frog. Especially in chicken brain, a relatively high concentration of ir-CNP (about 3 pmol/g) was detected. Although chicken α-natriuretic peptide (α-NP), which is more closely related to mammalian BNP than to ANP, has so far been identified in heart tissue (5), ir-chicken NP was not detected in brain at a significant concentration. These facts suggest the possibility that CNP-immunoreactive peptide functions as a neuropeptide in the chicken central nervous system. Thus, we undertook isolation of CNP-immunoreactive peptide from chicken brain, as reported in the present paper.

MATERIALS AND METHODS

Isolation: Fresh chicken brain (417 g obtained from 150 chickens) was diced and boiled for 10 min in 3 volumes (V/W) of water to inactivate intrinsic proteases. After cooling, glacial acetic acid was added to make a final concentration of 1M, and the tissue was homogenized with a Polytron mixer. The homogenates were centrifuged at 20,000 x g for 30 min, and resulting supernatants were subjected to acetone-precipitation (final concentration = 66%) at 4 ℃. The supernatant, obtained by centrifugation, was condensed by evaporation, and was loaded onto an LC-SORB SPW-C-ODS column (90 ml, Chemco) after two-fold dilution with water. The column was washed with 0.1% trifluoroacetic acid (TFA), and the adsorbed materials were eluted with 60% CH_3CN containing 0.1% TFA. After evaporation in vacuum, the adsorbed fraction was dissolved in 1M CH_3COOH and loaded onto an SP-Sephadex C-25 column (H^+-form, 1.4 × 15 cm, Pharmacia). Successive elutions with 1M CH_3COOH, 2M pyridine and 2M pyridine-acetate (pH 5.0) yielded three respective fractions of SP-Ⅰ, SP-Ⅱ and SP-Ⅲ. After lyophilization, the SP-Ⅲ fraction was separated by gel filtration on a Sephadex G-50 column (fine, 3.0 × 152 cm). The major CNP-immunoreactive fraction was then subjected to cation exchange HPLC on a TSK gel CM-2SW column (7.6 × 300 mm, Tosoh) eluting with a gradient of $HCOONH_4$ (pH 6.6) from 10 mM to 1.0 M in the presence of 10% CH_3CN. Fractions exhibiting main CNP immunoreactivity were lyophilized, dissolved in 0.1M sodium phosphate buffer (pH 7.4) containing 0.002% Triton X-100, and then loaded onto to an anti-α-ANP immunoglobulin G (IgG) immunoaffinity column (bed volume: 500 μl), as described previously for the purification of α-ANP [4-28] and α-ANP [5-28] (6). The adsorbed peptides on the column were eluted with a solution of 1M CH_3COOH containing 10% CH_3CN, and then finally purified by reverse phase HPLC on a C-18 column (μ-Bondasphere C-18, 300A, 3.9 × 150 mm, Waters) with a linear gradient elution of CH_3CN from 10% to 60% in 0.1% TFA for 60 min at a flow rate of 1.0 ml/min. Aliquots of all fractions in each purifiation step were submitted to RIA for CNP.

Radioimmunoassay: Details on preparation of an antiserum against porcine CNP and characterization RIA for porcine CNP will be reported in a separate paper. In brief, antiserum #171-4 was usable at a final dilution of 1:60,000, and peptides were measurable in a range of 1-100 fmol/tube (7). Chicken α-NP showed less than 0.5% crossreactivity in this RIA system.

Sequence analysis: Reductive S-carboxymethylation of chicken CNP was performed by the described method (2, 3), and reduced and S-carboxymethylated (RCM) CNP was purified by reverse phase HPLC under identical conditions as described above. The RCM-chicken CNP was sequenced by a gas-phase sequencer coupled with on-line HPLC identification system of the resulting phenylthiohydantoin (PTH) amino acids (Applied Biosystems 470A/120A). PTH-amino acids were detectable down to 0.5 pmol. In order to confirm the sequence, native and synthetic chicken CNP were subjected together to reverse phase HPLC on a C-18 column (#3063, 1.2 × 150 mm, Hitachi) with a linear gradient elution of CH_3CN from 0% to 60% in 0.1% TFA for 45 min at a flow rate of 50 μl/min.

Synthesis: Synthetic CNP was prepared by solid phase techniques using a peptide synthesizer (Applied Biosystems 430A). An intramolecular disulfide linkage was formed by oxidation with

potassium ferricyanide. The synthetic peptide was purified by reverse phase HPLC, and correct synthesis was confirmed by amino acid analysis and sequencing.

Bioassay: Chick rectum relaxant activity was assayed using freshly isolated chick rectum strips by the described method (2, 8). Diuretic-natriuretic and hypotensive responses were measured by the reported method using male Sprague-Dawley rats (weighing 300-400 g) anesthetized with pentobarbital (50 mg/kg) (2, 3). Test samples, dissolved in saline, were injected into assay rats through the femoral vein in one shot.

RESULTS AND DISCUSSION

In crude extracts of chicken brain, about 3 pmol/g wet weight of ir-CNP was detected by a newly established RIA system for porcine CNP (7), suggesting the presence in chicken brain of CNP-related peptides homologous to porcine CNP. Thus, we undertook isolation of chicken CNP from 417 g of brain. By acetone precipitation, reverse phase C-18 chromatography and SP-Sephadex ion exchange chromatography, more than 80% of CNP-immunoreactivity in the crude chicken brain extracts was effectively condensed in an SP-Ⅲ fraction mainly composed of basic peptides. The SP-Ⅲ fraction (dry weight: 140mg) was separated by gel filtration on a Sephadex G-50 column, and most of ir-CNP was observed in the region of molecular weight 2K-3K (Fig. 1). Fractions #55-61 exhibiting major CNP immunoreactivity (dry weight: 19 mg), were then subjected to cation exchange HPLC on a TSK gel CM-2SW column. As shown in Fig. 2, a major peak of ir-CNP emerged at an elution time of 42-44 min. After lyophilization, the CNP-immunoreactive fractions obtained above were loaded onto an anti-α-ANP IgG immunoaffinity column, which was more effective for immunoaffinity

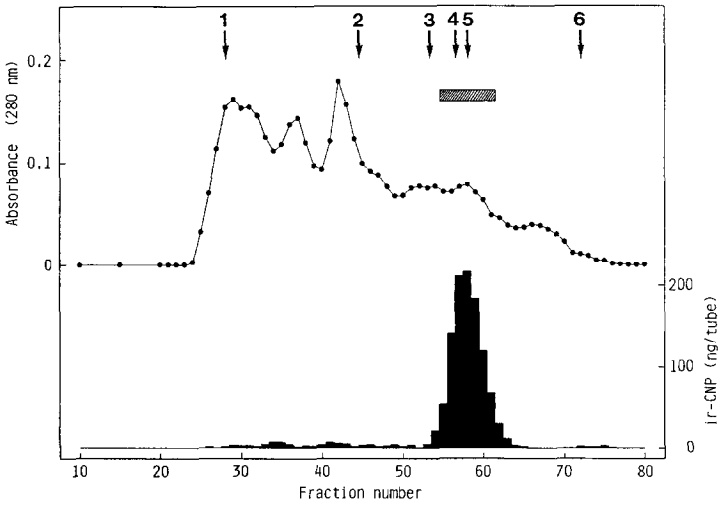

Figure 1. Sephadex G-50 gel filtration of chicken brain extracts.
Sample: Basic peptide fraction of chicken brain extracts (dry weight: 140 mg).
Flow rate: 20 ml/h. Fraction size: 15 ml/tube.
Column: Sephadex G-50 (fine, 3.0 × 152 cm, Pharmacia).
Arrows indicate elution positions of 1) bovine serum albumin, 2) porcine BNP-32, 3) porcine BNP-26, 4) human α-ANP [4-28], 5) porcine CNP and 6) NaCl.
Fraction marked with hatched bar was used for further purification.

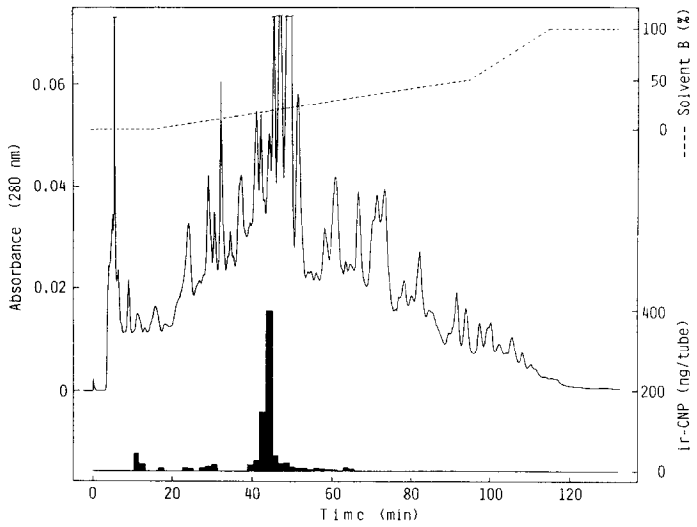

Figure 2. Cation exchange HPLC of basic peptide fraction of molecular weight 2K-3K.
Sample: Fractions #55-61 (hatched area) in Fig. 1 (dry weight: 19 mg).
Column: TSK gel CM-2SW (7.6 × 300 mm, Tosoh). Flow rate: 2.0 ml/min.
Solvent system: (A) 10mM $HCOONH_4$ (pH 6.6):CH_3CN = 90:10 (V/V),
(B) 1.0M $HCOONH_4$ (pH 6.6):CH_3CN = 90:10 (V/V).
Linear gradient elution from 0% B to 50% B for 80 min, followed by that from 50% B to 100% B for 20 min.

chromatography of chicken CNP than an anti-porcine CNP IgG column. The adsorbed peptides on the column were eluted and then separated by reverse phase HPLC on a C-18 column. As shown in Fig. 3a, most of CNP immunoreactivity completely coincided with a

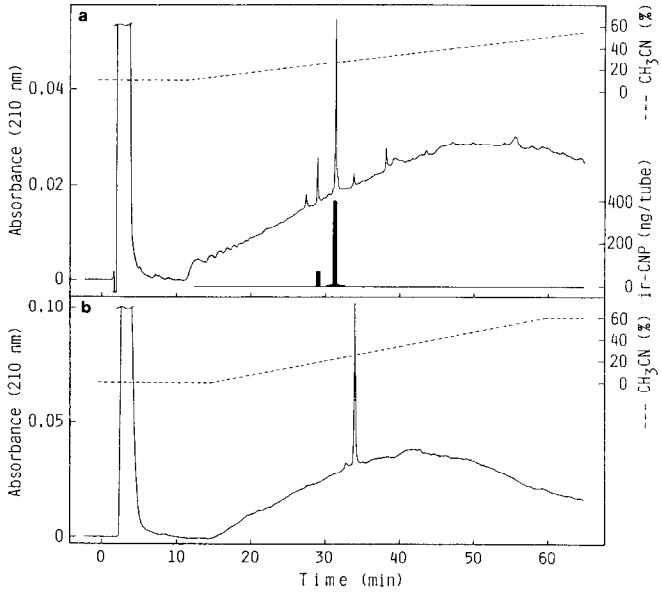

Figure 3. Final purification of chicken CNP by reverse phase HPLC.
Sample: Immunoaffinity-purified fraction of ir-CNP eluted at 42-44 min in Fig. 2.
Column: μ-Bondasphere C-18 (300A, 3.9 × 150 mm, Waters). Flow rate: 1.0 ml/min.
Solvent system: H_2O:CH_3CN:10%TFA = (A) 90:10:1, (B) 40:60:1 (V/V).
Linear gradient elution from (A) to (B) for 60 min.

main peptide peak eluted at 31 min on the chromatogram, indicating that the peptide was purified to a homogeneous state. Based on the peak height as well as CNP immunoreactivity in reverse phase HPLC (Fig. 3a), the isolation yield of the peptide was estimated to be approximately 180 pmol (400 ng), starting from 417 g of chicken brain.

A very limited amount of the peptide was obtained in the present purification, but three-fourths of the finally purified peptide was reduced and S-carboxymethylated, and then submitted to a gas-phase sequencer. PTH-amino acid liberated at each cycle of Edman degradation was definitely identified up to the 22nd step (Fig. 4). According to the amino acid sequence thus determined, we synthesized an identically sequenced peptide containing an intramolecular disulfide linkage and co-chromatographed with a native peptide on reverse phase HPLC. As shown in Fig. 3b, native and synthetic peptides were co-eluted completely as a single peak. Thus, the complete amino acid sequence of the 22-residue peptide was unambiguously established, as shown in Fig. 5.

The amino acid sequence of the peptide is identical to porcine CNP-22, except that 3 residues are replaced (Fig. 5). Both peptides are 22-amino acid residues in length and carry a 17-residue ring structure, which is thought to be essential for exerting diuretic-natriuretic and hypotensive activity. Chicken CNP, just as in the case of porcine CNP, terminates at the 22nd cysteine residue, lacking the C-terminal tail commonly found in A-type and B-type natriuretic peptides. Thus, the CNP-immunoreactive peptide isolated from chicken brain was designated chicken C-type natriuretic peptide. Chicken CNP showed chick rectum relaxant activity comparable to porcine CNP, i.e., 3~4 times more potent than human α-ANP. This peptide also induced diuretic-natriuretic and hypotensive responses in a manner similar to that of ANP and BNP (Table 1). However, its relative potencies were much lower than mammalian ANP

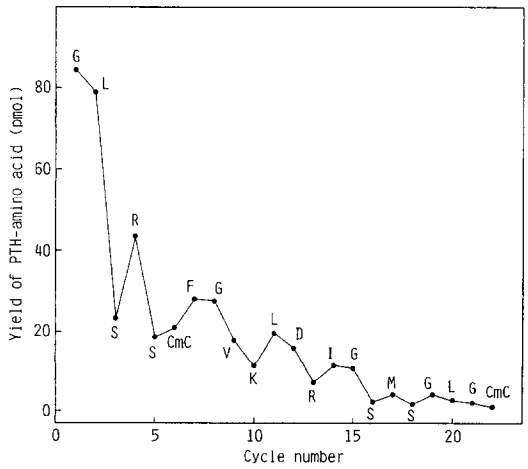

Figure 4. Yield of PTH-amino acid at each cycle of Edman degradation of RCM-chicken CNP. One letter amino acid notation is used. CmC; carboxymethylcysteine.

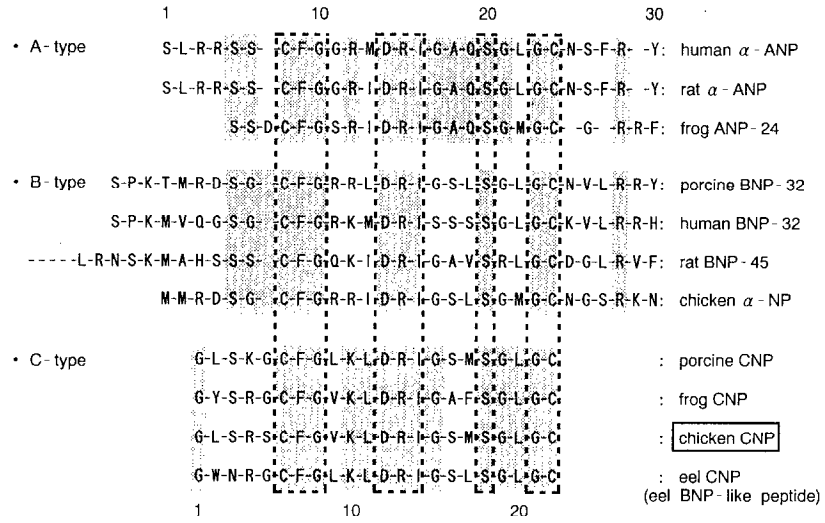

Figure 5. Amino acid sequence of chicken CNP, compared with those of known natriuretic peptides. Intramolecular disulfide linkage is formed between two cysteine residues in each peptide.

and BNP, and comparable to porcine CNP. These pharmacological data supported the present designation of chicken CNP.

Concurrent with the present study, we isolated a new natriuretic peptide from frog brain extracts and identified it as frog CNP (to be published). Frog CNP is also a 22-amino acid peptide having the 17-residue ring structure and lacking the C-terminal tail. As shown in Fig. 5, porcine, frog and chicken CNP exhibit remarkably high homology and structural features characteristic of CNP, such as deletion of the C-terminal tail. During preparation of this manuscript, Takei et al. reported isolation of a new natriuretic peptide from eel brain (9). Although they have designated it eel BNP-like peptide, the peptide shows extremely high structural homology to porcine, frog and chicken CNPs, including deletion of the C-terminal tail. Moreover, the peptide exhibits weak diuretic-natriuretic and hypotensive effects, when injected into anesthetized rats, in a manner similar to CNPs. Thus, the natriuretic peptide isolated from eel brain is probably eel CNP.

Table 1. Diuretic-natriuretic and hypotensive responses induced by chicken CNP and human α-ANP

peptide	dose	increase in excretion of				decrease in
	(μg/kg)	urine (%)	Na^+ (%)	K^+ (%)	Cl^- (%)	blood pressure (%)
human α-ANP	3.0	414 ± 46	461 ± 41	220 ± 22	362 ± 34	9.0 ± 2.5
chick CNP	30	153 ± 32	160 ± 9	145 ± 12	171 ± 2	4.4 ± 1.9
	90	261 ± 19	193 ± 6	163 ± 2	214 ± 5	10.3 ± 2.0
	200	293 ± 50	385 ± 50	174 ± 18	338 ± 107	11.5 ± 1.5

Diuretic-natriuretic responses were expressed as % change (mean ± s.e.m.) in urine output, and in excretions of Na^+, K^+ and Cl^- from 15 min urine samples collected before and after intravenous injection into anesthetized rats. Hypotensive responses are expressed as % decrease (mean ± s.e.m.) in systolic blood pressure after injection of the peptide. Three to six rats were used for each peptide.

By identification of CNP in most classes of vertebrates (mammal, avian, amphibian and piscine), CNP has now been established as a third type of the natriuretic peptide family. Regardless of phylogenic differences, CNPs have highly conserved amino acid sequences and uniform structures lacking the C-terminal tail, suggesting that CNP has exerted important physiological functions from an early evolutionary stage of the natriuretic peptide family. The structural features of CNP may be implicated in its unique pharmacological profile, such as strong effect on smooth muscles and weak effect on diuresis-natriuresis. The wide distribution of CNP in vertebrates as well as its pharmacological dissociation from ANP and BNP strongly suggests the presence of natriuretic peptide receptor(s) specific for CNP in addition to the so far known ANP-A (GC-A), ANP-B (GC-B) and the so-called clearance receptors (10-14). Therefore, the physiological significance of the natriuretic peptide family should be re-evaluated on the basis of diversity in the ligands, receptors and ligand-receptor combinations in each target tissue, although the three types of natriuretic peptides are assumed to function in a concerted manner in maintaining the homeostatic balance of body fluid volume and blood pressure.

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