Characterisation of C-type natriuretic peptide receptors in the gill of dogfish *Triakis scyllia*

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Abstract

Only C-type natriuretic peptide (CNP) has been identified in primitive elasmobranch fish. CNP is the most conserved molecule in the natriuretic peptide family, suggesting that it is the ancestral type. As a first step to investigating the ancestral type of natriuretic peptide receptors, CNP receptors were characterised in an elasmobranch (dogfish, *Triakis scyllia*) by radioligand-binding analysis using ^{125}I -[Tyr⁰]-dogfish (df)CNP. None of the modifications of the CNP molecule that occur at the time of iodination (addition of a Tyr residue at the N-terminus, introduction of iodine into Tyr⁰ or oxidation of Met¹⁷) affect the affinity of dfCNP for the receptors. Neither did oxidation of Met¹⁷ decrease the ability of CNP to stimulate cGMP production. In the tissues examined, CNP receptors were densest in the gill cells followed by the intestine, interrenal gland and rectal gland, all of which are involved in osmoregulation in elasmobranchs. CNP-stimulated guanylate cyclase (GC) activity was highest in the interrenal gland, intestine, brain and rectal gland, followed by the gill cells. Since the gill cells seem to contain both GC-coupled and uncoupled

receptors, this tissue was used to characterise dogfish CNP receptors. Scatchard analysis of the saturation isotherm revealed two classes of binding site: one has a K_d of 24.0 pM and $B_{\max}$ of 59.9 fmol/mg protein, and the other has low affinity ($K_d > 1\text{ nM}$) and high capacity ($B_{\max} > 200\text{ fmol/mg protein}$). The higher-affinity binding sites may represent GC-uncoupled receptors, because C-ANF, a specific ligand for GC-uncoupled receptors, almost completely displaced CNP binding. Affinity-labelling experiments showed that dogfish receptors have molecular masses of about 90, 170 and 340 kDa, and CNP binding to the former two receptors is inhibited by C-ANF. After reduction with 2-mercaptoethanol, most 170 kDa labelling was shifted to 90 kDa. It is concluded that GC-uncoupled receptors in the dogfish gill have higher molecular mass than those of mammals and eel (about 65 kDa), and are present mostly as monomers even in non-reducing conditions. However, a small population of GC-coupled receptors is also present, as demonstrated by an increase in cGMP production.

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Introduction

A-type (atrial) natriuretic peptide (ANP) was first discovered as a cardiac hormone with potent diuretic and natriuretic properties (de Bold 1985). It is now known that ANP, B-type (brain) natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) function as a family to maintain volume and pressure homeostasis. In teleost fish, a new type named ventricular natriuretic peptide (VNP) has been identified (Hagiwara *et al.* 1995). ANP, BNP and VNP are all cardiac hormones circulating in blood, while CNP is basically a paracrine factor in the brain and other peripheral tissues (Espiner 1994). In elasmobranch fish, however, only CNP and its mRNA have been detected in the heart as well as the brain (Suzuki *et al.* 1991, Schofield *et al.* 1991), and its concentrations in the heart and plasma are even higher than those of ANP, BNP and VNP in mammals and teleost fish (Suzuki *et al.* 1994). It seems therefore that CNP is the sole natriuretic peptide in

elasmobranchs, and it works not only as a paracrine factor but also as a circulating hormone. Thus CNP is considered to be an ancestral form of the natriuretic peptide family.

Natriuretic peptide receptors (NPRs) are also identified in selected vertebrate species (for a review, see Anand-Srivastava & Trachte 1993). NPR-A and NPR-B are membrane-bound guanylate cyclase (GC) with a molecular mass of 140 kDa, and thus utilise cGMP as an intracellular messenger. NPR-C and NPR-D have a molecular mass of 65 kDa and their intracellular messenger is as yet undetermined. NPR-D has only been identified in the eel (Kashiwagi *et al.* 1995). NPR-A, NPR-B and NPR-D are present as tetramers, but NPR-C is a dimeric receptor. ANP and BNP are selective ligands for NPR-A and CNP for NPR-B, while all natriuretic peptides exhibit similar affinity for NPR-C and NPR-D.

For experiments designed to try to understand the complicated natriuretic peptide system, dogfish is a useful material because only CNP has been identified in this

species. Since CNP is thought to be an ancestral form of the natriuretic peptide family, it is of interest to examine which types of receptor are present in the dogfish. To pursue this aim therefore we characterised NPRs in the dogfish *Triakis scyllia* using radioligand-binding studies in combination with cGMP assay and affinity labelling.

Materials and Methods

Drugs

Dogfish CNP (dfCNP), [Tyr⁰]-dfCNP, dogfish angiotensin II (dfANGII), C-ANF (rat des[Gln¹⁸,Ser¹⁹,Gly²⁰,Leu²¹,Gly²²]-ANP(4–23)-NH₂), pepstatin and leupeptin were obtained from Peptide Institute Inc. (Osaka, Japan). A protein molecular mass marker kit was purchased from Boehringer-Mannheim (Mannheim, Germany). Other reagents were from Wako Pure Chemical Industries Ltd (Osaka, Japan) or Sigma Chemical Co. (St Louis, MO, USA) unless otherwise specified. Na¹²⁵I (72 TBq/mmol) was purchased from Amersham International (Amersham, Bucks, UK). ¹²⁵I-[Tyr⁰]-dfCNP was prepared by the lactoperoxidase method, and purified by reverse-phase HPLC on an ODS-120T column (4.6 × 250 mm; Tosoh, Tokyo, Japan) with a linear gradient of CH₃CN from 15 to 60% for 60 min (Suzuki *et al.* 1994). Since the methionine residue at position 17 of dfCNP was oxidised during the iodination, [I-Tyr⁰,Met¹⁷(O)]-dfCNP was prepared as above using unlabelled NaI to test its ability for binding and stimulating cGMP production.

Preparation of membrane fraction

Dogfish (*Triakis scyllia*) of either sex caught in June 1996 at Koajiro Bay, Kanagawa, were purchased from fishermen, and kept in an aquarium with running seawater at Misaki Marine Biological Station, University of Tokyo, for more than 1 week before use. They weighed 0.8–2.5 kg at the time of experimentation. Membrane fractions were prepared from various tissues as described previously (Mishina & Takei 1997). The dogfish were anaesthetised by immersion in 1% tricaine methanesulphonate for 10 min and the heart was exposed. After collection of blood, any remaining blood was removed by perfusing 50 ml dogfish saline (240 mM NaCl and 423 mM urea) into the conus arteriosus after puncture of the sinus venosus. Tissues were quickly removed and frozen in liquid nitrogen. The gill cells were scraped from the gill arches and also frozen in liquid nitrogen until use. On the day of membrane preparation, the thawed gill cells were mixed thoroughly with 5 volumes of dogfish saline, filtered through a nylon mesh (pore size 40 µm) to remove contaminated blood vessels, and centrifuged at 800 g for 10 min to collect the cells. These procedures were repeated twice to remove the mucus secreted from the cell. The pellets of gill cells, or

diced other tissues, were homogenised in 8 volumes of buffer A (250 mM sucrose, 10 mM disodium EDTA, 50 mM Tris-HCl, pH 7.2) with a Polytron homogeniser at setting 6 for 30 s. The homogenates were centrifuged at 12 000 g for 20 min, and the supernatants were centrifuged again at 57 000 g for 30 min. The pellets were resuspended in 5 volumes of buffer A, and centrifuged at 57 000 g for 30 min. The pellets were suspended in buffer B (50 mM Tris-HCl, 5 mM MgCl₂, 125 mM NaCl, pH 7.2), and the protein concentration was determined by using a protein assay kit (Bio-Rad, Richmond, CA, USA) after treatment with 1 M NaOH. The membrane fractions thus prepared were stored in liquid nitrogen until use.

Binding assay

For the binding assay, 10^{−10} M ¹²⁵I-[Tyr⁰]-dfCNP and 25 µg membrane fraction were incubated in 100 µl buffer B containing 10^{−4} M EGTA, 10^{−4} M phenanthroline, 10^{−5} M phosphoramidon and 2 mg/ml BSA for 120 min at 25 °C unless otherwise stated. It was confirmed by HPLC analysis that free and bound ¹²⁵I-[Tyr⁰]-dfCNP were not metabolised after incubation under these conditions (Mishina & Takei 1997). After incubation, 1 ml ice-cold buffer B was added to each tube to terminate the reaction. The aliquots were then centrifuged at 14 000 g for 5 min at 4 °C and radioactivity in the pellets was counted with a Riasar γ-counting system (Packard Instrument Co., Meriden, CT, USA). Specific binding was defined as the difference between the amount of radioligand bound in the absence and presence of 10^{−6} M dfCNP. The time course of binding was examined at 4, 18, 25 and 37 °C. The saturation experiment was performed at 2 × 10^{−11}–2 × 10^{−7} M ¹²⁵I-[Tyr⁰]-dfCNP to determine the affinity (K_d) and binding capacity (B_{max}) of the receptors. To examine the specificity of the receptors, displacement experiments were performed with dfCNP, [Tyr⁰]-dfCNP, [I-Tyr⁰,Met¹⁷(O)]-dfCNP and C-ANF at a concentration of 10^{−12}–10^{−6} M. The acquired data were analysed using the LIGAND program (Munson & Rodbard 1980) to calculate the binding parameters.

Assay for cGMP production

GC activity was determined as described previously (Mishina & Takei 1997). Membrane fraction prepared from each tissue (30 µg) was incubated at 25 °C for 10 min in 80 µl 50 mM Tris-HCl buffer, pH 7.6, containing 1 mg/ml BSA, 0.5 mM isobutyl-1-methylxanthine, 15 mM phosphocreatine and 31.25 U/ml creatine kinase. Then 1 mM GTP, 4 mM MnCl₂ and 0 or 10^{−6} M dfCNP were added to the mixture to adjust the final volume to 100 µl, and the mixture was incubated for a further 10 min at 25 °C. The reaction was stopped by adding 900 µl 50 mM ammonium acetate buffer, pH 4.0, followed by boiling for 3 min. The suspension was centrifuged at

14 000 *g* for 5 min at 4 °C, and cGMP concentrations in the supernatant were determined by RIA using a cGMP assay kit (Yamasa, Tokyo, Japan) according to the manufacturer's protocol.

Affinity labelling

The membrane fraction of gill cells (30 µg) in buffer B was centrifuged at 14 000 *g* for 10 min at 4 °C, and the pellet was resuspended in 50 µl 20 mM phosphate buffer, pH 7.4, containing 130 mM NaCl, 1 mM EDTA, 1 mg/ml lysozyme, 0.2 mM phenylmethylsulphonyl fluoride, 10 µg/ml leupeptin and 10 µg/ml pepstatin. After the addition of 50 µl ^{125}I -[Tyr⁰]-dfCNP (3×10^{-10} M), the reaction mixture was incubated for 120 min at 25 °C in the absence or presence of 10^{-6} M unlabelled dfCNP or C-ANF. The bound ^{125}I -[Tyr⁰]-dfCNP was cross-linked to the receptors by incubation with 2 mM disuccinimidyl suberate (Pierce Chemical, Rockford, IL, USA) for 20 min at 25 °C. The reaction was terminated by adding 20 µl 2 M ammonium acetate. The mixture was then centrifuged at 14 000 *g* for 5 min at room temperature, and the pellet was suspended in 50 µl loading buffer (650 mM Tris-HCl, pH 6.8, containing 10% (w/v) glycerol, 1% SDS and 0.1 mg/ml bromophenol blue with or without 1% 2-mercaptoethanol) and boiled for 5 min. The mixture was centrifuged at 14 000 *g* for 5 min, and then 30 µl supernatant was electrophoresed on a 5.0% polyacrylamide slab gel. The proteins in the gel were fixed by incubation in 10% acetic acid and 25% methanol, and visualised by Coomassie brilliant blue staining. The dried slab gels were exposed to an imaging plate for 48 h for analysis in a BAS-1000 Mac system (Fuji Photo Film Co., Tokyo, Japan). The gel was later exposed to Kodak BioMax MS films with BioMax MS intensifying screen (Eastman Kodak, New Haven, CT, USA) at -80 °C for 1 week, and the film was developed in Kodak D-19 developer.

Results

We first determined the assay conditions for ^{125}I -[Tyr⁰]-dfCNP binding using the gill membrane fraction. Specific binding of ^{125}I -[Tyr⁰]-dfCNP increased linearly according to the amount of membrane protein in the incubation mixture, reaching a plateau at 80 µg (Fig. 1a). Binding was more rapid at higher temperatures, but the rate was not increased further above 25 °C (Fig. 1b). Equilibrium binding was attained in 60 min at 25 °C and was maintained for more than 180 min. Therefore the incubation was performed with 25 µg protein at 25 °C for 120 min in subsequent experiments.

Figure 2 shows the amount of specific CNP binding in dogfish tissues. Binding was greatest in the gill followed by the intestine, interrenal gland and rectal gland. The liver, heart, kidney and brain also showed significant binding, but the amount was much smaller.

Figure 1 (a) Relationship between ^{125}I -[Tyr⁰]-dfCNP binding and membrane protein, and (b) time course of ^{125}I -[Tyr⁰]-dfCNP binding at different temperatures (●, 4 °C; ■, 18 °C; ▲, 25 °C; ▼, 37 °C) in dogfish gills. Values are means of three measurements of one membrane preparation. Vertical bars represent S.E.M.

cGMP production was markedly stimulated by CNP in the intestine, brain, interrenal gland and rectal gland, and less so in the gill, kidney and liver (Fig. 3). C-ANF was not effective even at 10^{-6} M, but iodinated and methionine-oxidised [Tyr⁰]-dfCNP, [I-Tyr⁰,Met¹⁷(O)]-dfCNP was as effective as dfCNP in stimulating cGMP production in the gill membrane (data not shown).

A saturation isotherm showed that CNP binding was saturated initially between 10^{-9} and 10^{-8} M, but binding increased again above 10^{-8} M and reached a plateau at 5×10^{-8} M (Fig. 4a). Scatchard analysis demonstrated two classes of binding site (Fig. 4b): K_d and $B_{\max}$ for the high-affinity site were respectively 24.0 ± 5.8 pM and 59.9 ± 12.8 fmol/mg protein, and higher than 1 nM and more than 200 fmol/mg protein for the other site.

As shown in Fig. 5, CNP binding to the gill membrane was displaced in a concentration-dependent manner by unlabelled dfCNP and C-ANF but not by ANGII, demonstrating specificity for the natriuretic peptides. [I-Tyr⁰,Met¹⁷(O)]-dfCNP had an affinity similar to that of dfCNP. Scatchard analysis of the displacement experiment revealed a single class of binding site with K_d and $B_{\max}$ of 116.3 pM and 69.4 fmol/mg protein respectively. Since the ^{125}I -[Tyr⁰]-dfCNP concentration used in this

Figure 2 Specific ¹²⁵I-[Tyr⁰]-dfCNP-binding sites in various dogfish tissues. Each result represents the mean of three experiments using three membrane preparations for all tissue membranes except interrenal gland for which one preparation was used. Each membrane was prepared from two animals. Interrenal gland membrane was prepared from 12 animals. Vertical bars represent S.E.M.

Figure 3 CNP-stimulated GC activity in various dogfish tissues. Each value represents the mean of three experiments using the membrane preparations as described in Fig. 2. Vertical bars show S.E.M.

Figure 4 (a) Saturation isotherm of ^{125}I -[Tyr⁰]-dfCNP binding to the membrane fraction of dogfish gill (mean $\pm$ S.E.M.). (b) Scatchard plot of the isotherm.

Figure 5 Displacement of ^{125}I -[Tyr⁰]-dfCNP by dfCNP (●), [Tyr⁰]-dfCNP (■), [I-Tyr⁰, Met¹⁷]-dfCNP (▼), C-ANF (▲) and dfANGII (○). Each point is the mean $\pm$ S.E. of three experiments using the three membrane fractions described in the Fig. 2 legend.

experiment was 10^{-10} M, ligand bound exclusively to the high-affinity site, as demonstrated by the saturation binding experiment.

Affinity labelling with 3×10^{-10} M ^{125}I -[Tyr⁰]-dfCNP revealed that the receptor proteins had molecular masses of approximately 90, 170 and 340 kDa in both

non-reducing and reducing conditions (Fig. 6). Binding to the 340 and 170 kDa proteins was displaced almost completely by 10^{-6} M dfCNP, but some signal remained with the 90 kDa protein. C-ANF also displaced dfCNP binding similarly. After reduction with 2-mercaptoethanol, the signal became much weaker for

Figure 6 Affinity labelling of dogfish CNP receptors using 3×10^{-10} M ^{125}I -[Tyr⁰]-dfCNP. The CNP receptors were labelled in the absence (lanes 1 and 4) or presence of $1 \mu\text{M}$ unlabelled dfCNP (lanes 2 and 5) or C-ANF (lanes 3 and 6). The samples were analysed by SDS/PAGE under non-reducing conditions (lanes 1, 2 and 3) or after reduction with 2-mercaptoethanol (lanes 4, 5 and 6). This experiment was carried out twice using two separate membrane preparations.

the 340 and 170 kDa proteins, but became stronger for the 90 kDa protein.

Discussion

The present study demonstrates two types of CNP receptor, GC-coupled and uncoupled, in the gill cells of the dogfish *Triakis scyllia*. The population of GC-uncoupled receptors was more than 90%, as demonstrated by displacement with C-ANF, a specific ligand for GC-uncoupled receptors (Maack *et al.* 1987, Takashima *et al.* 1995, Kashiwagi *et al.* 1995), and by the weak stimulation of cGMP production by dfCNP. Similar results have recently been reported in another dogfish, *Squalus acanthias* (Donald *et al.* 1997). The function of dense GC-uncoupled receptors in the dogfish gill may be to regulate plasma CNP titre, as has been suggested for the trout (Olson & Duff 1993) and eel (Kaiya & Takei 1996). Most of the GC-uncoupled receptors may be homodimeric NPR-C, as observed in mammalian lung (Shimonaka *et al.* 1987) and teleost gill (Olson & Duff 1993, Donald *et al.* 1994, Mishina & Takei 1997), because most 170 kDa receptors are converted to 90 kDa after reduction with 2-mercaptoethanol. The presence of tetrameric NPR-D was not excluded in the dogfish gill, but its population is minor as shown in eel gill (Mishina & Takei 1997).

The affinity labelling experiment revealed that dogfish NPR-C-like receptors exist not only as homodimers or

homotetramers but also as monomers under non-reducing conditions, and the molecular mass of the monomer (~ 90 kDa) is higher than that of NPR-C or NPR-D (~ 65 kDa; Lowe *et al.* 1990, Takashima *et al.* 1995, Kashiwagi *et al.* 1995). The molecular mass of NPR-C-like receptors in *Squalus acanthias* is also higher (Donald *et al.* 1997). Mutation analysis of NPR-C showed that formation of a dimeric structure is not essential for ligand binding (Itakura *et al.* 1993). In fact, dfCNP binds to the monomeric dogfish NPR-C-like receptors. It is possible that the 90 kDa receptors are a deletion mutant of NPR-B which lacks GC activity identified in the rat (Ohyama *et al.* 1992). However, Donald *et al.* (1997) amplified the PCR product with NPR-C-specific primers. cDNA cloning of the full sequence of the dogfish receptors will give a definite answer to this question.

The saturation isotherm revealed that binding increased above 10^{-8} M dfCNP. The Scatchard analysis also identified two classes of binding site, one with a K_d of 24 pM and B_{max} of 59.9 fmol/mg protein, and the other with a very low affinity and high capacity. Since all binding experiments including displacement experiments and affinity labelling were performed with 10^{-10} M or 3×10^{-10} M ^{125}I -dfCNP, the labelled ligand did not bind to these low-affinity binding sites. Therefore the binding characteristics obtained in this study apply only to the high-affinity site, which satisfies criteria for identification of receptors, i.e. specificity, saturability and high affinity. These characteristics are those of NPR-C-like receptors, and it is possible that a small number of GC-coupled receptors present in the dogfish gill may have similar affinity or may be too minor to be detected by Scatchard analysis.

The K_d value, which represents the ligand concentration at which half of the ligand occupies the receptors, was 24 pM as shown in this study, while plasma CNP concentration in the dogfish was measured as 1.97 nM (Suzuki *et al.* 1994). The HPLC analysis showed that most circulating CNP in the dogfish plasma was prohormone. It is not known whether dogfish proCNP has an affinity similar to that of dfCNP-22 for the dogfish receptors, but, if so, all the receptors are occupied by the circulating proCNP. Therefore it is likely that proCNP is enzymically converted to mature forms in the circulation and generates biological responses only when proCNP titre increases above a certain level. In this respect, it seems reasonable to speculate that the CNP-binding protein with low affinity detected in the saturation experiment is a membrane-bound proCNP-processing enzyme, which cleaves proCNP when its concentration increases above about 10 nM (10^{-8} M).

It is of interest to note that modifications of the dfCNP molecule, i.e. addition of tyrosine residues at the N-terminus, oxidation of Met¹⁷, introduction of iodine into Tyr⁰, did not change the affinity of dfCNP for its receptors, nor decrease its ability to stimulate cGMP

accumulation. These results show that the N-terminus and the methionine residue in the intramolecular ring of dfCNP are not important for expression of biological activity. This is surprising because oxidation of Met¹³ in the intramolecular ring of rat ANP abolishes its biological activity (Watanabe *et al.* 1988). The low ligand selectivity of dogfish receptors may be explained in part by the presence of only CNP in this species.

In addition to the gill, significant CNP binding and stimulation of cGMP production were noted in the intestine, interrenal gland and rectal gland, indicating biological actions in these tissues. Significant CNP binding was also noted in the liver, but the binding may be due to NPR-C because of the lack of cGMP production in these tissues. CNP stimulated cGMP production significantly in the brain, although CNP-binding capacity was low. Since ANP inhibits drinking and neurohypophyseal hormone secretion (Brenner *et al.* 1990), CNP may also act on the brain to modulate these processes.

Since the plasma osmolality of marine teleost fish is almost one-third of that of seawater, these fish drink copiously to compensate for water lost osmotically through the body surfaces (Evans 1993). Thus intestinal absorption of ingested water is essential for teleost osmoregulation. However, the plasma osmolality of elasmobranchs is slightly hyperosmotic compared with seawater because of the accumulation of urea and trimethylamine oxide in the plasma. Therefore the contribution of the intestine to elasmobranch osmoregulation may not be so large. ANP potently inhibits intestinal ion and water transport in mammals and teleost fish (O'Grady *et al.* 1985, Matsushita *et al.* 1991, Ando *et al.* 1992, Loretz 1996). The presence of dense GC-coupled CNP receptors in the dogfish intestine, suggested in this study, is intriguing.

Dogfish interrenal gland secretes 1 α -hydroxycorticosterone (1 α -OHB) as a principal mineralocorticosteroid. The secretion of 1 α -OHB is stimulated by ANGII in *Scyliorhinus canicula* (Hazon & Henderson 1985), and dense ANGII receptors were identified in *Triakis* interrenal gland (Tierney *et al.* 1997). ANGII has been shown to stimulate mineralocorticoid secretion in species from teleost fish to mammals (see Kobayashi & Takei 1996), and ANP is known to modify its secretion in these vertebrate species (Racz *et al.* 1985, Lihrmann *et al.* 1988, Arnold-Reed & Balment 1991). Thus it is likely that CNP modulates 1 α -OHB secretion in the dogfish.

It has been reported that CNP promotes rectal gland secretion in the spiny dogfish, *Squalus acanthias* (Solomon *et al.* 1992). In this species, the action of CNP seems to be mediated by vasoactive intestinal peptide (Silva *et al.* 1996). The presence of GC-coupled receptors found in the present study also suggests CNP action on the *Triakis* rectal gland. In the rectal gland of spiny dogfish, two types of receptor have been identified, GC-coupled and uncoupled receptors, using porcine CNP, *Squalus* CNP, rat ANP, porcine BNP and C-ANF (Gunning *et al.* 1993).

The competitive binding assay revealed that half of the receptors were GC-coupled. The detection of GC-coupled and uncoupled receptors in *Triakis* rectal gland fits well with this result.

The substantial osmotic influx of water across the gills of elasmobranchs because of the slightly hypertonic body fluid is balanced by copious glomerular filtration, which approaches that of freshwater teleosts (Evans 1993). Although CNP binding was found to be low in this study, CNP-stimulated cGMP production was significant in the kidney. The relatively low receptor population may be due to the topical localisation of receptors in the glomerulus relative to the large dogfish kidney. Dense ANP receptors have been identified in the glomeruli of several vertebrate species by autoradiography (Bianchi *et al.* 1985, Kloas & Hanke 1992, Sakaguchi *et al.* 1996).

It is believed that CNP is the sole natriuretic peptide in elasmobranchs, the most primitive vertebrate species in which natriuretic peptides have been identified. This leads to the assumption that the ancestral form of natriuretic receptors is NPR-B because its specific ligand is CNP. However, the present study also demonstrated the presence of GC-uncoupled receptors in the dogfish gill. It is not known whether this type is NPR-C or a deletion mutant of GC-coupled receptors. Clarification of the identity of the ancestral gene of NPRs awaits cDNA cloning of the elasmobranch CNP receptor.

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