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Distribution and Characterization of Natriuretic Peptide Receptors in the Gills of the Spiny Dogfish, *Squalus acanthias*

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The distribution and nature of natriuretic peptide receptors (NPR) in the gills of dogfish, *Squalus acanthias*, were examined by tissue section autoradiography, competition analysis, protein electrophoresis, guanylate cyclase (GC) assays, and molecular cloning. Specific NP binding occurred on the gill filaments, but not on the interbranchial septum or gill arch. The binding was densest on the efferent edge of the gills. Higher resolution light-microscopic examination of emulsion-coated sections showed that specific binding occurred mainly on the secondary lamellae and filament body and not on the arterial circulation. At least two types of NPR were revealed. One is linked to GC since NP binding stimulates the production of cGMP. The GC receptor may be similar to the NPR-B mammalian receptor since only pCNP stimulated cGMP production. The second receptor is not linked to GC and binds the specific ligand C-ANF [rat des(Gln^{18}, Ser^{19}, Gly^{20}, Leu^{21}, Gly^{22})]. The sequence of a cDNA generated using primers based on conserved regions of vertebrate NPR-C had considerable homology with mammalian and eel NPR-C and eel NPR-D. The presence of GC-linked NPR and NPR-C/NPR-D suggests that the gills are an important target organ for NP action. © 1997 Academic Press

The heart of mammals contains two natriuretic peptides (NPs), namely atrial natriuretic peptide (ANP; Flynn *et al.,* 1983) and brain natriuretic peptide (BNP; Sudoh *et al.,* 1988). The third peptide of the family, C-type natriuretic peptide (CNP; Sudoh *et al.,* 1990), was initially thought to be a neuropeptide of the central nervous system, but recent studies have shown that it is expressed in a range of nonneural tissues of the periphery such as the rat heart (Vollmar *et al.,* 1993) and the endothelium (Inagami *et al.,* 1995). In contrast to the situation in mammals, CNP is the major cardiac NP in two species of elasmobranchs, *Squalus acanthias* (Schofield *et al.,* 1991) and *Triakis scyllia* (Suzuki *et al.,* 1991, 1992), and proCNP circulates in the plasma of *T. scyllia* (Suzuki *et al.,* 1994).

There are three receptors for NPs which are variously expressed in a large range of mammalian tissues (Ruskoaho, 1992). Two NP receptors (NPR) possess an intracellular guanylate cyclase (GC) domain, and binding of NPs stimulates the production of cGMP. However, the receptors have selective affinity for the different NPs: NPR-A is primarily activated by ANP and BNP whereas NPR-B is primarily activated by CNP. The third receptor, NPR-C, is a unique receptor that binds all NPs with high affinity but does not have an intracellular GC domain. A unique feature of the NPR-C is the ability to bind C-ANF, a truncated, ring-deleted peptide that does not bind to the GC receptors. This characteristic has been an important tool for describing NPR-C in a range of vertebrate tissues. It is generally considered that the NPR-C

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functions as a mechanism for clearing NPs from the circulation and tissues by internalization, since the receptor is distributed extensively in the vascular endothelium and most tissues in which NPR-A or NPR-B occurs. However, there is a growing database which shows that the NPR-C is coupled to the adenylyl cyclase and IP_3 signal transduction pathways in different tissues (Anand-Srivastava and Trachte, 1993). Recently, three types of NPR have been cloned from the Japanese eel, *Anguilla japonica*, an NPR-B (Katafuchi *et al.*, 1994), an NPR-C (Takashima *et al.*, 1994), and a new NPR which is similar to NPR-C and has been termed NPR-D (Kashiwagi *et al.*, 1995). Similarly to NPR-C, NPR-D binds C-ANF and does not show GC activity.

In recent years, the biological role of NPs in the physiology of fish has received much attention and NPs have been shown to affect vascular smooth muscle and gill epithelial function in a variety of species (Evans and Takei, 1992). In elasmobranchs, high-affinity CNP receptors mediate vasodilatation in the branchial arteries of *Scyliorhinus canicula* (Bjenning *et al.*, 1992) and the ventral aorta of *S. acanthias* (Evans *et al.*, 1993) and potently stimulate chloride efflux from the rectal gland of *S. acanthias* (Solomon *et al.*, 1992). Gunning *et al.* (1993) have characterized the NPR in the rectal gland of *S. acanthias* and found that the high-affinity receptor that bound CNP and stimulated cGMP was similar to NPR-B, but had some distinctive features. In addition to a GC receptor, the rectal gland contained a low-molecular-weight receptor that bound C-ANF and was concluded to be similar to mammalian NPR-C.

The control of gill blood flow and epithelial function in fish is critical in osmoregulatory and cardiovascular homeostasis since all blood pumped from the heart must pass through the gill vasculature. Many studies have shown that both neuronal and hormonal mechanisms regulate the vascular smooth muscle and the epithelium of teleost fish (Nilsson and Holmgren, 1993). In contrast, the gills of elasmobranch fish are not innervated by autonomic nerves (Nilsson and Holmgren, 1993) and, therefore, regulation of gill function must occur by humoral and/or local paracrine mechanisms alone. The presence of CNP in the heart of elasmobranchs suggests that this peptide could be an important regulator of gill function. In the present study we have examined the distribution and nature of NPR in the gills of the spiny dogfish, *S. acanthias*, to determine the role of NPs in gill function. These studies were performed using the following techniques: tissue section autoradiography to determine anatomical locations; *in vitro* receptor binding assays to determine kinetics and specificity of the sites; affinity cross-linking followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) to determine molecular weights of receptor proteins; GC assays to determine if binding sites in the gills are GC-linked receptors; and molecular analysis of putative NPR-C. In this study, iodinated porcine CNP (pCNP) was used as a ligand since it has been shown that pCNP and *S. acanthias* CNP bind to NPR in the ventral aorta and rectal gland with equal affinity (Evans *et al.*, 1993; Gunning *et al.*, 1993).

MATERIALS AND METHODS

Animals

Spiny dogfish *S. acanthias* (3–5 kg) were netted in Frenchman Bay, Maine, and were maintained in live cars with running sea water (12–15°) at the Mount Desert Island Biological Laboratory, Salisbury Cove, Maine. Animals were sacrificed by pithing, and the required tissues were removed and used immediately or were frozen in liquid N_2 and shipped to the University of Florida.

Autoradiography

For autoradiography, gills were freeze mounted in Tissue Tek (Miles Inc., Elkhart, IN) in a microtome cryostat (Minotome, IEC, Needham Heights, MA), and 20-μm sections were cut and mounted on gelatin-chromium-aluminum-coated slides and dried overnight under vacuum at 4°. The sections were used immediately or were stored in sealed boxes at −20° until used.

Preincubation of the gill sections was performed at room temperature (22–24°) for 15 min in 50 m*M* tris-(hydroxymethyl)aminomethane (Tris) · HCl buffer (pH 7.4), 50 m*M* NaCl, 5 m*M* $MgCl_2$, 0.1% bovine serum albumin (BSA), and 0.05% bacitracin. This was

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followed by incubation for 60 min in the same buffer supplemented with 4 µg/ml of leupeptin, 2 µg/ml chymostatin, 2 µg/ml pepstatin, 10^{-6} *M* phenylmethylsulfonyl fluoride (PMFS), and porcine ^{125}I-Tyr^{0}-CNP (termed ^{125}I-pCNP here). Displacement of specific binding was examined in consecutive sections in the presence of 1 µ*M* porcine CNP (pCNP), 1 µ*M* Tyr^{0}-CNP (the peptide used for iodination), 1 µ*M* rat (3–28) ANP, and 1 µ*M* rat C-ANF, the truncated ANP that binds only to NPR-C of mammals (Maack, 1992). To test the specificity of C-ANF in *S. acanthias*, a set of preliminary pharmacological experiments was performed with vascular smooth muscle of the ventral aorta. In this tissue, C-ANF did not cause vasodilatation and did not act as an antagonist to pCNP-facilitated relaxation by prior occupation of the receptor protein which mediated this response (Evans *et al.*, 1993). Therefore, it is probable that C-ANF can be used as a NPR-C-specific ligand in the shark gill.

After incubation, the slides were washed (2×10 min at 4°) in preincubation buffer, fixed for 20 min in 4% formaldehyde in 0.1 *M* phosphate buffer (pH 7.4, 4°), washed in 0.1 *M* phosphate buffer (pH 7.4, 4°), and then in distilled water (1 min) and dehydrated in alcohol and dried overnight at 60°. The sections were then apposed to Hyperfilm-βmax for 5–21 days at room temperature. The film was developed in Kodak GBX developer for 4 min, washed in water for 2 min, and fixed in Kodak GBX fixer for 5 min.

For higher resolution autoradiography, sections were dipped in nuclear track emulsion (Kodak NTB.2) at 43°. After drying the sections were stored at 4° for 7–14 days and then developed in Kodak D 19 for 3 min, washed in water, and fixed in Kodak Rapid Fixer diluted 1:1 for 7 min. Subsequently they were stained in 1% toluidine blue. Sections were viewed under an Olympus BH-2 microscope, and photomicrographs were taken with a Wild Leitz MPS 46 Photoautomat camera using Kodak T-max 100 black and white film. Specific binding was determined by comparing the silver grain densities of sections incubated with ^{125}I-pCNP alone to those of sections incubated with ^{125}I-pCNP and 1 µ*M* cold ligand.

In vitro NP Binding Assays

The gill arch was dissected off and the gill filaments and interbranchial septum were placed in a 50-ml centrifuge tube in 10 vol of 50 m*M* Tris and 1 m*M* $NaHCO_3$ (pH 7.4) and homogenized with a Tissue-Tearor (Biospec, Bartlesville, OK) at setting 7 for 1 min. The homogenate was diluted 1:1 with 50 m*M* Tris, 1 m*M* EDTA, and 1 m*M* $MgCl_2$ (pH 7.4) and centrifuged at 800*g* for 10 min at 4°. The supernatant was collected and centrifuged at 30,000*g* for 20 min. The pellet was washed with 50 m*M* Tris (pH 7.4) and 250 m*M* sucrose and resuspended in the same buffer and the protein concentration was determined with a BCA protein assay kit, calibrated against BSA standards. Membranes were stored at −70° until use.

For competition binding assays, 25–50 µg protein of the gill membrane fraction was incubated in the same buffer used for the sections and 20–35 p*M* ^{125}I-pCNP. Competition for the iodinated ligands was performed using the unlabeled peptides pCNP and C-ANF in increasing concentrations (10^{-12}–10^{-6}). The incubation volume was either 0.125 or 0.25 ml. Binding reactions were performed for 4 hr at 23°; preliminary experiments showed that maximal binding was achieved after 2 hr. Binding was terminated by dilution with 2 ml of ice-cold 50 m*M* Tris · HCl (pH 7.4) and bound ligand was separated from free ligand by filtration through 1% polyethylenimine-treated Whatman GF/C filters. Filters were washed with 5 ml of ice-cold 50 m*M* Tris · HCl (pH 7.4), and the radioactivity on the filter was determined in a Beckman gamma counter with 78% efficiency.

Affinity Cross-Linking and Electrophoresis of Gill Membranes

Membranes were isolated as previously described and incubated in binding assay buffer with ^{125}I-CNP alone or in combination with 1 µ*M* pCNP for 2 hr. Affinity cross-linking was performed according to Martin *et al.* (1989). Following incubation, the covalent cross-linking agent disuccimidyl suberate in dimethylsulfoxide was added to a final concentration of 1 m*M* and the incubation continued for 20 min at 4°. The cross-linking reaction was stopped by addition of an equal volume of quench buffer containing 400 m*M* EDTA and 1 *M* Tris · HCl (pH 6.8). Membranes were centrifuged in an Eppendorf centrifuge at 13,000*g* for 20 min to separate unbound hormone, and the resulting pellet was suspended in 100 µl of sample buffer for SDS-PAGE, containing 62.5 m*M* Tris base, 2% SDS, 5%

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glycerol, 0.01% bromophenol blue, 2% β-mercaptoethanol, pH 6.8, and then boiled for 3 min. The solubilized membranes were again centrifuged at 13,000*g* to remove particulate matter, and electrophoresis was performed on a 7.5% unidimensional polyacrylamide slab gel using a mini-gel apparatus and stained with Coomassie brilliant blue (Bio-Rad, Richmond, CA). The gels were dried and apposed to Hyperfilm MP for 1 week. Identification of the molecular weights of the labeled bands was done by use of reference standards (30,000–200,000) on the Coomassie blue-stained gel. A linear relationship between the relative mobility (R_f) and the log relative molecular mass of the standards was constructed and the unknown values were calculated.

Guanylate Cyclase (GC) Assays

Gill membranes were isolated and the protein concentration was determined. Membranes were kept on ice until needed. For determination of GC activity, 50 µg of gill protein was added to 50 m*M* Tris · HCl, 2 m*M* isobutyl methylxanthine (IBMX), 10 m*M* creatinine phosphate, 100 U/ml creatine phosphokinase, 4 m*M* $MnCl_2$, 1 m*M* GTP, and increasing concentrations of pCNP in a final volume of 100 µl. The basal rate of cGMP generation was determined in tubes without ligand. The incubations were performed for 15 min at 24° and were terminated by the addition of 4 m*M* EDTA. The tubes were boiled for 3 min and centrifuged at 2300*g* for 15 min. The supernatant was collected and frozen until the cGMP content was determined by radioimmunoassay.

Molecular Cloning

Gill total RNA was isolated by the acid-phenol technique (Chomczynski and Sacchi, 1987), and mRNA was selected using magnetic separation (Polyattract mRNA Isolation System, Promega). Double-stranded cDNA was synthesized using the Promega Riboclone system. Primers based on conserved regions identified from aligned bovine, human, and eel NPR-C cDNA sequences were used for polymerase chain reaction (PCR), and computer analysis predicted amplification of a 470-base pair (bp) product. PCR was carried out using the following 100-µl reaction mixture: 1× PCR buffer, dNTPs, 0.8 µ*M* forward and reverse primers, 2.5 U AmpliTaq, 10 m*M* $MgCl_2$, and 50 ng gill cDNA. An initial cycle of 3 min at 95°, 2 min at 45°, and 2 min at 72° was followed by 34 cycles of 1 min at 95°, 2 min at 45°, and 2 min at 72°. Following the last cycle, the PCR products were subject to blunt-ending by the addition of 2.5 U *Pfu* polymerase (Stratagene) and incubation at 72° for 15 min. Ninety microliters of the PCR was run on a 1% agarose gel and an approximately 470-bp product was excised and the cDNA recovered using glass milk (Bresaclean, Bresatec). The cDNA was cloned into a pCRscript vector (Stratagene) and sequenced on an Applied Biosystems automated sequencer (Westmead Hospital, Sydney). The sequence was analyzed using BLAST (National Centre for Biotechnology Information).

Data Analysis

Binding data were analyzed using the EBDA and LIGAND computer programs (McPherson, 1985). Analysis of variance was performed using Statview SE on a Macintosh computer.

Materials

Hyperfilm-βmax, Hyperfilm MP, and the cGMP [^{3}H] assay system were purchased from Amersham (Arlington Heights, IL). Porcine ^{125}I-Tyr^0-CNP (1400–1600 Ci/mmol) and porcine Tyr^0-CNP were obtained from Peninsula (Belmont, CA). Rat ANP (3–28), porcine CNP, and C-ANF [rat des(Gln^{18}, Ser^{19}, Gly^{20}, Leu^{21}, Gly^{22})ANP-(4-23)-NH_2] were obtained from Bachem (Torrance, CA). The BCA protein assay kit was purchased from Pierce (Rockford, IL). GTP and IBMX were purchased from Sigma. All PCR reagents were purchased from Perkin–Elmer. All other chemicals were reagent grade.

RESULTS

Autoradiographic Studies

Autoradiograms of gill sections showed high-density ^{125}I-pCNP binding on the gill filaments (Figs. 1 and 2), but not on the interbranchial septum or gill arch. Binding was distributed over the filaments along

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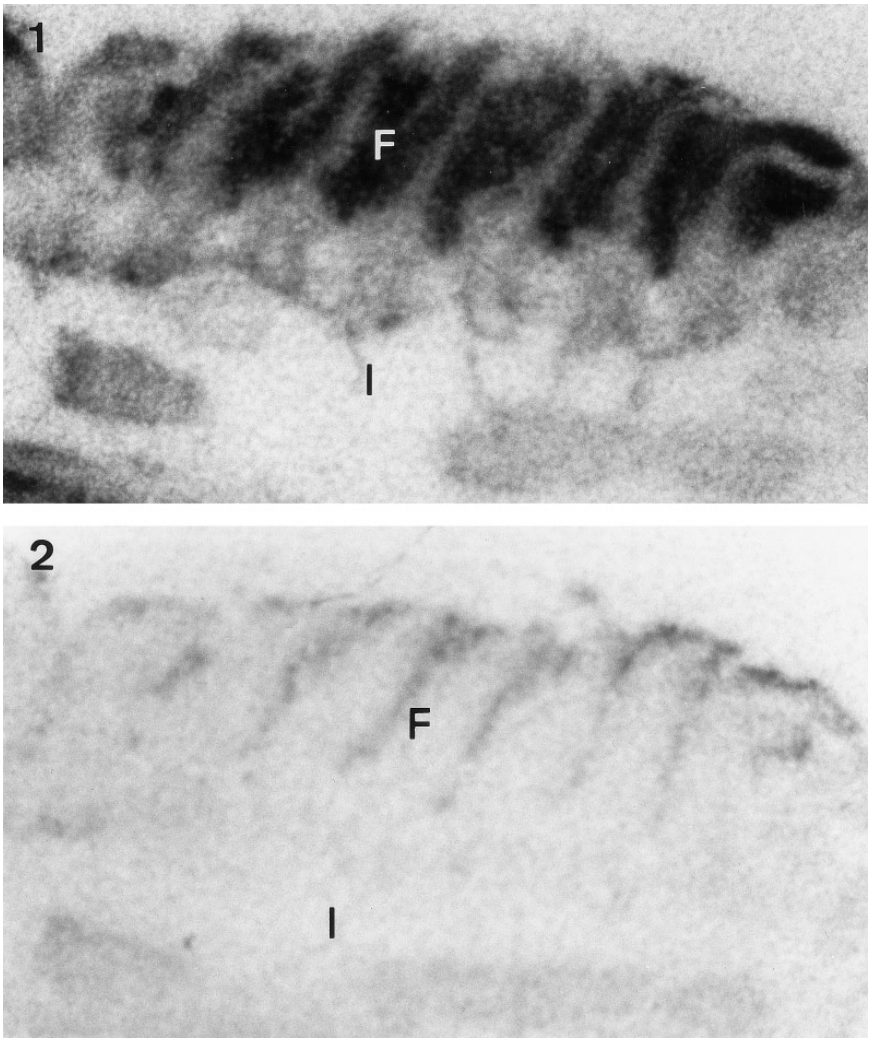

FIGS. 1 and 2. Autoradiograms of shark gill exposed to X-ray film showing the distribution of ^{125}I-pCNP binding sites. ×15.
FIG. 1. A transverse section of the holobranch showing total binding of ^{125}I-pCNP.
FIG. 2. An adjacent section showing the nonspecific binding when the section is incubated with ^{125}I-pCNP and 1 μ*M* cold pCNP. A comparison of the sections shows that dense specific binding is present on the gill filaments. F, gill filaments; I, interbranchial septum; ×15.

the entire length of each hemibranch and showed a gradation in density such that the binding was densest on the efferent (relative to blood flow) edge of the gills. The specific binding of both radioligands was displaced by incubation of sections with 1 μ*M* pCNP (Fig. 2), ^{0}Tyr-CNP, rANP, or C-ANF. Higher resolution light-microscopic examination of emulsion-coated sections showed that specific binding of ^{125}I-pCNP occurred mainly on the secondary lamellae and filament body, with little, if any, specific binding being found on the afferent filamental artery, corpus cavernosum, afferent lamellar arterioles, efferent lamellar arterioles, efferent filamental artery, and the branchial arteries.

Characterization of NP-Binding Sites

The binding sites in the gills were analyzed by competitive radioligand binding assays, electrophoresis of membrane proteins affinity cross-linked to radioligand, and determination of GC activity.

Competition studies. The ability of the peptides pCNP and C-ANF to compete for the ^{125}I-pCNP bind-

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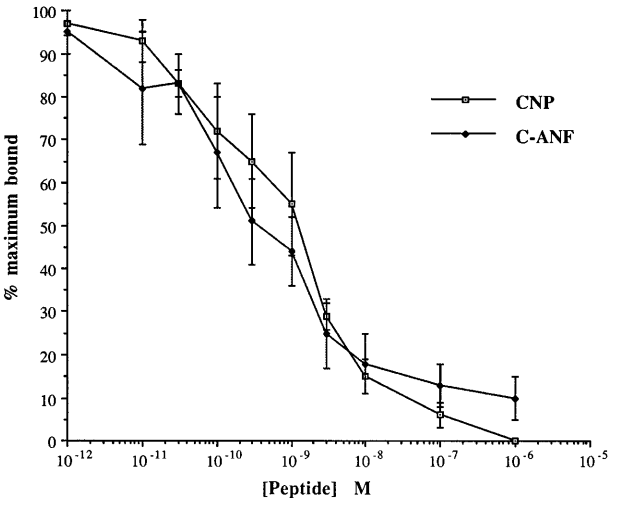

FIG. 3. Competition binding of pCNP and C-ANF to 50 µg of shark gill membranes incubated to equilibrium with 35 p*M* ^{125}I-pCNP in the presence of increasing concentrations of cold peptide. Each point is the mean ± SE of five experiments.

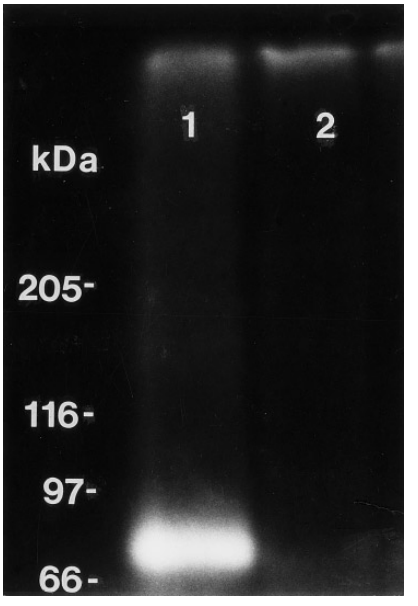

FIG. 4. Autoradiogram of SDS–PAGE of 100 µg shark gill membranes incubated with ^{125}I-pCNP. The gel was stained with Coomassie blue, dried, and exposed to X-ray film for 1 week. The X-ray film was exposed directly to the photographic paper. A single band at approximately 80 kDa (lane 1) was displaced by 1 µ*M* pCNP (lane 2).

ing sites was determined with competitive radioreceptor assays. The relative IC_{50} values for percentage displacement were as follows: 1 n*M* for pCNP and 0.4 n*M* for C-ANF. pCNP displaced all the binding; however, about 10% of residual binding was observed at 1 µ*M* C-ANF (Fig. 3). The mean dissociation constant (K_d) and receptor density (B_{max}) were 1.98 ± 0.56 n*M* and 987.5 ± 187.1 fmol/mg protein, respectively.

Electrophoresis. Affinity cross-linking of ^{125}I-pCNP to gill membranes and subsequent SDS–PAGE showed a single band at approximately 80 kDa; this binding was displaced by 1 µ*M* pCNP (Fig. 4).

Stimulation of cGMP production by pCNP. pCNP stimulated cGMP in a dose-dependent manner. Significant increases ($P < 0.05$) in cGMP production above basal level were observed at 0.1 and 1 µ*M* pCNP (Fig. 5). rANP and C-ANF did not stimulate cGMP production (Fig. 5).

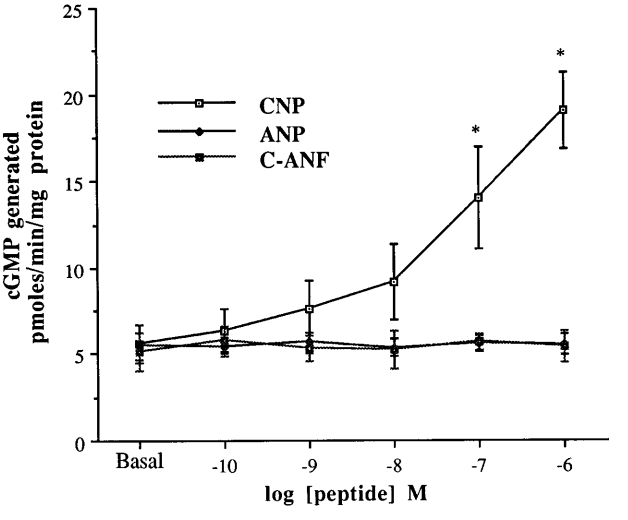

FIG. 5. Effects of pCNP, rANP, and C-ANF on stimulation of cGMP production. Significant increases ($P < 0.05$) in cGMP production above basal level were observed at 0.1 and 1 µ*M* pCNP (shown by asterisks). Each point is the mean ± SE of five experiments for pCNP and three experiments for rANP and C-ANF.

Molecular Cloning

PCR amplification using NPR-C-specific primers produced a 468-bp product as predicted; the cDNA sequence and deduced amino acid sequence are shown in Fig. 6. Computer analysis of the nucleotide sequence

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GAGAGGAATTGTTACTTCACTGTGGAGGGGGTCCATATGGCCTTCGTTGGTCAAGATTAT 60
E  R  N  C  Y  F  T  V  E  G  V  H  M  A  F  V  G  Q  D  Y

TCCATGCATTCAATTAGTATCTATGATGAGGAGGACCAGGTCGACCACATCGTGCAGGAG 120
S  M  H  S  I  S  I  Y  D  E  E  D  Q  V  D  H  I  V  Q  E

ATCCAGGACAATGCCAGGATTGTGATTATGTGTGCAAGTAGTCACACCATCCGGAAAATC 180
I  Q  D  N  A  R  I  V  I  M  C  A  S  S  H  T  I  R  K  I

ATGCTTGCAGCACACCGCCAGGGTATGACCAATGGAGACTATGTATTCTTCAACATCGAA 240
M  L  A  A  H  R  Q  G  M  T  N  G  D  Y  V  F  F  N  I  E

CTCTTCAACAGCAGCCTATATGGAAATGGCTCATGGAAACAAGGAGATAAATTTGATCCT 300
L  F  N  S  S  L  Y  G  N  G  S  W  K  Q  G  D  K  F  D  P

GAAGCCAAACAAGCATACCAGTCNCTTCAGACCGTGACTCTACTGAGGACAGCAAAGCCA 360
E  A  K  Q  A  Y  Q  S  L  Q  T  V  T  L  L  R  T  A  K  P

GAGTTTCACAAGTTTGCCGCTGATATGAGAAATAAATCACGTCATCAGTATGATTTGACC 420
E  F  H  K  F  A  A  D  M  R  N  K  S  R  H  Q  Y  D  L  T

TCTGAAGGTGACAATGTGAACATGTTCGTCGAAGGCTTTCACGACGCG             468
S  E  G  D  N  V  N  M  F  V  E  G  F  H  D  A
```

FIG. 6. Nucleotide (top line) and deduced single-letter amino acid sequence of the 468-bp PCR product generated using NPR-C specific primers; the amino acid sequences used for primer design are underlined. The nucleotides are numbered on the right side.

showed that it was homologous to bovine NPR-C (60%), eel NPR-C (49%), and eel NPR-D (51%). Alignment of the six-frame translation of the nucleotide sequence against a protein database generated an amino sequence that was homologous to bovine NPR-C (65%), eel NPR-C (51%), and eel NPR-D (53%). The putative shark NPR-C amino acid sequence contained two cysteine (C) residues and an N-glycosylation site which are conserved in bovine and eel NPR-C and eel NPR-D (Fig. 7).

```
sNPR-C/D     ERNCYFTVEGVHMAFVGQDYSMHSISI-YDEEDQVD--HIVQEIQDNARI
bNPR-C 206   ERNCFFTLEGVHEVF--QEEGLHTSAYNFDETKDLDLEDIVRHIQASERV
eNPR-C 175   ERNCYFTIEGVHSSLHVEGYKVDSVVIH--KDHRVETDEIIKDIYKTEAV
eNPR-D 176   QRNCYFTLEGVHNILKTENVHIDALNIH-SKDNKVDSDEIIKLIYDSE-V
              *** ** ****                          *   *

sNPR-C/D     VIMCASSHTIRKIMLAAHRQGMTNGDYVFFNIELFNSSLYGNGSWKQGDK
bNPR-C 254   VIMCASSDTIRGIMLAAHRHGMTSGDYAFFNIELFNSSFYGDGSWKRGDK
eNPR-C 223   VVMCAGGDTVRDIMLAAHRRRLTSGGYIFFNIELFNSSSYGDGSWRRGDK
eNPR-D 224   IIMCAGADIIRDIMLAAHRRRLTNGSYIFFNIELFNSSSYGNGSWKRGDK
               ***     * *******   * * * ********** ** ***  ***

sNPR-C/D     FDPEAKQAYQSLQTVTLLRTAKPEFHKFAADMRNKSRHQYDLTSEGDNVN
bNPR-C 304   HDFEAKQAYSSLQTITLLRTAKPEFEKFSMEVKSSVEKQ-GL-SEEDYVN
eNPR-C 272   YDAEAKLAYSALNVVTLMRTAKAEFETFTTEVKKSIQRAGIGP-DSANVN
eNPR-D 274   FDMDAKQAYATLNTVTLLRTVKPEFEDFSMEVKKSLQKAGIRHCDSDNIN
              *  ** **  *   ** ** * **  *                    *

sNPR-C/D     MFVEGFHDA
bNPR-C 352   MFVEGFHDA
eNPR-C 321   MFMEGFHDA
eNPR-D 324   VFVEGFHDA
              * ******
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FIG. 7. Alignment of the putative shark NPR-C/D amino acid sequence with bovine NPR-C (bNPR-C), eel NPR-C (eNPR-C), and eel NPR-D (eNPR-D), using single-letter codes. The numbers on the left side correspond to the amino acid sequence of the complete mRNA of the bovine and eel receptors. Identical amino acids are shown by asterisks. The conserved cysteine (C) residues and N-glycosylation sites are shown in boldface and underlined respectively.

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DISCUSSION

The gills are the major site of osmotic water influx in elasmobranchs because the plasma is hyperosmotic to seawater and the gill epithelium is very permeable to water (Evans, 1993). The net flux will be determined by a combination of the permeability of the gill epithelium and the blood flow profile of the gill which determines the amount of plasma available for molecular exchanges at the epithelium. The gills of elasmobranchs lack any autonomic neural elements (Nilsson and Holmgren, 1993); therefore, vascular and epithelial regulation must occur through endocrine, paracrine, or autocrine mechanisms. For example, the presence of a circulating catecholamine system in elasmobranchs is well established as are the effects of amines on the branchial circulation (Nilsson and Holmgren, 1993). It follows that CNP released from the heart (Schofield *et al.,* 1991; Suzuki *et al.,* 1991, 1992), or possibly the vascular endothelium (Inagami *et al.,* 1995), could have important actions on vascular and epithelial effector tissues that affect branchial exchanges and contribute to blood volume homeostasis.

Recently, the presence of CNP in the plasma has been established in *T. scyllia* (Suzuki *et al.,* 1994) and it is assumed that the source of this peptide is the heart and that CNP circulates in the plasma of other species of elasmobranchs. Several studies have shown that CNP has quite potent effects in organs important in salt and water balance. For example, CNP stimulates chloride secretion in the rectal gland epithelium of *S. acanthias,* indicating an epithelial action of the hormone in this tissue (Solomon *et al.,* 1992). In addition, the vascular smooth muscle of the ventral aorta of *S. acanthias* (Evans *et al.,* 1993) and the afferent branchial artery of *S. canicula* (Bjenning *et al.,* 1992) is relaxed by low concentrations of CNP without prior preconstriction, suggesting that CNP is involved in the control of gill hemodynamics. Our study provides evidence that two types of CNP receptors are present in the gills of *S. acanthias,* which could be important in regulation of the gill vasculature and / or the branchial epithelium.

The ^{125}I-pCNP binding in the gills, as shown by autoradiography, was displaced by 1 μ*M* unlabeled pCNP, Tyr-CNP, rANP, and the NPR-C-specific ligand, C-ANF. This observation suggests that an NPR-C-like receptor is the sole NPR in the branchial tissues. However, subsequent experiments provided evidence that two types of NPR are present in the gills: one receptor linked to GC and a receptor which binds C-ANF and is homologous to vertebrate NPR-C and eel NPR-D (termed NPR-C / NPR-D hereafter).

The evidence for a GC receptor is primarily based on the experiments in which CNP stimulated the production of cGMP in gill membranes in a dose-dependent manner. In parallel assays, both rANP and C-ANF failed to stimulate cGMP, indicating the presence of a receptor whose intracellular GC domain is activated only by CNP binding. These data are very similar to the findings with rectal gland tissues which showed that only CNP stimulated the production of cGMP (Gunning *et al.,* 1993). Further evidence for a small population of GC receptors was obtained from the competition binding assays in which C-ANF did not completely displace the specific binding compared to the displacement by CNP (see Fig. 5); the specific binding not displaced by C-ANF may represent GC receptors. SDS–PAGE of gill membranes affinity cross-linked to ^{125}I-pCNP failed to show a protein of a molecular weight in the range of GC receptors (130–150 kDa), which may be due to the small number of GC receptors. It seems, therefore, that a GC receptor specific for CNP is present in dogfish tissues. However, until cloning of a GC receptor from *S. acanthias* is performed it will not be possible to conclude whether those receptors are NPR-A or NPR-B.

The anatomical location of the gill GC receptors, represented by the small proportion of binding sites that do not bind C-ANF, could not be determined with autoradiography. The autoradiographical binding data would suggest that the GC receptors reside on the filament epithelium and secondary lamellae. However, it is also possible that GC receptors occur on vascular endothelium or vascular smooth muscle cells but were not discerned with the autoradiography technique used in this study. Interestingly, a number of studies using mammalian tissues have demonstrated cGMP activity in tissues, although no binding sites for GC receptors were visualized (Leitman and Murad, 1990). In addition, the afferent branchial artery of *S. canicula* is relaxed by low concentrations of CNP (Bjenning *et al.,* 1992), which suggests that GC receptors may reside on similar vessels of *S. acanthias.*

The second type of NPR in the shark gill is an

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NPR-C/NPR-D. The partial cDNA generated with primers designed against conserved regions of known NPR-C sequences showed considerable homology with bovine and eel NPR-C and eel NPR-D. The shark cDNA sequence contained two Cys residues and an N-glycosylation site which are conserved in bovine and eel NPR-C and eel NPR-D (see Kashiwagi *et al.*, 1995). Further evidence for the presence of an NPR-C/NPR-D in shark gills was obtained with the use of C-ANF, a truncated NP which binds to specifically to NPR-C (Maack, 1992) and eel NPR-D (Kashiwagi *et al.*, 1995). In both autoradiography and competition displacement experiments, C-ANF displaced the majority of binding. In addition, affinity cross-linking of ^{125}I-pCNP to gill membranes followed by SDS–PAGE under reducing conditions revealed a band of approximately 80 kDa, which parallels that of reduced mammalian and eel NPR-C and eel NPR-D. There is, therefore, strong evidence for the presence of an NPR-C/NPR-D in shark gill tissues.

Emerging evidence shows that C-ANF-binding sites are present in a range of fish tissues; in particular, they are abundant in the gills (Donald *et al.*, 1994; Takashima *et al.*, 1994; Toop *et al.*, 1995). In the rectal gland of *S. acanthias*, C-ANF was able to displace about 50% of CNP binding, indicating the presence of a NPR-C-like receptor in that tissue (Gunning *et al.*, 1993). NPR-C has been cloned from the gill of the Japanese eel, *A. japonica* (Takashima *et al.*, 1994), and NP ligand binding and electrophoretic studies strongly supported the presence of NPR-C-like receptors in the gills of toadfish, *Opsanus beta* (Donald *et al.*, 1994). The consistent presence of NPR-C in the gills of fish raises interesting questions about their function. Mammalian NPR-C binds and internalizes NPs for metabolic degradation (Anand-Srivastava and Trachte, 1993); however, such a mechanism has not yet been demonstrated in fish. Whole animal studies using rainbow trout, *Oncorhynchus mykiss*, showed that the gills had an enormous capacity to bind circulating ANP and that this effect was reversed by prior treatment with C-ANF, indicating that NPR-C was binding the ANP (Olson and Duff, 1993). In fish, NPs released from the heart will pass through the gills prior to reaching more distal tissues, and, therefore, the regulated expression of NPR-C in the gills could possibly influence the downstream plasma concentration of the hormone. Interestingly, distension of the right atrium of mammals is the major stimulus for ANP release into blood flowing to the lungs, in which there is a preponderance of NPR-C on the vascular endothelium (Anand-Srivastava and Trachte, 1993). It seems, therefore, that in both fish and mammals significant changes in the plasma concentration of circulating NPs could occur prior to reaching target tissues in the periphery. In fish, such a mechanism may be present to compensate for excessive release of NPs due to large variations in the inotropic state of the heart associated with activities such as exercise (see e.g., Cousins and Farrell, 1996).

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