Serum-Free Culture of Cells of the Endocrine System" Ed by DW Barnes, DA Sirbasku, GH Sato, Alan R. Liss, Inc, New York, pp 1–13

12 Ikawa H, Irahara M, Matsuzaki T, Saito S, Sano T, Aono T (1992) Impaired induction of prolactin secretion from the anterior pituitary by suckling in streptozotocin-induced diabetic rat. Acta Endocrinol 126: 167–172

13 Keech CA, Gutierrez-Hartmann A (1991) Insulin activation of rat prolactin promoter activity. Mol Cell Endocrinol 78: 55–60

14 Keefer DA (1980) In vivo estrogen uptake by individual cell types of the rat anterior pituitary after short-term castration-adrenalectomy. Cell Tissue Res 209: 167–175

15 Kirkland JL, Barrett GN, Stancel GM (1981) Decreased cell division of the uterine luminal epithelium of diabetic rats in response to 17 β-estradiol. Endocrinology 109: 316–318

16 Kudlow JE, Kobrin MS (1984) Secretion of epidermal growth factor-like mitogens by cultured cells from bovine anterior pituitary glands. Endocrinology 115: 911–917

17 Michels KM, Lee W-H, Seltzer A, Saavedra JM, Bondy CA (1993) Up-regulation of pituitary [^{125}I] insulin-like growth factor-I (IGF-I) binding and IGF binding protein-2 and IGF-I gene expression by estrogen. Endocrinology 132: 23–29

18 Murphy LJ, Bell GI, Friesen HG (1987) Tissue distribution of insulin-like growth factor I and II messenger ribonucleic acid in the adult rat. Endocrinology 120: 1279–1282

19 Nelson KG, Takahashi T, Bossert NL, Walmer DK, McLachlan JA (1991) Epidermal growth factor replaces estrogen in the stimulation of female genital-tract growth and differentiation. Proc Natl Acad Sci USA 88: 21–25

20 Nelson KG, Takahashi T, Lee DC, Luetteke NC, Bossert NL, Ross K, Eitzman BE, McLachlan JA (1992) Transforming growth factor-α is a potential mediator of estrogen action in the mouse uterus. Endocrinology 131: 1657–1664

21 Olchovsky D, Bruno JF, Gelato MC, Song J, Berelowitz M (1991) Pituitary insulin-like growth factor-I content and gene expression in the streptozotocin-diabetic rat: evidence for tissue-specific regulation. Endocrinology 128: 923–928

22 Sakuma S, Shirasawa N, Yoshimura F (1984) A histometrical study of immunohistochemically identified mitotic adenohypophysial cells in immature and mature castrated rats. J Endocrinol 100: 323–328

23 Shirasawa N, Yoshimura F (1982) Immunohistochemical and electron microscopical studies of mitotic adenohypophysial cells in different ages of rats. Anat Embryol 165: 51–61

24 Sirbasku DA (1978) Estrogen induction of growth factors specific for hormone-responsive mammary, pituitary, and kidney tumor cells. Proc Natl Acad Sci USA 75: 3786–3790

25 Stanley FM (1992) An element in the prolactin promoter mediates the stimulatory effect of insulin on transcription of the prolactin gene. J Biol Chem 267: 16719–16726

26 Starus DS (1984) Growth-stimulatory actions of insulin *in vitro* and *in vivo*. Endocr Rev 5: 356–369

27 Sutherland RL, Watts CKW, Clarke CL (1988) Oestrogen action. In "Hormones and their actions, Part I" Ed by BA Cooke, RJB P King, HJ van der Molen, Elsevier Science Publishers BV, Amsterdam pp 197–215

28 Takahashi S, Okazaki K, Kawashima S. (1984) Mitotic activity of prolactin cells in the pituitary glands of male and female rats of different ages. Cell Tissue Res 235: 497–502

29 Takahashi S, Kawashima S (1987) Proliferation of prolactin cells in the rat: effects of estrogen and bromocriptine. Zool Sci 4: 855–860

30 Tesone M, Ladenheim RG, Charreau EH (1985) Alterations in the prolactin secretion in streptozotocin-induced diabetic rats. Correlation with pituitary and hypothalamus estradiol receptors. Mol Cell Endocrinol 43: 135–140

31 Tomooka Y, DiAugustine RP, McLachlan JA (1986) Proliferation of mouse uterine epithelial cells *in vitro*. Endocrinology 118: 1011–1018

32 Webster J, Scanlon MF (1991) Growth factors and the anterior pituitary. Bailliere's Clin Endocrinol Metab 5: 699–726

33 Weisenberg L, Fridman O, Libertun C, De Nicola AF (1983) Changes in nuclear translocation of estradiol-receptor complex in anterior pituitary and uterus of rats with streptozotocin diabetes. J Steroid Biochem 19: 1737–1741

34 Yamauchi K, Shiino M (1986) Pituitary prolactin cells in diabetic rats induced by the injection of streptozotocin. Exp Clin Endocrinol 88: 81–88

ZOOLOGICAL SCIENCE 11: 451–454 (1994)

Molecular Evolution of Shark C-type Natriuretic Peptides

Masayoshi Takano[1], Yuichi Sasayama[2]
and Yoshio Takei[3]

Ocean Research Institute, University of Tokyo, Minamidai, Nakano, Tokyo 164 and [2]Department of Biology, Faculty of Science, Toyama University, Gofuku, Toyama 930, Japan

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.

Accepted June 7, 1994
Received April 4, 1994

[1] Present address: Research Institute, Zenyaku Kogyo Co. Ltd., Ohizumimachi, Nerima, Tokyo 178, Japan

[3] To whom all correspondence should be addressed

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×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

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 CH_3CN 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

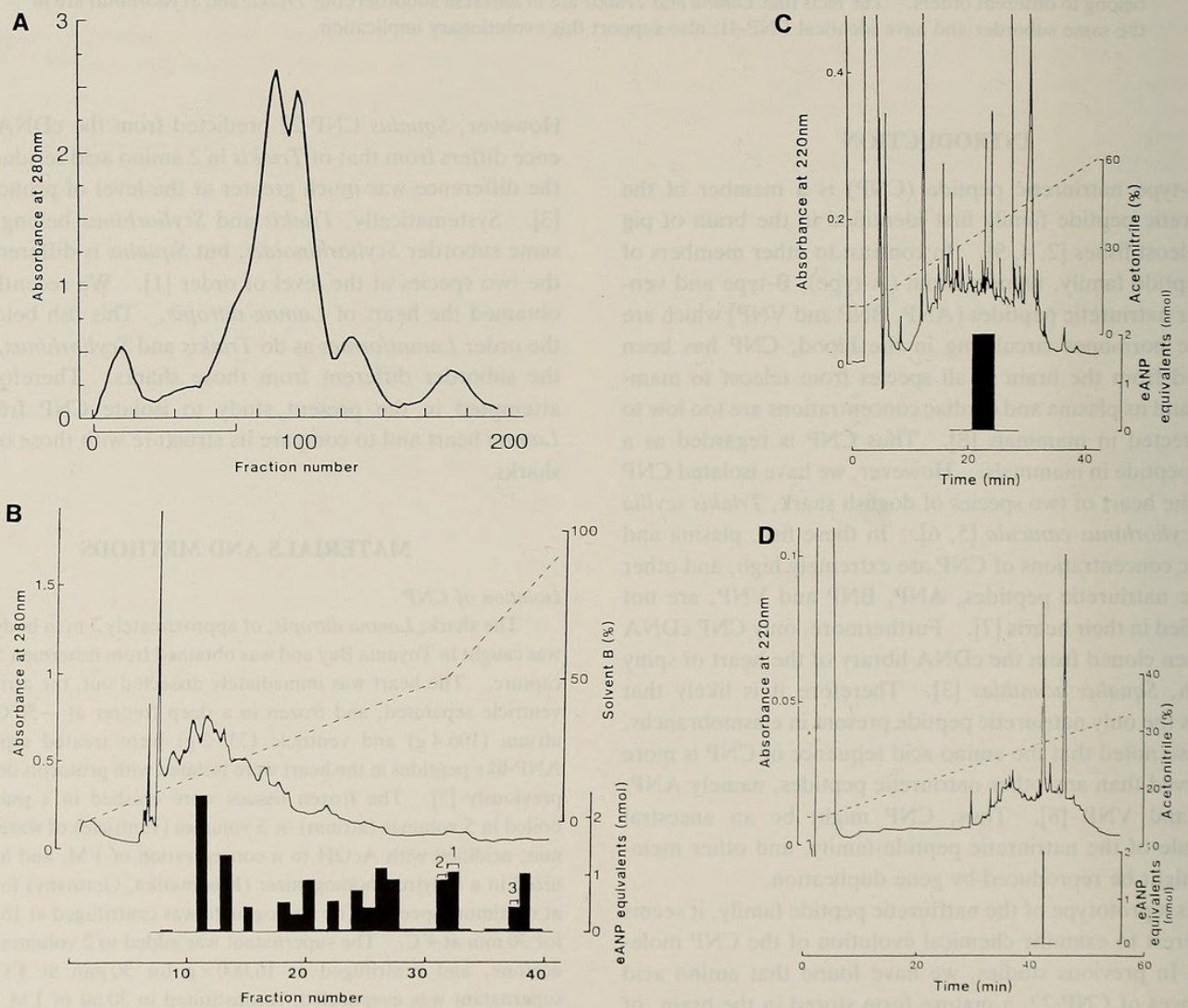

Fig. 1. Purification of C-type natriuretic peptide (CNP) from *Lamna* atrium. Solid columns represent relaxant activity in the chick rectum expressed as equivalents to eel atrial natriuretic peptide (eANP). A: Sephadex G-25 chromatography of crude atrial extract. Fractions marked by square bracket were 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 NH_4OAc : CH_3CN=9:1) against solvent A (10 mM NH_4OAc : CH_3CN=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 CH_3CN concentrations.