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LOCALISATION AND CHARACTERISTICS OF NATRIURETIC PEPTIDE RECEPTORS IN THE GILLS OF THE ATLANTIC HAGFISH *MYXINE GLUTINOSA* (AGNATHA)

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Summary

Specific binding of iodinated natriuretic peptides ^{125}I-ANP and ^{125}I-CNP was examined in the gill of the Atlantic hagfish *Myxine glutinosa* by tissue section autoradiography, saturation and competition analysis of binding to membrane preparations, affinity cross-linking, followed by SDS–PAGE and guanylate cyclase assays. Autoradiographs showed specific, saturable binding on the respiratory lamellar epithelium. *In vitro* analysis of the binding sites demonstrated that ^{125}I-ANP bound to two receptor sites with the same affinity (K_d=15.4±1.6 pmol l^{-1}; B_{max}=45.9±3.0 fmol mg^{-1} protein). ^{125}I-CNP bound to high- and low-affinity receptor sites; variables for the high-affinity site (K_d=12.9±4.7 pmol l^{-1}; B_{max}=23.4±6.5 fmol mg^{-1} protein) did not differ from those for the ^{125}I-ANP sites. The low-affinity site had an apparent K_d and B_{max} of 380±80 pmol l^{-1} and 120±21 fmol mg^{-1} protein, respectively. All receptors had an apparent molecular mass of approximately 150 kDa, with no indication of a mammalian type NPR-C at a lower apparent molecular mass. 1 nmol l^{-1} unlabelled rANP and 20 and 30 nmol l^{-1} unlabelled pCNP and C-ANF, respectively, competed for 50 % of ^{125}I-ANP sites. 0.1 nmol l^{-1} rANP and pCNP and 8 nmol l^{-1} C-ANF competitively inhibited 50 % of ^{125}I-CNP binding. Both rANP and pCNP stimulated cyclic GMP production, although rANP was a more potent stimulator than was pCNP. C-ANF did not stimulate cyclic GMP production. These data suggest the existence of an ANP guanylate-cyclase-linked receptor similar to the mammalian NPR-A and an ANP/CNP receptor that may be similar to, although not structurally homologous with, the mammalian NPR-C clearance receptor.

Key words: hagfish, *Myxine glutinosa*, Agnatha, natriuretic peptides, natriuretic peptide receptors.

Introduction

Natriuretic peptides (NPs: ANP, BNP and CNP) are a family of peptide hormones found principally in the heart and central nervous system. They reduce blood pressure and hypervolaemia in mammals by decreasing cardiac output, reducing peripheral vascular resistance (partly by relaxation of vascular smooth muscle) and decreasing intravascular volume. In addition, blood volume is reduced directly by the potent diuretic and natriuretic effects of NPs in the kidney and secondarily by the inhibition of the release of aldosterone from the adrenal glands and renin from the juxtaglomerular cells (Brenner *et al.* 1990; Ruskoaho, 1992). The functions of NPs in the central nervous system appear to be the control of water intake and salt preference and the inhibition of vasopressin secretion (Samson, 1990).

The effects of NPs are mediated through two membrane-bound receptor types (NPRs); guanylate-cyclase-coupled receptors (approximate molecular mass 130 kDa) that activate the guanosine 3′,5′-cyclic monophosphate (cyclic GMP) intracellular second-messenger system (Martin *et al.* 1989; Koller and Goeddel, 1992), and the ‘clearance’ receptor (NPR-C, a homodimer of a 65 kDa protein) that is not coupled to guanylate cyclase activity. NPR-C was originally named because it was believed to modulate circulating concentrations of NPs by their removal from the blood (Chinkers and Garbers, 1991; Maack, 1992). Some studies suggest that NPR-C is linked with second-messenger systems other than cyclic-GMP-mediated mechanisms (Levin, 1993). At least two guanylate-cyclase-linked receptors have been identified to date: NPR-A and NPR-B. NPR-A appears preferentially to bind ANP, but will also bind BNP and to a lesser extent to CNP; whereas NPR-B binds CNP preferentially (Koller and Goeddel, 1992). These receptors require an intact cysteine ring for NPs to bind. However, NPR-C can bind a diversity of NPs, including truncated and ring-deleted analogues (Brenner *et al.* 1990).

Since the role of ANP in mammalian salt and water balance was discovered, interest has developed in the possible action

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of NPs in fish osmoregulation (for a review, see Evans, 1990, 1993, 1995; Evans and Takei, 1992). Immunohistochemical studies and radioimmunoassays indicate that all classes of fishes have NP systems (Reinecke *et al.* 1985, 1987; Evans *et al.* 1989; Vallarino *et al.* 1990; Donald and Evans, 1992; Donald *et al.* 1992), and various NPs have been isolated and sequenced from both teleosts and elasmobranchs (Takei *et al.* 1989, 1990, 1991; Price *et al.* 1990; Schofield *et al.* 1991; Suzuki *et al.* 1991, 1992). There is some evidence to suggest that NPs may function as a saltwater hormone in fishes, since plasma NP immunoreactivity is greater in some fishes adapted to higher salinities (Westenfelder *et al.* 1988; Evans *et al.* 1989; Freeman and Bernard, 1990; Smith *et al.* 1991); the eel (*Anguilla japonica*), however, appears to be an exception since ANP plasma concentrations decline in higher salinities (Takei and Balment, 1993).

There have been a few studies on NP binding sites in teleosts and elasmobranchs. ^{125}I-ANP binding sites have been found in the heart of *Conger conger* (Cerra *et al.* 1992), in the kidney, gills and heart of two species of Antarctic fish (Uva *et al.* 1993) and in chondrocytes of gill cartilage and parenchymal cells of the secondary lamellae of the Japanese eel *Anguilla japonica* (Sakaguchi *et al.* 1993). ^{125}I-CNP binding has been observed in the rectal gland of the dogfish shark *Squalus acanthias*, and CNP stimulates cyclic GMP production in this tissue (Gunning *et al.* 1993). ANP has been found to stimulate cyclic GMP production in isolated rainbow trout nephrons (Perrott *et al.* 1993). In a series of studies, Duff and Olson (1992) and Olson and Duff (1993) have identified NPR-C-like receptors in the gills of the rainbow trout. These binding sites are capable of removing 60 % of ^{125}I-ANP from the circulation in a single pass through the gills.

Unlike all other fishes that are obligatory osmoregulators, the hagfish, whose blood is virtually iso-osmotic with sea water, does not have an osmotic problem and behaves physiologically like an osmoconforming marine invertebrate (Hardisty, 1979). However, hagfish do have an NP system: the heart, brain and plasma of *Myxine glutinosa* have NP-like immunoreactivity (Reinecke *et al.* 1987; Evans *et al.* 1989; Donald *et al.* 1992), and NP binding sites have been located in the archinephric ducts and glomeruli of the kidney and in the ventral aorta (Kloas *et al.* 1988). In addition, rat ANP and CNPs, which dilate the ventral aortic vascular smooth muscle of both the toadfish *Opsanus beta* and the dogfish shark *Squalus acanthias*, also dilate that of the hagfish *Myxine glutinosa* (Evans *et al.* 1989, 1993; Price *et al.* 1990; Evans, 1991). Dilation of the ventral aorta could alter the dynamics of gill blood perfusion. Because hagfishes diverged from other vertebrate groups at least 500 million years ago (Forey and Janvier, 1993), they are an interesting group in which to study the evolution of NPs and their receptors. Similarities between the NP system of hagfish and higher vertebrates are presumably indicative of the ancestral vertebrate condition. However, the converse is not necessarily true: hagfish characteristics that are not shared with higher vertebrates may not be primitive, but may be derived characteristics resulting from the separate NP evolution since the separation of myxinoids from the main vertebrate line. The present study examines the presence and character of NP binding sites in the gill of the Atlantic hagfish *Myxine glutinosa* using autoradiography, radioligand binding assays and guanylate cyclase assays.

Materials and methods

Animal maintenance

Hagfish were collected from the Bay of Fundy, supplied by Huntsman Marine Laboratory, St Andrews, NB, Canada, and maintained in running sea water (SW, 12–14 °C) at the Mount Desert Island Marine Laboratory, Maine, or in 10 °C tanks aerated through charcoal/fibre filters at the University of Florida, Gainesville. Animals were allowed to equilibrate for 3 weeks before experimentation. Hagfish were anaesthetised in MS 222 (1:1000, Sigma, St Louis, MO) before dissection.

Autoradiography

After dissection, tissues were freeze-mounted in Tissue Tek (Miles Inc. Elkhart, Indiana) and sectioned in a microtome cryostat (Minotome, IEC, Massachusetts). 18 μm sections were mounted on gelatin-chromium-aluminium-coated slides and dried overnight under vacuum at 4 °C. The sections were stored in sealed boxes at −20 °C until used.

Sections were preincubated for 15 min at room temperature (22–24 °C) in 50 mmol l^{-1} Tris–HCl buffer (pH 7.4), 50 mmol l^{-1} NaCl, 5 mmol l^{-1} $MgCl_2$, 0.1 % bovine serum albumin (BSA) and 0.05 % bacitracin. The sections were then incubated for 90 min in the same buffer supplemented with 4 μg ml^{-1} leupeptin, 2 μg ml^{-1} chymostatin, 2 μg ml^{-1} pepstatin, 1 μmol l^{-1} PMFS (phenylmethylsulphonylfluoride) and rat (3-[^{125}I]iodotyrosol^{28}) atrial natriuretic peptide (74 TBq mmol^{-1}; Amersham, Illinois) or human, porcine, rat (^{125}I-[Tyr^{0}]) C-type natriuretic peptide-22 (55.5 TBq mmol^{-1}; Peninsula Laboratories, California). Nonspecific binding was determined in adjacent sections in the presence of 1 μmol l^{-1} unlabelled rat 3-28 ANP (rANP, Bachem, California) for ^{125}I-ANP-incubated sections and 1 μmol l^{-1} porcine CNP (pCNP; Bachem, California) for ^{125}I-CNP-incubated sections. The ability of 1 μmol l^{-1} pCNP (^{125}I-ANP-labelled sections), 1 μmol l^{-1} rANP (^{125}I-CNP-labelled sections) and 1 μmol l^{-1} rat des[Gln^{18}, Ser^{19}, Gly^{20}, Leu^{21}, Gly^{22}]ANP-(4–23)-NH_2 (C-ANF; Bachem, California) to displace specific radiolabelled NP binding was also determined in adjacent sections. C-ANF is a truncated ANP that binds only to NPR-C in mammals (Maack *et al.* 1987).

Following incubation, the slides were washed (2×10 min at 4 °C) in 50 mmol l^{-1} Tris–HCl buffer, fixed for 20 min in 4 % formaldehyde in 0.1 mol l^{-1} phosphate buffer (pH 7.4, 4 °C), washed in 0.1 mol l^{-1} phosphate buffer (pH 7.4, 4 °C) and then in distilled water (1 min), dehydrated through alcohols and dried overnight at 60 °C. Sections were apposed to Hyperfilm-βmax (Amersham, Illinois) for 5 days at room temperature. The film was processed using Kodak GBX developer (4 min),

rinsed in water (2 min) and fixed with Kodak GBX fixer (5 min).

For examination of binding sites with light microscopy, some sections were dipped in nuclear track emulsion (Kodak NTB.2) at 43 °C. After drying, the sections were stored for 10 days at 4 °C and then developed in Kodak D 19 (3 min), washed in water and fixed in Kodak Rapid Fixer diluted 1:1 (7 min). Subsequently, they were stained in 1 % Toluidine Blue, examined with an Olympus BH-2 microscope and photomicrographs made with a Wild Leitz MPS 46 Photoautomat camera on Kodak T-max 100 black and white film.

Membrane preparation

Gill membranes were prepared from individual hagfish for saturation and competition binding studies, affinity cross-linking followed by SDS–polyacrylamide electrophoresis and for guanylate cyclase assays. The gill pouches were removed from anaesthetised hagfish, placed in a 50 ml centrifuge tube in 5 ml of ice-cold 50 mmol l^{-1} Tris–HCl and 1 mmol l^{-1} $NaHCO_3$ (pH 7.4) and quickly homogenised with a Tissue-Tearor (Biospec, Bartlesville, Oklahoma). The homogenate was diluted with 5 ml of 50 mmol l^{-1} Tris–HCl, 1 mmol l^{-1} EDTA and 1 mmol l^{-1} $MgCl_2$ (pH 7.4) and centrifuged at 800 ***g*** for 10 min at 4 °C. The supernatant was collected and centrifuged at 30 000 ***g*** for 20 min. The pellet was washed with 50 mmol l^{-1} Tris–HCl (pH 7.4) and 250 mmol l^{-1} sucrose and resuspended in 400 μl of the same sucrose buffer. Protein concentration was determined with a BCA protein assay kit (Pierce), calibrated against BSA standards. Membranes were stored at −70 °C until use.

Radioligand binding assays

For the ANP saturation curves, 75 μg of gill membrane protein was incubated in 250 μl of incubation buffer (the same as was used for sections, see above) in the presence of increasing concentrations (5–300 pmol l^{-1}) of ^{125}I-rANP. Binding reactions were performed for 90 min at 23 °C. The reaction was terminated by the addition of 2 ml of ice-cold 50 mmol l^{-1} Tris–HCl (pH 7.4) and the preparation was filtered through 1 % polyethylenimine-treated Whatman GF/C filters. Filters were washed with 5 ml of 50 mmol l^{-1} Tris–HCl (pH 7.4), and the radioactivity was measured in a Beckman gamma counter with 78 % efficiency. For competition binding studies, 75 μg of gill membrane protein was incubated in 250 μl of the incubation buffer and 25 pmol l^{-1} of ^{125}I-rANP or ^{125}I-pCNP with the unlabelled peptides rANP, C-ANF and pCNP present in increasing concentrations (10^{-12} to 10^{-6} mol l^{-1}). Binding reactions were performed as above.

Affinity cross-linking

Hagfish gill membranes were isolated as described above and 100–125 μg of protein was placed in incubation buffer with 0.25 nmol l^{-1} iodinated peptide in the presence or absence of excess unlabelled rANP, pCNP or C-ANF. The final incubation volume was 250 μl. Affinity cross-linking was performed according to Martin *et al.* (1989). Following incubation, the covalent cross-linking agent disuccimidyl suberate (DSS; Pierce) in dimethylsulphoxide was added to a final concentration of 1 mmol l^{-1} and the mixture was shaken gently for 20 min at 23 °C. The cross-linking reaction was stopped by the addition of an equal volume of quench buffer (400 mmol l^{-1} EDTA and 1 mol l^{-1} 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 pellet was resuspended in 30 μl of sample buffer, for SDS–polyacrylamide gel electrophoresis (SDS–PAGE), containing 62.5 mmol l^{-1} Tris base, 2 % SDS, 5 % glycerol, 0.01 % Bromophenol Blue, 2 % β-mercaptoethanol, pH 6.8. The suspended material was then boiled for 4 min. Samples, including one of molecular mass markers (30–200 kDa), were loaded onto a 7.5 % unidimensional polyacrylamide slab gel and electrophoresed at 200 V. Gels were stained with Coomassie Brilliant Blue (Bio-Rad), dried and apposed to Hyperfilm MP (Amersham) with intensifying screens for 1–2 weeks at −70 °C. Films were developed as for section autoradiography. Molecular masses of subsequent bands were determined as predicted values from the regression equations of the negative logarithm of relative mobility *versus* molecular mass standards for each gel.

Guanylate cyclase assays

Gill membranes were isolated as described above and used immediately. For determination of guanylate cyclase activity, 50 μg of gill protein was added to 50 mmol l^{-1} Tris–HCl, 2 mmol l^{-1} isobutylmethylxanthine (IBMX), 10 mmol l^{-1} creatine phosphate, 1000 i.u. ml^{-1} creatine phosphokinase, 4 mmol l^{-1} $MnCl_2$, 1 mmol l^{-1} GTP and increasing concentrations of rANP, pCNP and C-ANF in a final volume of 100 μl. The basal rate of cyclic GMP generation was determined in tubes without ligand. The incubations were performed for 15 min at 23 °C and were terminated by the addition of 4 mmol l^{-1} EDTA. The tubes were boiled for 3 min and centrifuged at 2300 ***g*** for 15 min. The supernatant was collected and frozen and the cyclic GMP content was determined by radioimmunoassay (cyclic GMP RIA kit, Amersham, Arlington Heights, Illinois).

Data analysis

The values of K_d and B_{max} were determined from the saturation binding data using EBDA and LIGAND computer programs (McPherson, 1985). Additional statistics were computed using the Statview SE program (Abacus Concepts, 1988). Data are presented as means ± S.E.M.

Results

Autoradiography

The gross morphology of hagfish gills is different from that of other fishes. The gill lamellae are contained in ovoid muscular pouches through which water flows in countercurrent to the blood; *M. glutinosa* has six gill pouches on either side

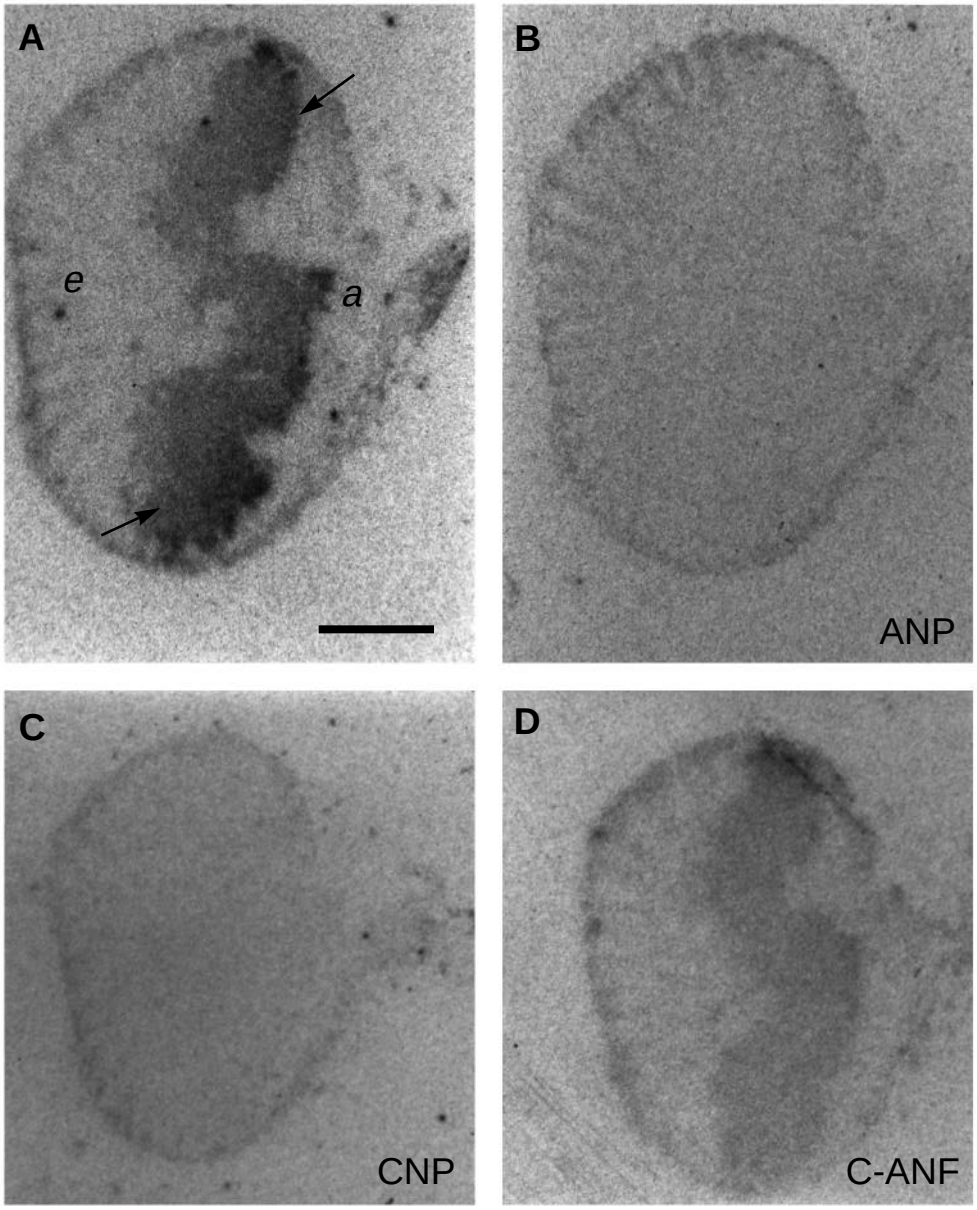

Fig. 1. Autoradiographs of four serial longitudinal sections of a single hagfish gill pouch incubated with ^{125}I-ANP alone (A) or in the presence of various unlabelled natriuretic peptides (NPs) (B–D). (A) Specific binding in the respiratory lamellar region of the gill (arrows); *a*, region of the afferent vasculature supplying the lamellae; *e*, region of the efferent vasculature draining the lamellae. (B) Displacement of the specific binding on the lamellae by 1 μmol l^{-1} rANP, revealing the level of non-specific binding. (C) Displacement of the specific binding by 1 μmol l^{-1} pCNP. (D) Partial displacement of the specific binding by 1 μmol l^{-1} C-ANF. Results (not shown) for ^{125}I-CNP binding were similar, with the exception that C-ANF displaced all specific binding. Scale bar, 1 mm.

of the oesophagus. Fig. 1 shows longitudinal sections through a whole gill pouch; there are three main regions, afferent, lamellar and efferent (Fig. 1A). The afferent section (relative to blood flow) includes a multilayered epithelium containing ionocytes (cells structurally similar to the ion-transporting chloride cells of higher fish) separated by connective tissue from the arterio-arterial vasculature. The vasculature consists of a network of radial vessels and afferent cavernous tissue surrounded by smooth muscle (Fig. 1A: *a*). Arranged between the afferent and efferent sections of the gill are the respiratory lamellae (Fig. 1A: indicated by arrows). These are characterised by a bilayered epithelium in which ionocytes are present. There is no smooth muscle in this section of the gill, although there are pillar cells. The lamellar portion of the gill is drained by efferent lamellar arterioles and the efferent cavernous tissue. The efferent portion of the gill is also characterised by a multilayer epithelium; however, ionocytes are absent here (Fig. 1A: *e*) (Elger, 1987).

Both ^{125}I-ANP- and ^{125}I-CNP-specific binding were observed on the respiratory lamellae of the hagfish gill (Fig. 1A; Fig. 1 shows autoradiographs of gill sections incubated with ^{125}I-ANP, ^{125}I-CNP-specific binding is not shown, but was similar); specific binding of radioligands was displaced by both 1 μmol l^{-1} rANP and 1 μmol l^{-1} pCNP (Fig. 1B,C, ^{125}I-CNP not shown). 1 μmol l^{-1} C-ANF did not completely displace ^{125}I-ANP binding, but displaced virtually all ^{125}I-CNP binding (Fig. 1D, ^{125}I-CNP not shown). Examination of radiotrack emulsion-dipped sections indicated that specific binding sites for both radioligands were scattered generally in the lamellar region over the thin bilayered epithelium (Fig. 2A,B, ^{125}I-CNP binding only shown, ^{125}I-ANP binding was similar). The exact cell types to which the

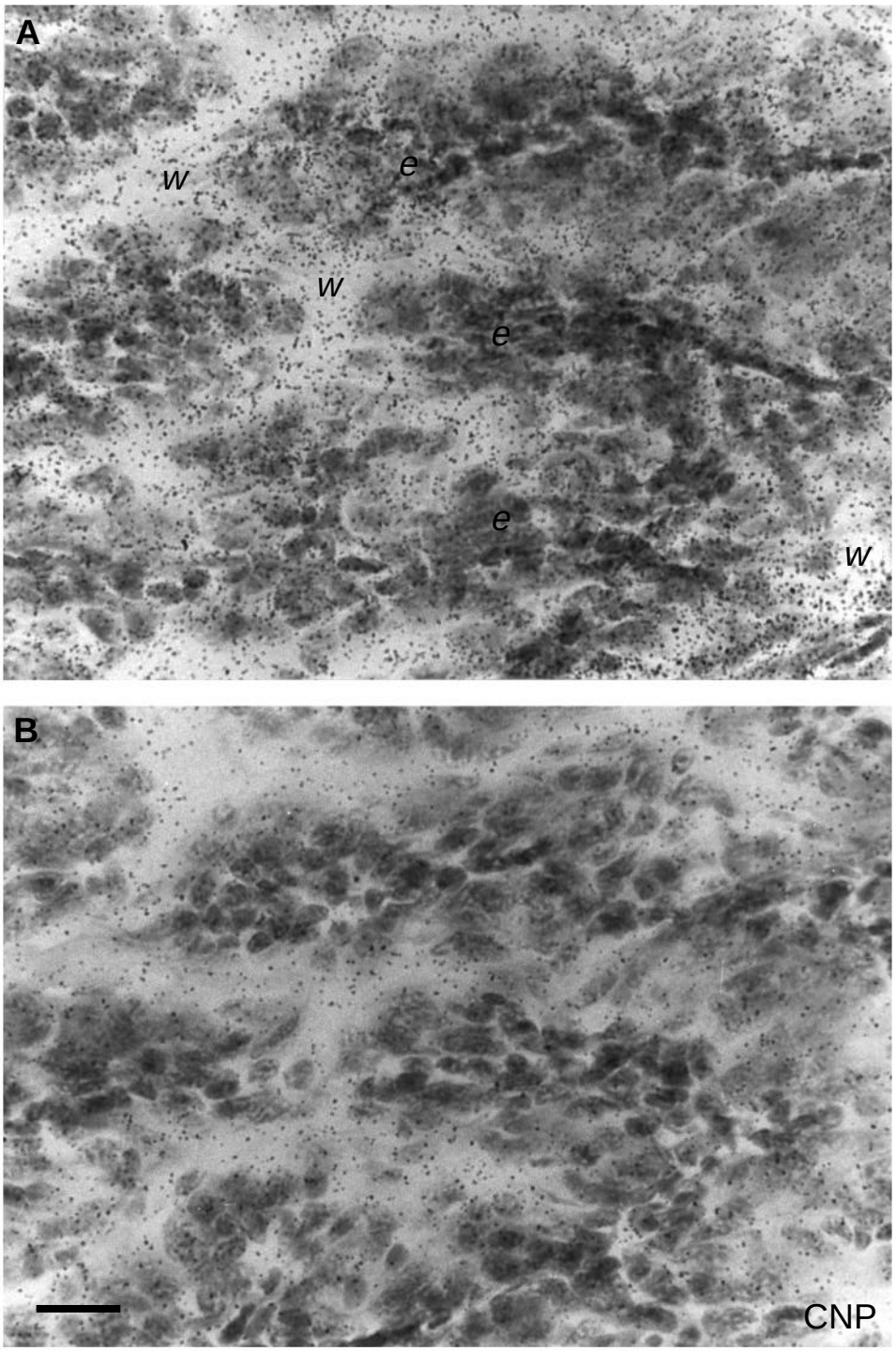

Fig. 2. Light micrograph of longitudinal gill sections dipped in X-ray-sensitive emulsion showing the distribution of specific binding in the lamellar region. Scale bar, 25 μm. (A) ^{125}I-CNP-specific binding to the lamellar epithelial cells (*e*), specific binding indicated by the density of the silver grains; *w* indicates water channels. Orientation and magnification are the same in B. (B) ^{125}I-CNP with the addition of 1 μmol l^{-1} pCNP, showing the level of non-specific binding; the density of silver grains is reduced. Results (not shown) were similar for ^{125}I-ANP binding.

radioligands were binding could not be determined. Specific binding sites were not observed in either the afferent or efferent gill regions.

Radioligand binding assays

Saturation binding

Both ^{125}I-ANP and ^{125}I-CNP bound specifically and saturably to hagfish gill membranes. Maximum binding for both radioligands was reached by 200 pmol l^{-1} (Figs 3 and 4). EBDA and LIGAND analysis indicated that ^{125}I-ANP binding fitted a single site model with an apparent K_d of 15.4±1.6 pmol l^{-1} and a B_{max} of 45.9±3.0 fmol mg^{-1} protein or, alternatively, multiple sites with equal affinities (Fig. 3). Analysis of the ^{125}I-CNP binding site indicated a two-site model with a high- and a low-affinity site (Fig. 4). The high-affinity site was not statistically different from the ^{125}I-ANP site, with an apparent K_d of 12.9±4.7 pmol l^{-1} and a B_{max} of 23.4±6.5 fmol mg^{-1} protein. The low-affinity site had an

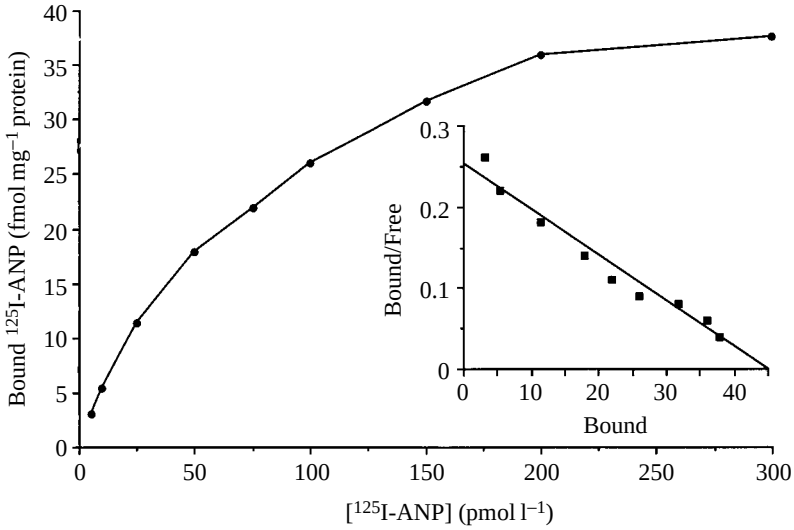

Fig. 3. Example of a typical saturation plot of ^{125}I-ANP-specific binding to gill membranes; inset Scatchard plot of the same data showing linear distribution. Axes: Bound/Free *versus* Bound (fmol mg^{-1} protein). K_d, 15.4±1.6 pmol l^{-1}; B_{max}, 45.9±3.0 fmol mg^{-1} protein.

apparent K_d and B_{max} of 380±80 pmol l^{-1} and 120±21 fmol mg^{-1} protein, respectively.

Competition binding

1 nmol l^{-1} unlabelled rANP, and 20 and 30 nmol l^{-1} unlabelled pCNP and C-ANF, respectively, competed for 50 % of ^{125}I-ANP-specific sites. 100 nmol l^{-1} rANP bound virtually all ANP sites. 1 μmol l^{-1} pCNP and 1 μmol l^{-1} C-ANF competed for all but 5 % and 10 % of binding sites, respectively (Fig. 5A). 0.1 nmol l^{-1} rANP and pCNP and 8 nmol l^{-1}M C-ANF competitively inhibited 50 % of ^{125}I-CNP-specific binding. 1 nmol l^{-1} rANP, 10 nmol l^{-1} pCNP and 1 μmol l^{-1} C-ANF bound 100 % of ^{125}I-CNP-specific sites (Fig. 5B). Rat ANP and pCNP competed equally for ^{125}I-CNP binding sites above 50 % binding. However, below 50 % binding, rANP was a more effective competitive inhibitor than pCNP, suggesting that these sites are low-affinity CNP binding sites (in accordance with the saturation analysis above) that bind ANP with greater affinity.

Affinity cross-linking and SDS–PAGE

Affinity cross-linking followed by SDS–PAGE under reducing conditions of ^{125}I-ANP and ^{125}I-CNP binding to gill membranes indicated an apparent single binding site with an approximate molecular mass of 150 kDa (Fig. 6, lane 1; only ^{125}I-ANP binding shown, results of ^{125}I-CNP binding were identical). Binding was prevented by the addition of 0.1 μmol l^{-1} rANP and pCNP to the incubation reaction (Fig. 6, lanes 2 and 3). The addition of 0.1 μmol l^{-1} C-ANF did not completely inhibit radioligand binding (Fig. 6, lane 4) although, in other experiments (not shown here), visible binding was prevented by 1 μmol l^{-1} C-ANF. The mean apparent molecular masses from three experiments for each ligand were 150±2.5 kDa for ^{125}I-ANP and 153±16 kDa for ^{125}I-CNP. In neither case did a second lower band appear, contrasting with mammals and teleosts, both of which show a lower band indicative of the NPR-C homodimer breaking into the monomeric species under reducing conditions (Martin *et al.* 1989; Donald *et al.* 1994).

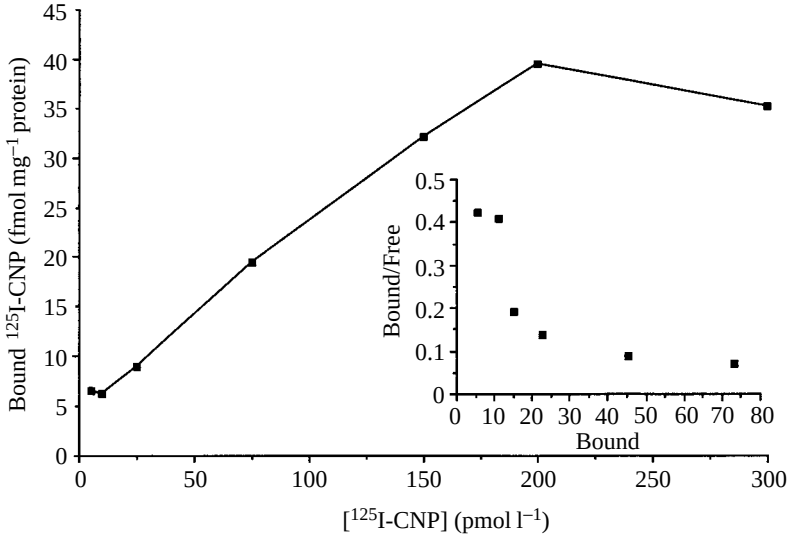

Fig. 4. Example of a typical saturation plot of ^{125}I-CNP-specific binding to gill membranes; inset Scatchard plot of the same data showing non-linear distribution indicative of high- and low-affinity binding sites. Axes: Bound/Free *versus* Bound (fmol mg^{-1} protein). High-affinity site: K_d, 12.9±4.7 pmol l^{-1}; B_{max}, 23.4±6.5 fmol mg^{-1} protein. Low-affinity site: K_d, 380±80 pmol l^{-1}M; B_{max}, 120±21 fmol mg^{-1} protein.

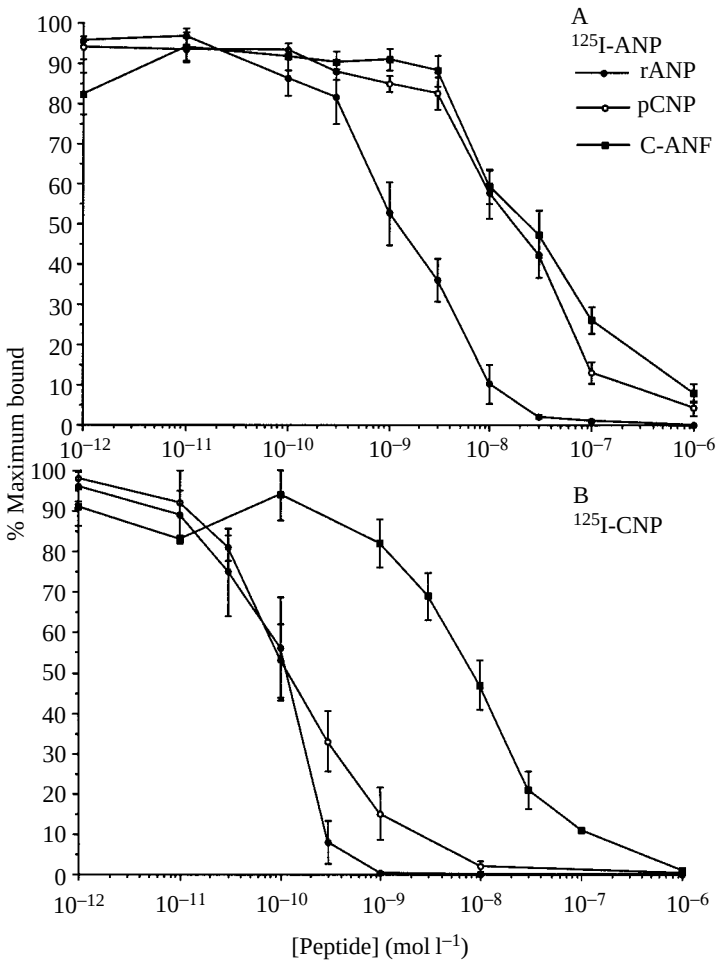

Fig. 5. Competition study indicating the relative abilities of rANP, pCNP and C-ANF at increasing concentrations to compete for ^{125}I-ANP- (A) or ^{125}I-CNP- (B) specific binding sites. 25 pmol l^{-1} ^{125}I-ANP or ^{125}I-CNP was added to each membrane incubation reaction. Each point is the mean ± S.E.M. of gill membrane preparations from 10 separate hagfish.

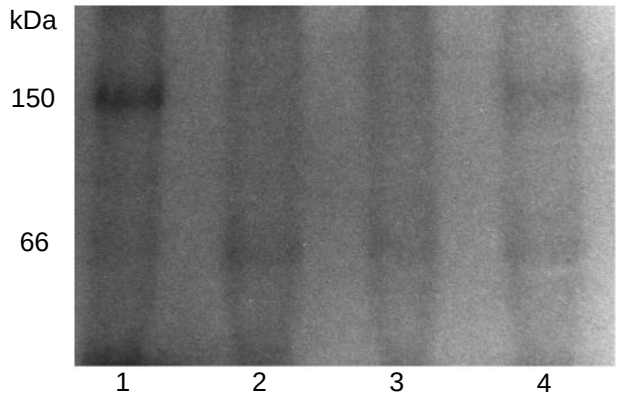

Fig. 6. Autoradiograph of SDS–polyacrylamide gel electrophoresis of ^{125}I-ANP binding sites with ^{125}I-ANP in hagfish gill membranes under reducing conditions. Specifically labelled band (lane 1) indicates an apparent molecular mass of 150 kDa. Cross-linking of radiolabelled ligand was inhibited by the presence of 0.1 μmol l^{-1} rANP (lane 2), 0.1 μmol l^{-1} pCNP (lane 3) and only partially inhibited in the presence of 0.1 μmol l^{-1} C-ANF (lane 4). Affinity-cross linking and SDS–PAGE of ^{125}I-CNP binding (not shown) was similar, with the exception that 0.1 μmol l^{-1} C-ANF inhibited all visible ^{125}I-CNP binding.

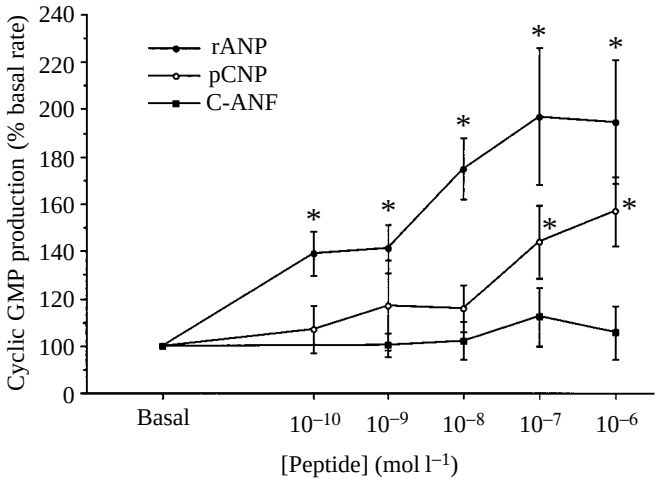

Fig. 7. Relative effects of increasing concentrations of rANP, pCNP and C-ANF on cyclic GMP production in gill membrane preparations. Data points are mean ± S.E.M. of membrane preparations from five individual hagfish. *Significant increase above basal levels (P<0.05).

Guanylate cyclase assays

The basal cyclic GMP accumulation rate was 2.9±0.4 pmol l^{-1} cyclic GMP mg^{-1} protein min^{-1}. Both rANP and pCNP stimulated cyclic GMP production in a dose-dependent manner; 0.1 nmol l^{-1} rANP and above, and 10 μmol l^{-1} pCNP and above, significantly stimulated cyclic GMP production above the basal rate (Fig. 7). A maximum rANP-stimulated rate of 6.2±1.6 pmol l^{-1} cyclic GMP mg^{-1} protein min^{-1} was reached in the presence of 0.1 μmol l^{-1} rANP. The maximum pCNP-stimulated rate, 4.2±0.6 pmol l^{-1} cyclic GMP mg^{-1} protein min^{-1}, was approached at 1 μmol l^{-1} pCNP. C-ANF did not stimulate cyclic GMP production at any concentration.

Discussion

The present study shows that there are both ANP and CNP receptors on the lamellar epithelium of the gills of *M. glutinosa* (Figs 1 and 2). Because the epithelium in the lamellar region does not contain smooth muscle, receptors may be involved in epithelial cell function (perhaps ionocyte function) or in NP clearance from the circulation, rather than in the regulation of blood flow. However, blood flow in the lamellar regions could be influenced by possible pillar cell contraction: teleost pillar cells contain myosin, but whether they have contractile properties has yet to be demonstrated (Farrell, 1993). Although, at this point, there is no evidence that NPs are

directly regulating local lamellar blood flow, the presence of NPRs in the ventral aorta (Kloas *et al.* 1988) and the ability of NPs to dilate this tissue (Evans, 1991; Evans *et al.* 1993) suggest that NPs can regulate blood flow to the branchial vasculature as a whole.

The presence of NP receptors in the gill agrees with teleost studies; binding is predominantly located on the chloride cells of two species of Antarctic fishes, *Chionodraco hamatus* and *Pagothenia bernacchii* (Uva *et al.* 1993), and Broadhead *et al.* (1992) suggest that binding occurs on chloride cells of the eel *Anguilla anguilla*. In the Japanese eel *Anguilla japonica*, NP binding is localised mainly on chondrocytes of gill cartilage and on parenchymal cells, which include chloride cells, of the secondary lamellae (Sakaguchi *et al.* 1993). Other studies have shown binding in the arterio-arterial gill vasculature in trout, where receptors appear to perform a clearance function (Olson and Duff, 1993) and on the afferent and efferent branchial arteries and arterioles of the gulf toadfish *Opsanus beta* (Donald *et al.* 1994). In the latter study, affinity cross-linking experiments showed high (140 kDa) and low (75 kDa) molecular mass binding sites, suggesting a population of NPR-C and guanylate cyclase receptors similar to those found in mammals.

Saturation analysis indicates that there are saturable ANP and CNP binding sites in the gill (Figs 3 and 4). Interpretation of the Scatchard plots suggests that ANP binds to a minimum of one high-affinity site, whereas CNP binds to two sites of differing affinity. The competition data demonstrate that ANP and CNP are capable of competing for the binding sites of both radioligands, but with different efficiencies. CNP is less able to compete with ANP for ^{125}I-ANP sites (Fig. 5A), but ANP and CNP compete equally well for ^{125}I-CNP sites (Fig. 5B), suggesting that there are two ANP sites, one that binds ANP in preference to CNP (the low-affinity CNP site) and one that binds ANP and CNP with equal affinity (the high-affinity CNP site). In addition, the similarity in the upper 50 % of the competition curves of rANP and pCNP for ^{125}I-CNP binding sites (Fig. 5B) is consistent with the presence of a high-affinity ANP/CNP site, whereas the dissimilarity between the competing ligands in the lower 50 % of the curves suggests the presence of a high-affinity ANP/low-affinity CNP site. A similar situation was found in shark rectal gland competition studies, where CNP, rather than ANP, displaced specific binding more readily in the lower 50 % of the curve (Gunning *et al.* 1993). The stimulation of cyclic GMP production by ANP (Fig. 7), and to a lesser extent by CNP, indicates that it is probably the high-affinity ANP/low-affinity CNP receptor that is coupled to guanylate cyclase activity. The high-affinity ANP/low-affinity CNP receptor appears to resemble the NPR-A of mammals. Mammalian guanylate-cyclase-linked NPR-A receptors bind ANP with much greater affinity than they bind CNP (Koller and Goeddel, 1992). There is no evidence in the hagfish gill of a guanylate-cyclase-linked NPR-B-like receptor that preferentially binds CNP (Koller and Goeddel, 1992).

C-ANF competitively inhibits the majority of both ^{125}I-ANP and ^{125}I-CNP binding (Fig. 5), but it is unclear to which site this NP analogue preferentially binds. It appears only partially to displace ^{125}I-ANP, but to displace all of ^{125}I-CNP from tissue sections. Therefore, it is tempting to suggest that C-ANF binds mainly to the high-affinity ANP/CNP receptor (Fig. 1D). C-ANF has been constructed to bind specifically to the clearance (NPR-C) receptor in mammals (Maack *et al.* 1987); however, whether this specificity for NPR-C is maintained in other vertebrate classes is unknown.

Both receptor types in the hagfish gill have an apparent molecular mass of approximately 150 kDa (Fig. 6). This molecular mass is slightly heavier than that of the mammalian NPR-A, and the homodimer form of the NPR-C, which both appear at approximately 130 kDa. In the presence of reducing conditions, mammalian NPR-C, and toadfish NPR-C-like receptors, separate into the monomeric species and are visible as lower molecular mass bands at 65 kDa (mammals, Brenner *et al.* 1990) and 75 kDa (toadfish, Donald *et al.* 1994). Sakaguchi *et al.* (1993) discovered a 68 kDa band under reducing conditions in Japanese eel gill membranes; biochemical characterisation demonstrated that this receptor was of the NPR-C type. The absence of a lower molecular mass band in hagfish gills strongly suggests that, unlike the situation in mammals and teleosts, the high-affinity ANP/CNP receptor is not a homodimeric NPR-C type. Consequently, it appears unlikely that this ANP/CNP receptor is homologous in structure to the NPR-C of mammals. It is also unlikely that this receptor is linked to guanylate cyclase activity, because CNP did not stimulate cyclic GMP production as effectively as did ANP (Fig. 7). Whether the high-affinity ANP/CNP hagfish receptor functions as a clearance receptor has yet to be determined; however, it is possible that the hagfish gill contains receptors to modulate circulating NP concentrations, since teleost gills perform a clearance function for NPs and other humoral factors (Olson and Duff, 1993). It is also unknown whether the ANP/CNP receptor is linked to other second-messenger pathways, as has recently been demonstrated for the mammalian NPR-C (Levin, 1993).

It is now evident that hagfish have a well-developed NP system. Previous research has shown that not only do the heart, brain and plasma of *Myxine glutinosa* have NP-like immunoreactivity (Reinecke *et al.* 1987; Evans *et al.* 1989; Donald *et al.* 1992), but also that NPs are vasoactive, dilating the vascular smooth muscle of the ventral aorta (Evans, 1991; Evans *et al.* 1993), where binding sites have also been located (Kloas *et al.* 1988). The discovery of NP immunoreactivity and the localisation of NP binding sites in the hagfish brain indicate that NPs are neuropeptides, functional in the central nervous system (Donald *et al.* 1992; J. A. Donald and T. Toop, unpublished observations). The presence of binding sites in the glomeruli and archinephric ducts shows that the hagfish kidney is also a target organ (Kloas *et al.* 1988; T. Toop, unpublished observations).

The present study extends NP function to the gills of hagfish. Although the present study utilizes heterologous mammalian peptides and not the native hagfish NPs, this research clearly indicates that guanylate-cyclase-linked activity in NPRs is a

phylogenetically ancient vertebrate characteristic. In addition, the presence of ANP-specific receptors, rather than CNP-specific ones, suggests that CNP is not the primitive circulating vertebrate NP, in spite of its importance as a heart hormone in elasmobranchs (Schofield *et al.* 1991; Suzuki *et al.* 1991; Solomon *et al.* 1992; Karnaky *et al.* 1992). The ancestral presence of the NPR-C, or clearance receptor, is less clear. Further investigations are needed before the relationship between the hagfish ANP/CNP receptor and the NPR-C can adequately be determined.

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