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CHARACTERIZATION OF IMMUNOREACTIVE HUMAN C-TYPE NATRIURETIC PEPTIDE IN BRAIN AND HEART

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SUMMARY: Amino acid sequence of human C-type natriuretic peptide (CNP) has recently been deduced to be identical to those of porcine and rat CNPs in the bioactive unit of C-terminal 22 residues (CNP-22) (1). Thus, tissue concentrations and molecular forms of immunoreactive (ir-) CNP in human brain and heart were determined or characterized using a radioimmunoassay established for porcine CNP. In human brain (hypothalamus and medulla-pons), ir-CNP was detected at a concentration of 1.04 pmol/g, being about 25 times or 70 times higher than ir-atrial (A-type) natriuretic peptide (ANP) or ir-brain (B-type) natriuretic peptide (BNP). CNP was present mainly as CNP-53, with CNP-22 as well as 13K CNP (presumed to be pro-CNP) as minor components. In heart, 1~5 pmol/g of ir-CNP was detected in both atrium and ventricle, but this ir-CNP was shown to be derived from crossreactivity of ANP. These results demonstrated that human CNP functions exclusively in the central nervous system in contrast to ANP and BNP which mainly function in the circulation system.

C-type natriuretic peptide (CNP) is a third member of the natriuretic peptide family recently identified in porcine brain (1). CNP was first isolated as 22-residue CNP-22, but CNP-53 of 53 residues is now also known to be an endogenous molecular form in porcine brain (2,3). Amino acid sequences of rat and human CNP precursors have very recently been deduced from nucleotide sequence analyses, indicating that the amino acid sequence of CNP-22 is identical among these three mammalian species (4,5). CNP has also been isolated from piscine, amphibian and avian brain, and is shown to retain a highly conserved amino acid sequence during vertebral evolution (6-9).

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Abbreviations: CNP, C-type natriuretic peptide; ANP, atrial or A-type natriuretic peptide; BNP, brain or B-type natriuretic peptide; RIA, radioimmunoassay; ir, immunoreactive; MW, molecular weight; TFA, trifluoroacetic acid; HPLC, high performance liquid chromatography; CNP-22, pro-CNP[82-103]; CNP-53, pro-CNP[51-103]; γ-ANP, pro-ANP; α-ANP, pro-ANP[99-126].

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On the other hand, CNP shows pharmacological dissociation from atrial natriuretic peptide (A-type natriuretic peptide, ANP) and brain natriuretic peptide (B-type natriuretic peptide, BNP), and has been demonstrated to interact with a natriuretic peptide receptor distinct from that of ANP and BNP (1,10,11). In studies of the regional distribution of the three natriuretic peptides, a relatively high concentration of ir-CNP was found in porcine brain, and rat CNP mRNA was specifically expressed in brain and not in peripheral tissues including heart, in contrast to ANP and BNP which are mainly expressed and present in porcine and rat heart (3,4). Furthermore, in lower vertebrates, CNP is detected in brain in an extremely high concentration (6,8). These data strongly suggest that CNP is a natriuretic peptide designed for functioning in the central nervous system. In order to help elucidate functions of CNP, we measured tissue concentration of ir-CNP and characterized CNP-immunoreactive substances in human brain and heart.

MATERIALS AND METHODS

Peptides: CNP-22, [Tyr^0] -CNP-22 and human BNP-32 were synthesized in our laboratory by the solid phase method. Homogeneity of synthetic peptides was confirmed by reverse phase and CM ion exchange high performance liquid chromatography (HPLC) as well as amino acid analysis and sequencing. Human CNP-53 was kindly donated by Mr. Y. Minamitake, Suntory Institute for Biomedical Research. Human γ-ANP was purified from human cardiac atrium as reported previously (12).

Radioimmunoassay (RIA) procedures for CNP: RIA for CNP was performed as reported (3). Standard CNP-22 or unknown sample (100 μl) was incubated with antiserum (#171-4) diluent (1/20,000, 100 μl) for 24 hr, then tracer solution (15,000~18,000 cpm in 100 μl), which was prepared by the lactoperoxidase method followed by HPLC purification, was added. After additional incubation for 36 hr, free and bound tracers were separated by the polyethyleneglycol method. Assay was routinely performed in duplicate at 4 ℃. In this RIA system, peptides were measurable in a range of 1~50 fmol/tube, and human α-ANP and BNP-32 showed 0.015% and less than 0.001% crossreactivity, respectively. Synthetic human CNP-53 and chicken CNP showed an affinity comparable to CNP-22, while crossreactivity of frog CNP was only 2.3%.

RIAs for ANP and BNP: RIAs for human ANP and BNP were performed as reported previously (13,14). CNP-22 showed less than 0.001% crossreactivity in these RIA systems.

Human tissue: Human brain tissue, mainly composed of hypothalamus and medulla-pons (more than 90% wet wt), was obtained from the following subjects; No. 1) 25 y (years of age), male (sex), traffic accident (cause of death), No. 2) 33 y, female, heart failure, and No. 3) 72 y, female, liver cirrhosis. In these patients, no pathological finding was detected in brain tissue. Human cardiac atrium (auricle portion) was obtained from subject No. 4) 70 y, female, multiple myeloma. Cardiac contents of ir-CNP, ir-ANP and ir-BNP were measured for 12 other subjects described in our previous report (14).

Tissue extraction: After removing hematoma, each brain tissue or cardiac atrium was diced and boiled in 10 volumes of water for 10 min to inactivate intrinsic proteases. After cooling, acetic acid was added (final concentration = 1M), and boiled tissue was homogenized with a Polytron mixer for 4 min. Homogenates were then centrifuged at 20,000 x *g* for 25 min, and supernatants were filtered and stored. Tissue extracts were loaded onto Sep-pak C-18 cartridge (2.5 ml, Waters), washed with 0.1% trifluoroacetic acid (TFA), and then adsorbed materials were eluted with 60% CH_3CN containing 0.1% TFA. After lyophilization, the eluates were dissolved in RIA standard buffer and submitted to RIAs for CNP, BNP and ANP. Recovery yield of ir-CNP was estimated by adding ^{125}I-[Tyr^0]-CNP, and more than 95% of radioactivity was recovered.

Characterization of ir-CNP: Human brain extracts (20 g wet wt equally from 3 subjects) were desalted and condensed with a reverse phase C-18 column (90 ml, LC-SORB ODS, Chemco). Atrial extracts were also treated with the Sep-pak C-18 cartridge. The adsorbed materials on

the C-18 column or cartridge was separated by Sephadex G-50 fine column (1.9 x 134 cm for brain extracts or 1.8 x 134 cm for atrial extracts) using 1M acetic acid as solvent. An aliquot of each fraction was submitted to RIAs for ANP, BNP and CNP. Fractions containing ir-CNP in brain and atrium as well as ir-BNP in brain were further separated by reverse phase HPLC and CM ion exchange HPLC. Reverse phase HPLC was performed on a Chemcosorb 5C18-300 (4.6 x 250 mm, Chemco) or Hi-Pore RP-318 (4.6 x 250 mm, Bio-Rad) column with a linear gradient elution of CH_3CN from 10% to 60% or from 0% to 60% in 0.1% TFA. CM ion exchange HPLC was carried out on a TSK gel CM-2SW column (4.6 x 250 mm, Tosoh) with a linear gradient elution of $HCOONH_4$ (pH 6.6) from 10mM to 1.0M or from 10mM to 0.5M each containing 10% CH_3CN.

RESULTS AND DISCUSSION

Characterization of ir-CNP in human brain: Acid extracts of human brain tissue, mainly composed of hypothalamus and medulla-pons regions, were condensed with a C-18 column and subjected to Sephadex G-50 gel filtration. As shown in Fig. 1, four peaks of ir-CNP were observed on the chromatogram, while ir-BNP was eluted as one major peak. The third peak of ir-CNP of molecular weight (MW) 6K, corresponding to CNP-53, constituted 62.5% of the total ir-CNP. The three other ir-CNP peaks were eluted at the regions of MW >20K, 12K and 3K, and exhibited comparable amounts of immunoreactivity. The four ir-CNPs of different MWs were then analyzed by reverse phase HPLC. As shown in Figs. 2a, 2b and 2d, three ir-CNPs of 12K, 6K and 3K were eluted at almost the same retention time. Among them, 6K and 3K CNP emerged at the elution times of CNP-53, CNP-22 and its methionine sulfoxide

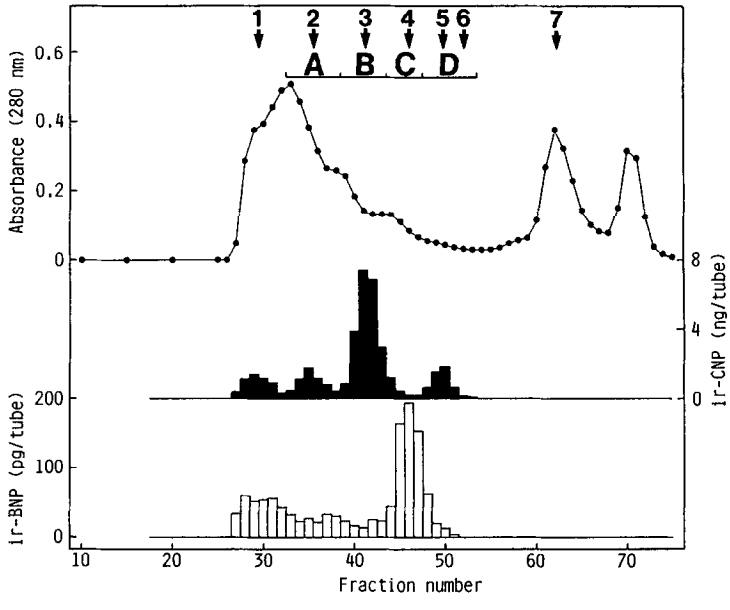

Figure 1. Sephadex G-50 gel filtration of human brain extracts.
Sample: Acid extracts of three human brain tissues (20 g wet wt equivalents in total), pre-treated with reverse phase C-18 column. Solvent: 1M acetic acid.
Column: 1.9 x 134 cm, fine, Pharmacia. Flow rate: 12 ml/hr. Fraction size: 6 ml/tube.
Arrows indicate elution positions of 1) Vo, 2) γ-ANP, 3) CNP-53, 4) BNP-32, 5) CNP-22, 6) α-ANP[4-28] and 7) Vt.

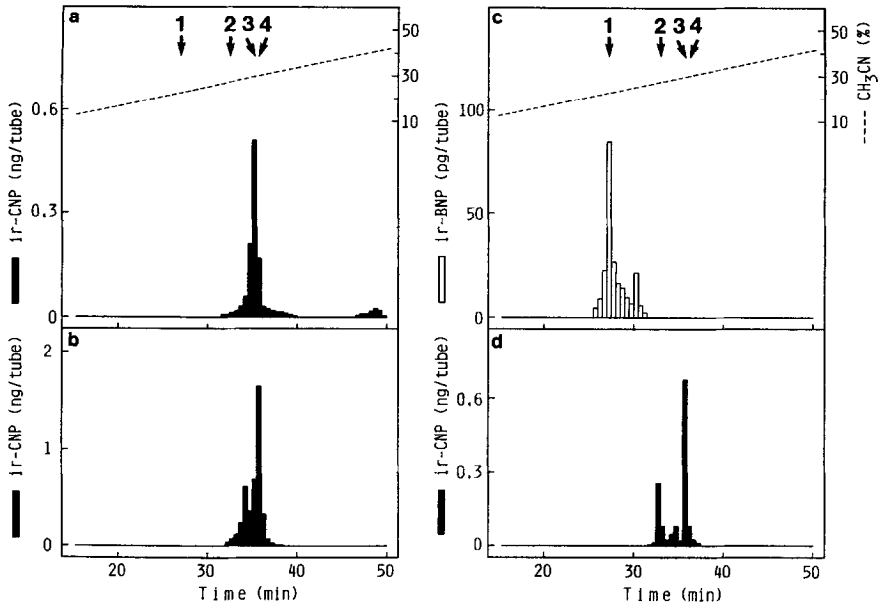

Figure 2. Reverse phase HPLC of ir–CNP and ir–BNP in human brain extracts.
Sample: 1.5 ml aliquots of (**a**) fraction A (#31–38), (**b**) fraction B (#39–43), (**c**) fraction C (#44–47) and (**d**) fraction D (#48–55) in Fig. 1.
Column: Chemcosorb 5C18–300 (4.6 x 250 mm, Chemco). Flow rate: 1.0 ml/min.
Solvent system: Linear gradient elution of CH_3CN from 10% to 60% in 0.1% TFA over 60 min.
Arrows indicate elution times of 1) BNP–32, 2) methionine sulfoxide form of CNP–22, 3) CNP–22 and 4) CNP–53.

form. 12K CNP was deduced to be composed of a single component with hydrophobicity comparable to that of CNP–53 and CNP–22. Only ir–CNP observed at the void volume region of the gel filtration was eluted at more hydrophobic conditions as a wide band, as in the case of porcine ir–CNP of MW>20K (3), suggesting that this immunoreactivity was derived from non–specific disturbance of antibody–antigen interaction.

Immunoreactive CNPs of MW 12K, 6K and 3K were further analyzed by CM ion exchange HPLC. As shown in Figs. 3**a** and 3**c**, 6K and 3K CNPs were each eluted as a single peak at elution times identical to those of CNP–53 and CNP–22, respectively. 12K CNP were not recovered from ion exchange HPLC probably due to its high basicity and low solubility. Based on these results, CNP is shown to be present mainly as CNP–53 in brain, along with CNP–22 and 12K CNP as minor components, in a manner quite similar to that in porcine brain. 12K CNP may be a pro–CNP of 103 residues, since 12K CNP emerged as a single peak in reverse phase HPLC and has an MW comparable to that of pro–CNP (5), although we have not been able to isolate pro–CNP in any species.

Characterization of ir–BNP in human brain: Immunoreactive BNP was eluted as one major peak of MW 4K in Sephadex G–50 gel filtration (Fig. 1). Succeeding reverse phase HPLC and CM ion exchange HPLC indicated that 4K BNP consisted of a single component

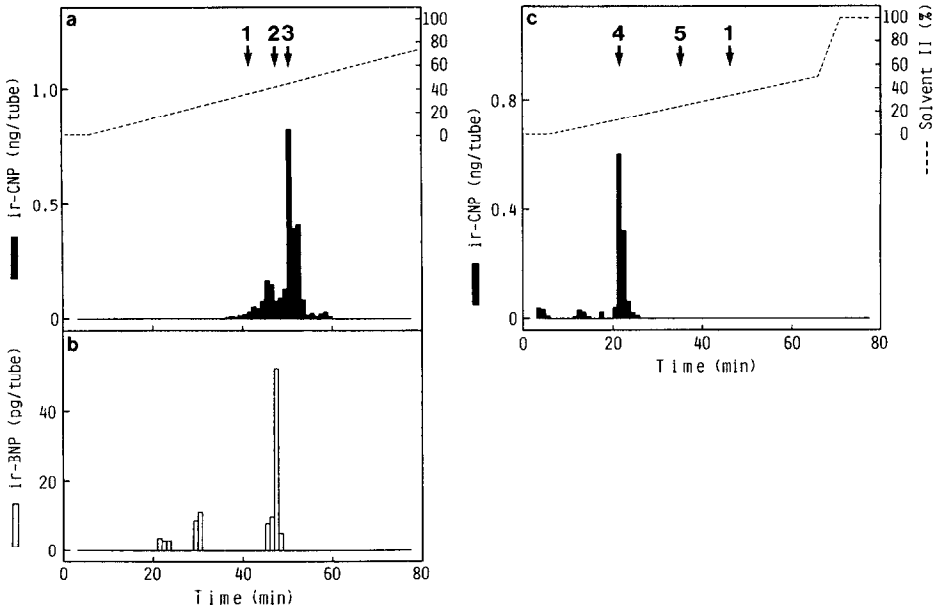

Figure 3. CM ion exchange HPLC of ir–CNP and ir–BNP in human brain extracts.
Sample: 1.5 ml aliquots of (**a**) fraction B (#39–43), (**b**) fraction C (#44–47) and (**c**) fraction D (#48–55) in Fig. 1.
Column: TSK gel CM–2SW (4.6 x 250 mm, Tosoh). Flow rate: 1.0 ml/min.
Solvent system: (I) 10 mM $HCOONH_4$ (pH 6.6) : CH_3CN = 90 : 10 (V/V),
(II) 1.0 M $HCOONH_4$ (pH 6.6) : CH_3CN = 90 : 10 (V/V).
(**a**,**b**) Linear gradient elution from (I) to (II) for 100 min,
(**c**) linear gradient elution from (I) to 50%(II) for 60 min.
Arrows indicate elution times of 1) α–ANP, 2) BNP–32, 3) CNP–53, 4) CNP–22 and 5) α–ANP[4–28].

indistinguishable from BNP–32. Thus, human BNP is found to function mainly as a molecular form of BNP–32, indicating a common proteolytic processing system of human BNP both in neuronal tissue and in heart tissue (14).

Tissue concentration of three natriuretic peptides in human brain: After characterization of ir–CNP and ir–BNP, tissue concentrations of three types of natriuretic peptides in human brain (hypothalamus and medulla–pons regions) were measured. A mean concentration of ir–CNP in brain was determined to be 1.04 ± 0.19 pmol/g (mean ± SD), while those of ir–ANP and ir–BNP were 0.039 ± 0.002 and 0.015 ± 0.001 pmol/g, respectively. The tissue concentration of ir–CNP was, therefore, found to be 27 times and 69 times higher than that of ir–ANP and ir–BNP. Based on these results, CNP was concluded to be the major natriuretic peptide functioning in human brain, at least among the three natriuretic peptides so far identified.

Characterization of ir–CNP in human cardiac atrium: A significant concentration of ir–CNP (1~5 pmol/g) was found in human heart, including atrium and ventricle, as was the case in porcine heart. Immunoreactive CNP in human cardiac atrium was precisely characterized, although we have not fully identified ir–CNP in porcine heart yet. In Sephadex G–50 gel filtration of human cardiac atrial extracts, ir–CNP was mainly observed in two MW regions corresponding to γ–ANP and α–ANP (fractions **A** and **B**) in a profile similar to that of ir–ANP

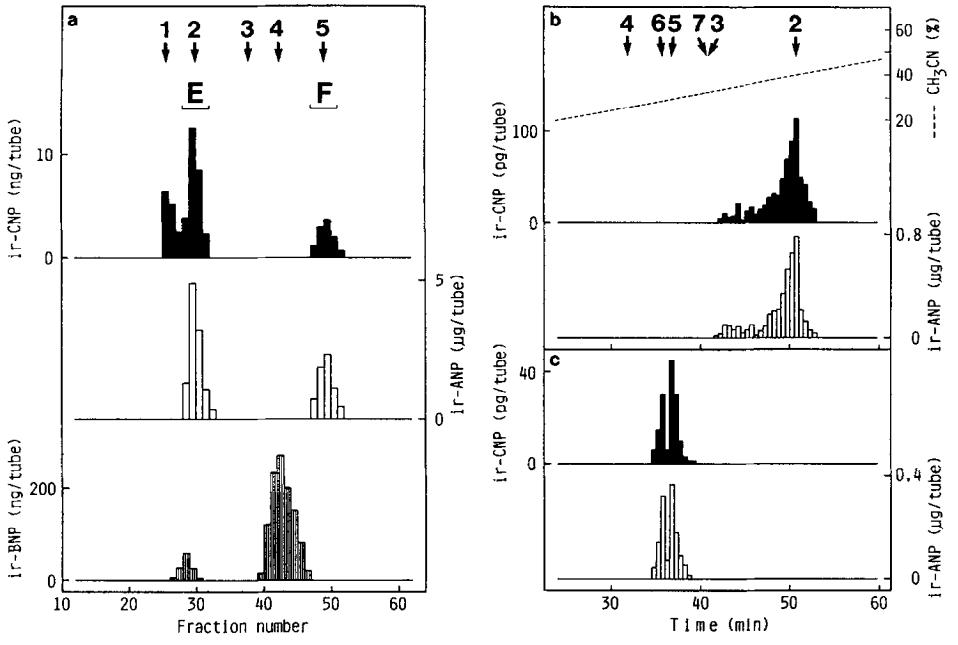

Figure 4. (a) Sephadex G-50 gel filtration of human cardiac atrial extracts.
Sample: Acid extracts of human right cardiac atrium (450 mg wet wt equivalents) of subject No. 4, pre-treated with reverse phase C-18 column. Solvent: 1M acetic acid. Column: 1.8 x 134 cm, fine, Pharmacia. Fraction size: 5 ml/tube. Flow rate: 8 ml/hr.
(b,c) Reverse phase HPLC of high MW and low MW ir-CNP in human cardiac atrium.
Sample: 1 ml aliquots of (b) fraction E (#29-32) and (c) fraction F (#48-51) in (a).
Column: Hi-Pore RP-318 (4.6 x 250 mm, Bio-Rad). Flow rate: 1.0 ml/min.
Solvent system: Linear gradient elution of CH_3CN from 0% to 60% in 0.1% TFA over 60 min.
Arrows indicate elution positions and/or times of 1) Vo, 2) γ-ANP, 3) CNP-53, 4) BNP-32, 5) α-ANP, 6) methionine sulfoxide form of α-ANP and 7) CNP-22.

(Fig. 4a). On the other hand, most ir-BNP was eluted at the position of BNP-32. Fractions **A** and **B** were each separated by reverse phase HPLC. High MW ir-CNP (fraction **A**) emerged at the elution time of γ-ANP in a profile quite similar to that of ir-ANP (Fig. 4b). Low MW ir-CNP (fraction **B**) was eluted at the elution time of α-ANP and its methionine sulfoxide form also in a profile similar to that of ir-ANP (Fig. 4c). Furthermore, ir-CNP levels measured in each fraction were almost equal to those calculated from the level of ir-ANP and its crossreactivity in the CNP RIA system. Thus, apparent CNP immunoreactivity detected in atrium is concluded to be derived from the crossreactivity of ANP, which is present in atrium at 10^4 times higher concentration.

In the present study, CNP is shown to be present in human brain at a high concentration (1.04 pmol/g), and its tissue concentration is far higher than those of ANP and BNP. We have recently reported that porcine brain contains CNP at a high concentration (0.79 pmol/g), and this concentration is 1.5~10 times higher than those of ANP and BNP (3). In rat, CNP mRNA was detected only in brain and not in heart and other peripheral tissues (9), but ir-CNP concentration in brain (1.8 pmol/g) is between those of ANP and BNP (to be published). These

data indicate that CNP is a major natriuretic peptide existing in brain, and that its tissue concentration is rather constant regardless of species differences. In contrast, tissue concentrations of ANP and BNP in brain vary by species (3,15), with human brain containing the lowest levels of these two peptides. As a result, CNP is assigned to be the primary natriuretic peptide functioning in human brain.

CNP immunoreactivity detected in heart was verified to be derived from crossreactivity of ANP. Thus, expression of the CNP gene is restricted to the central nervous system and is not present in human heart, where ANP and BNP are found in extremely high concentrations. These results suggest that CNP exerts its physiological functions only as a neuropeptide, although both ANP and BNP are also present in the central nervous system. Processing pathways and endogenous molecular forms of the three types of natriuretic peptides are summarized in Fig. 5. The major molecular form of CNP in human brain is identified to be CNP–53, which is larger than that of ANP and BNP, such as α–ANP[4–28] and BNP–32. Although nothing is known about functional differences between CNP–53 and CNP–22, the larger molecular form commonly found in human and pig might have some physiological meaning. In the CNP–53 structure, the CNP–22 unit is flanked by a typical processing signal, Lys–Lys. However, processing of ANP and BNP precusors as well as conversion of pro–CNP into CNP–53 all take place after a single Arg residue, which is recognized as a signal, even in the central nervous system. These facts suggest that processing enzyme(s) of the natriuretic peptide system do not preferentially cleave after the Lys–Lys signal and that the production rate of CNP–22 may be reduced in comparison with that of α–ANP[4–28] and BNP–32.

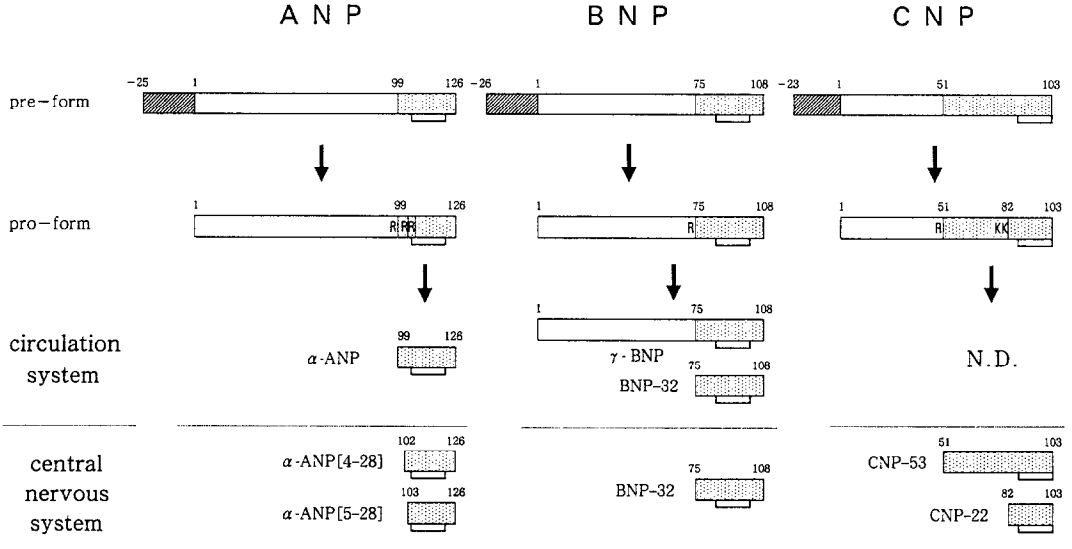

Figure 5. Proteolytic processing pathways and endogenous molecular forms of three types of natriuretic peptides in human central nervous system and circulation system. N.D.) not detected in significant concentration.

The three natriuretic peptides, ANP, BNP and CNP, have a highly homologous structure, but CNP interacts with a B–type bioactive receptor carrying a guanylate cyclase domain, while ANP and BNP bind to an A–type bioactive receptor (11). In the 3'–untranslated regions of the cDNAs encoding ANP, BNP and CNP precursors, a characteristic nucleotide sequence (ATTTA), which changes turnover rates of mRNA, is found at a different frequency in each cDNA (4). In our series of studies on the distribution of CNP in human and pig, we found CNP to be localized exclusively in the central nervous system, in contrast to ANP and BNP which are mainly present in heart and the circulation system. These differences in the receptors, cDNA structures and distribution of the three types of natriuretic peptides are thought to reflect their functional differences in the regulation of body fluid volume and blood pressure.

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