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Molecular Evolution of Shark C-type Natriuretic Peptides

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ABSTRACT—C-type natriuretic peptides (CNP) of varying length were isolated from the atrium or ventricle of a shark, Lamna ditropis and their amino acid sequences were determined. Although the sequence of Lamna CNP was highly homologous to those of other CNPs sequenced to date, the Lamna CNP-41, the longest CNP identified in this study, has one amino acid replacement from those of Triakis scyllia and Scyliorhinus canicula, and three amino acid replacements from that of Squalus acanthias. The degree of similarity of CNP molecules coincides well with their systematic positions in the cladogram of elasmobranchs; Lamna, Triakis and Scyliorhinus belong to the same order, but Lamna and Squalus belong to different orders. The facts that Lamna and Triakis are in different suborders but Triakis and Scyliorhinus are in the same suborder and have identical CNP-41, also support this evolutionary implication.

INTRODUCTION

C-type natriuretic peptide (CNP) is a member of the natriuretic peptide family first identified in the brain of pig and teleost fishes [2, 4, 9]. In contrast to other members of the peptide family, namely atrial (A-type), B-type and ventricular natriuretic peptides (ANP, BNP and VNP) which are cardiac hormones circulating in the blood, CNP has been isolated from the brain in all species from teleost to mammals, and its plasma and cardiac concentrations are too low to be detected in mammals [8]. Thus CNP is regarded as a neuropeptide in mammals. However, we have isolated CNP from the heart of two species of dogfish shark, Triakis scyllia and Scyliorhinus canicula [5, 6]. In these fish, plasma and cardiac concentrations of CNP are extremely high, and other cardiac natriuretic peptides, ANP, BNP and VNP, are not identified in their hearts [7]. Furthermore, only CNP cDNA has been cloned from the cDNA library of the heart of spiny dogfish, Squalus acanthias [3]. Therefore it is likely that CNP is the only natriuretic peptide present in elasmobranchs. It is also noted that the amino acid sequence of CNP is more conserved than any other natriuretic peptides, namely ANP, BNP and VNP [8]. Thus, CNP might be an ancestral molecule of the natriuretic peptide family, and other members might be reproduced by gene duplication.

As a prototype of the natriuretic peptide family, it seems of interest to examine chemical evolution of the CNP molecule. In previous studies, we have found that amino acid sequences of CNP-22, a mature form stored in the brain, of *Triakis* and *Scyliorhinus* are identical, and even proCNP differs in only 3 out of 115 amino acid residues [5,6]. However, Squalus CNP-22 predicted from the cDNA sequence differs from that of Triakis in 2 amino acid residues, and the difference was much greater at the level of prohormone [3]. Systematically, Triakis and Scyliorhinus belong to the same suborder Scyliorhinoidei, but Squalus is different from the two species at the level of order [1]. We recently have obtained the heart of Lamna ditropis. This fish belongs to the order Lamniformes as do Triakis and Scyliorhinus, but to the suborder different from those sharks. Therefore, we attempted in the present study to isolate CNP from the Lamna heart and to compare its structure with those of other sharks.

MATERIALS AND METHODS

Isolation of CNP

The shark, Lamna ditropis, of approximately 3 m in body length was caught in Toyama Bay and was obtained from fishermen 5 h after capture. The heart was immediately dissected out, the atrium and ventricle separated, and frozen in a deep freezer at -50° C. The atrium (106.4 g) and ventricle (333.2 g) were treated separately. ANP-like peptides in the heart were isolated with protocols described previously [5]. The frozen tissues were crushed in a pulverizer, boiled in 5 volumes (atrium) or 3 volumes (ventricle) of water for 10 min, acidified with AcOH to a concentration of 1 M, and homogenized in a Polytron homogenizer (Kinematika, Germany) for 90 sec at maximum speed. The homogenate was centrifuged at $16,000 \times g$ for 30 min at 4°C. The supernatant was added to 2 volumes of cold acetone, and centrifuged at 16,000×g for 30 min at 4°C. The supernatant was evaporated, reconstituted in 30 ml of 1 M AcOH, and added to 2 liters of cold acetone. After centrifugation, the pellet was dissolved in 30 ml of 1 M AcOH, and applied onto a column (5×85 cm) of Sephadex G-25 fine (Pharmacia, Sweden) for desalting. The fractions which contain molecules with Mr > ca. 2,000 were applied onto a column of SP-Sephadex C-25 (1.6×20 cm), and adsorbed materials were eluted successively with 150 ml each of 1 M AcOH, 2 M pyridine, and 2 M pyridine-AcOH, pH 5.0. Each fraction was evaporated and assayed for relaxant activity in the

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chick rectum as described below. Bioactive fractions were subjected to cation-exchange high performance liquid chromatography (HPLC) in an IEC-CM column (7.5×75 mm, Jasco, Japan). Each bioactive fraction was then subjected to reverse-phase HPLC in an ODS-120T column (4.6×250 mm, Tosoh, Japan) with different gradients of CH₃CN concentrations. The detailed chromatographic conditions are described in the legend of each figure. The purified material was subjected to amino acid sequencing in a protein sequencer (477A, Applied Biosystems, USA). Validity of the amino acid sequence was examined by mass spectrometry (JMS-HX110, JEOL, Japan).

ANP-like activity was assayed at each step of purification using a relaxant activity in the chick rectum [10]. New-born male chicks were purchased from Kanagawa Poultry Cooperation (Yokohama) and reared under a infra-red lamp with free access to food and water. The chick was decapitated, rectum immediately isolated, and set up in a trough whose temperature was controlled at 37°C. The rectum

was precontracted with 2×10^{-6} M carbachol (Sigma, USA), and the relaxation was quantified by a displacement transducer connected to a transducer amplifier (1829 and 45347, NEC-Sanei, Japan). ANP-like activity was expressed as equivalents to eel ANP which was used as standard.

RESULTS

Same molecules were isolated from atrial and ventricular extracts. After Sephadex G-25 chromatography, fractions of 1–70, which contain molecules larger than CNP-22 [5], were pooled and subjected to SP-Sephadex C-25 chromatography (Fig. 1a). Since only the fraction eluted with pyridine-AcOH exhibited rectum-relaxant activity, this fraction was subjected to cation-exchange HPLC. The bioactive



FIG. 1. Purification of C-type natriuretic peptide (CNP) from Lamna atrium. Solid columns represent relaxant activity in the chick rectum expressed as equivalents to eel atrial natriuretic peptide (eANP). A: Sephadex G-25 chromatography of crude atrial extract. Fractions marked by square bracket were subjected to SP-Sephadex C-25 chromatography. B: cation-exchange high-performance liquid chromatography (HPLC) of the fraction eluted with pyridine-AcOH in SP-Sephadex C-25 chromatography. Broken lines show gradient of solvent B (1 M NH₄OAc : CH₃CN=9:1) against solvent A (10 mM NH₄OAc : CH₃CN=9:1). CNP-29, CNP-38 and CNP-41 were recovered, respectively, from fractions marked with bracket 1, 2, and 3. C and D: reverse-phase HPLC of fraction 37 of panel B and a fraction with bioactivity in panel C, respectively. Sequence analysis of bioactive peak in panel D revealed that the peak is that of CNP-29. Broken lines show gradient of CH₃CN concentrations.



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