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EVIDENCE OF A GUANYLYL CYCLASE NATRIURETIC PEPTIDE RECEPTOR IN THE GILLS OF THE NEW ZEALAND HAGFISH *EPTATRETUS CIRRHATUS* (CLASS AGNATHA)

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Summary

Natriuretic peptide binding sites were examined in the gills of the hagfish *Eptatretus cirrhatus* (Class Agnatha, subfamily Eptatretinae) using radio-ligand binding techniques, molecular cloning and guanylyl cyclase assays. Iodinated rat atrial natriuretic peptide (^{125}I-rANP) and iodinated porcine C-type natriuretic peptide (^{125}I-pCNP) bound specifically to the lamellar folds and cavernous tissue of *E. cirrhatus* gills, and 0.3 nmol l^{-1} rat ANP competed for 50 % of specific ^{125}I-rANP binding sites. Affinity cross-linking of ^{125}I-rANP to gill membranes followed by sodium dodecylsulphate–polyacrylamide gel electrophoresis revealed a single binding site of 150 kDa. In the presence of Mn^{2+}, 0.1 nmol l^{-1} rANP inhibited cGMP production, whereas 1 μmol l^{-1} rANP stimulated cGMP production rates. At 1 μmol l^{-1}, pCNP also stimulated cGMP production. The production of cGMP was also measured in the presence and absence of ATP with either Mn^{2+} or Mg^{2+}. Reverse transcriptase polymerase chain reaction (RT-PCR) of hagfish gill RNA, followed by cloning and sequencing of PCR products, produced a partial cDNA sequence of a natriuretic peptide guanylyl cyclase receptor. The deduced amino acid sequence indicated 87–91 % homology with other natriuretic peptide guanylyl cyclase receptors. This study indicates the presence of a natriuretic peptide guanylyl cyclase receptor in the gills of *E. cirrhatus* that is similar to the natriuretic peptide guanylyl cyclase receptors in higher vertebrates. These observations demonstrate that the coupling of natriuretic peptide receptors with guanylyl cyclase has a long evolutionary history.

Key words: Agnatha, New Zealand hagfish, natriuretic peptide receptor, guanylyl cyclase, *Eptatretus cirrhatus*.

Introduction

The natriuretic peptides are a family of polypeptide hormones that act to reduce blood volume. Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are principally of cardiac origin (Hagiwara et al., 1995), although a number of studies have demonstrated the presence and synthesis of ANP in other tissues (Awazu and Ichikawa, 1993). Both ANP and BNP reduce blood volume by stimulating natriuresis (excretion of NaCl *via* the kidneys) and diuresis (excretion of H_2O) and by inhibiting the renin–angiotensin–aldosterone system (Nicholls, 1994). In contrast, C-type natriuretic peptide (CNP) appears to act primarily as a paracrine agent in the vasculature and may also possess neuromodulatory functions (Barr et al., 1996).

Although the natriuretic peptides have traditionally been regarded as potent vasodilators, they can also stimulate vasoconstriction. The vasoconstrictive effects of natriuretic peptides in mammalian efferent glomerular arterioles contribute to the hypotensive actions of natriuretic peptides by increasing glomerular pressure and, hence, glomerular filtration rate (Brenner et al., 1990). Natriuretic-peptide-mediated vasoconstriction has also been observed in the splanchnic and mesenteric circulation of dogs (Woods, 1998, 1999) and in the branchial circulation of fishes. Natriuretic peptides infused into trout cause a transient increase in blood pressure prior to hypotensive effects (Olson and Duff, 1992; Olson et al., 1997; McKendry et al., 1999). A similar response is observed in the spiny dogfish *Squalus acanthias* (McKendry et al., 1999). A recent study by Baustian and Beyenbach (1999) has demonstrated that heart and kidney fractions isolated from the toadfish (*Opsanus tau*) are capable of constricting aortic ring preparations, although vasodilation is also observed with heart extracts. In addition, ANP and CNP are capable of inducing both vasoconstriction and vasodilation in the branchial vasculature of the hagfish *Eptatretus cirrhatus* (Glover, 1996).

Receptor proteins present in the plasma membranes of target cells mediate the physiological effects of natriuretic peptides. There are two types of receptor: those that are coupled to

guanylyl cyclase activity and those that are not. Natriuretic peptide receptor A (NPR-A) and natriuretic peptide receptor B (NPR-B) contain a guanylyl cyclase that forms part of the cytoplasmic domain (Anand-Srivastava and Trachte, 1993). These receptors elicit intracellular effects by catalysing the conversion of guanosine triphosphate into guanosine-3′,5′-cyclic monophosphate (cGMP). In mammals, ATP regulates the natriuretic-peptide-dependent guanylyl cyclase activities of NPR-A and NPR-B. This regulation may be inhibitory or stimulatory, depending on whether Mg^{2+} or Mn^{2+} is present as a cofactor (Duda et al., 1996). The non-guanylyl-cyclase-linked receptors are natriuretic peptide receptor C (NPR-C) and natriuretic peptide receptor D (NPR-D). It was originally believed that NPR-C modulated the plasma concentrations of natriuretic peptides by clearing them from circulation (Maack et al., 1987; Inagami, 1989; Maack, 1992). However, several studies link NPR-C to other intracellular second-messenger systems such as the adenosine-3′,5′-cyclic monophosphate or the inositol trisphosphate pathways (Anand-Srivastava et al., 1990; Anand-Srivastava and Trachte, 1993; Levin, 1993). While NPR-C binds all natriuretic peptides with equal affinity, the guanylyl cyclase receptors exhibit different affinities for the different natriuretic peptide ligands. While NPR-A binds ANP preferentially but will also bind BNP and to a lesser extent CNP, NPR-B binds CNP almost exclusively. The second non-guanylyl-cyclase receptor, NPR-D, which displays considerable homology to NPR-C, has been cloned from the eel *Anguilla japonica* (Kashiwagi et al., 1995), and there is some evidence that this receptor also occurs in the lamprey *Geotria australis* (Toop et al., 1998).

The natriuretic peptides are implicated in fish osmoregulation (Evans, 1990). Natriuretic peptide systems are present in all classes of fish (Reinecke et al., 1985, 1987; Evans et al., 1989; Vallarino et al., 1990; Donald and Evans, 1992; Donald et al., 1992; Toop et al., 1995a,b), and natriuretic peptide binding sites have been identified in the gills, kidneys, heart and vasculature of all fishes examined (Kloas et al., 1988; Cerra et al., 1992; Duff and Olson, 1992; Donald et al., 1994, 1997; Toop et al., 1995a,b, 1998). Natriuretic peptide receptors have been cloned from the eel *Anguilla japonica* (Katafuchi et al., 1994; Kashiwagi et al., 1995, 1999; Takashima et al., 1995) and the dogfish *Squalus acanthias* (Aller et al., 1999). From these studies, it is clear that the structure of piscine natriuretic peptides and natriuretic peptide receptors is similar to that of mammalian receptors. A number of studies have indicated that the NPR-C-type receptors predominate in the gills (for example, Donald et al., 1994, 1997); in addition, Olson and Duff (1993) demonstrated that the gills of rainbow trout *Oncorhynchus mykiss* are capable of removing 60 % of ^{125}I-ANP from the circulation in a single pass. These studies suggest that the gill natriuretic peptide receptors clear natriuretic peptides from the circulation and that this clearance is probably mediated by the NPR-C-type receptors. Several studies have shown that natriuretic peptides can also stimulate guanylyl cyclase activity in the gills (Donald et al., 1994, 1997; Toop et al., 1995a, 1998; Mishina and Takei, 1997). The cloning of natriuretic peptide guanylyl cyclase receptors from the gills of the Japanese eel (Katafuchi et al., 1994; Kashiwagi et al., 1999) and the rectal gland of the dogfish (Aller et al., 1999) confirms the presence of guanylyl-cyclase-linked receptors in fishes.

The hagfishes (family Myxinidae) diverged from other vertebrate groups at least 500 million years ago (Forey and Janvier, 1993) and consist of two subfamilies, the Myxininae and the Eptatretinae (Fernholm, 1998). The hagfishes are osmotically at odds with the obligatory osmoregulators that comprise all other vertebrate classes since hagfish plasma is virtually iso-osmotic with sea water (Hardisty, 1979). In spite of the lack of significant osmoregulation, hagfishes have well-developed natriuretic peptide systems. The heart, brain and plasma of *Myxine glutinosa* possess natriuretic-peptide-like immunoreactivity (Reinecke et al., 1987; Evans et al., 1989; Donald et al., 1992), and rat ANP dilates the ventral aorta (Evans, 1991). Kloas et al. (1988) located natriuretic peptide binding sites in the kidney and ventral aorta of *M. glutinosa*, and Toop et al. (1995a,b) localised and characterised both ANP and CNP binding sites in the kidneys, gills and aorta of the same species. It was demonstrated that both ANP and CNP stimulated cGMP production, although ANP was the more potent stimulator. While it was suggested that two receptor populations are present in the gills of *M. glutinosa*, affinity cross-linking of radiolabelled ligand to membrane preparations and subsequent sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) indicated a single band with a molecular mass of approximately 150 kDa. There was no indication of a mammalian-type NPR-C with a lower apparent molecular mass (Toop et al., 1995a).

Using a variety of receptor–ligand binding and molecular techniques, the present study aimed to characterise natriuretic peptide receptors in the gills of the New Zealand hagfish *Eptatretus cirrhatus*, a member of the subfamily Eptatretinae. One of the main purposes of this study was to ascertain whether the unique characteristics of the branchial natriuretic peptide receptor in *M. glutinosa* (subfamily Myxininae) are shared by other hagfishes. A further aim of this study was to examine whether the guanylyl cyclase system could potentiate the vasoconstrictive responses to natriuretic peptides observed by Glover (1996). In addition, we examined the role of ATP in regulating natriuretic-peptide-stimulated guanylyl cyclase activity to assess how similar the regulation of the guanylyl cyclase is to that reported for mammals.

Materials and methods

Animal maintenance

Hagfishes *Eptatretus cirrhatus* (Forster, 1801) were collected from the coastal waters of the South Island of New Zealand. They were transported to the University of Canterbury, Christchurch, and held in a recirculating seawater system at approximately 12 °C. Animals were unfed and exposed to a daily light:dark regime of 14 h:10 h. No animal was held in captivity for longer than 4 weeks. Animals were

anaesthetised in 0.04 % benzocaine (ethyl-*p*-aminobenzoate) and 0.04 % MS222 (ethyl-*m*-aminobenzoate methane sulphate) prior to being killed and dissected. Whole gill pouches were snap-frozen in liquid nitrogen and transported to Deakin University, Australia, where they were stored at −70 °C until use.

Autoradiography

Gill pouches were freeze-mounted in OCT embedding medium (Tissue-Tek, Sakura) and sectioned in a cryostat (Reichert-Jung, at −15 °C). Sections (20 μm thick) were thaw-mounted onto gelatin/chromium/aluminium-coated microscope slides, air-dried for approximately 30 min and stored at −20 °C until use.

Slides were preincubated for 15 min at room temperature (20–25 °C) in 50 mmol l^{-1} Tris-HCl (pH 7.4), 100 mmol l^{-1} NaCl, 5 mmol l^{-1} $MgCl_2$, 0.25 % bovine serum albumin (BSA), 0.05 % bacitracin (pH 7.4). 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 pmol l^{-1} phenylmethylsulphonylfluoride (PMFS) and either 2 nmol l^{-1} ^{125}I-labelled rat ANP (3-[^{125}I]iodotyrosyl^{28} rat atrial natriuretic peptide, 200 pmol l^{-1}, 2000 Ci $mmol^{-1}$ (74 TBq $mmol^{-1}$), Amersham) or 2 nmol l^{-1} ^{125}I-labelled CNP (^{125}I[Tyr^0] C-type natriuretic peptide-22 (porcine), 100 pmol l^{-1}, 1118 Ci $mmol^{-1}$ (41.4 TBq $mmol^{-1}$), Peninsula) (Toop et al., 1995b). Non-specific binding was determined in adjacent sections in the presence of 1 μmol l^{-1} rat $ANP_{1–28}$ (rANP, Auspep, Australia) for ^{125}I-rANP-incubated sections and 1 μmol l^{-1} porcine $CNP_{1–22}$ (pCNP, Auspep, Australia) for ^{125}I-pCNP-incubated sections. The ability of 1 μmol l^{-1} pCNP (for ^{125}I-rANP-incubated sections), 1 μmol l^{-1} rANP (for ^{125}I-pCNP-incubated sections) and 1 μmol l^{-1} rat des[Gln^{18}, Ser^{19}, $Gly^{20,22}$, Leu^{21}]$ANF_{4–23}$-NH_2 (C-ANF, Peninsula, USA) to displace specific radiolabelled natriuretic peptide binding was also determined in adjacent sections. C-ANF is a truncated ANP that in mammals binds only to NPR-C (Maack et al., 1987).

Following incubation, slides were washed (2×10 min at 4 °C) in 5 mmol l^{-1} Tris-HCl and 10 mmol l^{-1} NaCl (pH 7.4), fixed for 20 min in 4 % formaldehyde in 0.1 mmol l^{-1} phosphate buffer (4 °C, pH 7.4), washed in 0.1 mmol l^{-1} phosphate buffer and then in distilled water (1 min), dehydrated through an ethanol series, dried for 30 min at 40 °C and dipped in nuclear track silver emulsion (LM-1, Amersham) at 43 °C. After drying, the slides were stored for 14 days at 4 °C and then processed using Ilford Phenisol X-ray developer and Ilford Hypam rapid paper and film fixer according to the manufacturer's instructions. Subsequently, they were stained with haematoxylin and eosin. Photomicrographs were made with a digital colour system (Spot 35 Camera System, Diagnostic Instruments, Michigan, USA).

Membrane preparation

Gill membranes were prepared from individual animals for guanylyl cyclase assays, affinity cross-linking SDS–PAGE and competition binding studies, following Toop et al. (1995a). Single gill pouches were homogenised with a Teflon pestle in 10 ml of ice-cold 50 mmol l^{-1} Tris-HCl and 1 mmol l^{-1} $NaHCO_3$, (pH 7.4) in 50 ml glass tubes. The homogenate was diluted with 10 ml of 50 mmol l^{-1} Tris-HCl, 1 mmol l^{-1} EDTA and 1 mmol l^{-1} $MgCl_2$ (pH 7.4, 4 °C) and centrifuged for 15 min at 1200 ***g*** at 4 °C (Beckman J2-21 M/E centrifuge). The pellet was discarded, and the supernatant was centrifuged at 20 700 ***g*** for 20 min at 4 °C. The new pellet was resuspended in 400 μl of 50 mmol l^{-1} Tris-HCl, 250 mmol l^{-1} sucrose buffer (pH 7.4, 4 °C). Protein concentration was determined using a bovine serum albumin (BSA) protein assay kit calibrated with BSA standards (Pierce). Membrane preparations were frozen in liquid nitrogen and stored at −20 °C until required. This technique results in a mixture of plasma and intracellular membranes.

Competition binding assays

A solution consisting of 75 μg of hagfish gill protein, 25 pmol l^{-1} ^{125}I-rANP, and rANP, pCNP or C-ANF at increasing concentration (1 pmol l^{-1} to 1 μmol l^{-1}) was made up to a total volume of 250 μl with incubation buffer and incubated at room temperature for 90 min. The reaction was halted by the addition of 2 ml of ice-cold 50 mmol l^{-1} Tris-HCl (pH 7.4), and bound ligand was separated from free ligand by vacuum-filtration through 1 % polyethyleneimine-treated Whatman GF/C filters. Filters were washed with 50 mmol l^{-1} Tris-HCl (pH 7.4), and radioactivity was measured in a Minaxi γ auto-gamma 5000 series gamma counter (United Technologies, Packard Instrument Company) with 79 % efficiency.

Affinity cross-linking and SDS–PAGE

Gill membranes were isolated as described above, and 100 μg of gill protein was added to a reaction mixture consisting of incubation buffer together with 0.25 nmol l^{-1} ^{125}I-rANP alone or together with an excess (1 μmol l^{-1}) of unlabelled rANP, pCNP or C-ANF. The total volume was 250 μl. Following a 2 h incubation period (to allow ligand–receptor interaction), the covalent cross-linking agent disuccimidyl suberate (Pierce) in dimethylsulphoxide was added to a final concentration of 1 mmol l^{-1}, and the incubation was continued for a further 20 min. The cross-linking reaction was halted by the addition of 250 μl of a solution containing 400 mmol l^{-1} EDTA and 1 mmol l^{-1} Tris-HCl (pH 6.8), and the total mixture was centrifuged at 13 000 ***g*** for 20 min (Biofuge 13, Heraeus Instruments). The pellet was resuspended in 25 μl of reducing sample buffer, containing 62.5 mmol l^{-1} Tris-HCl, 10 % glycerol, 2 % sodium dodecylsulphate, 5 % 2-β-mercaptoethanol and 0.001 % Bromophenol Blue (pH 6.8), for SDS–PAGE, and the sample tubes were boiled for 3 min. The samples were loaded onto a 7.5 % unidimensional polyacrylamide slab gel and electrophoresed alongside molecular mass standards (30–200 kDa) at 90 V for 15 min and at 150 V for 1 h. Gels were stained with Coomassie Blue, destained, and dried overnight at 37 °C. Dried gels were

apposed to Hyperfilm MP (Amersham) with intensifying screens for 14 days at −70 °C. Films were developed as described for emulsion-dipped slides.

Guanylyl cyclase assays

Gill membranes were isolated as described above, and 50 μg of gill protein was added to a reaction mixture containing 1 mmol l^{-1} guanosine triphosphate (GTP), 10 mmol l^{-1} creatine phosphate, 2 mmol l^{-1} isobutylmethylxanthine, 50 mmol l^{-1} Tris-HCl, 100 units ml^{-1} creatinine phosphokinase and either 4 mmol l^{-1} $MnCl_2$ or 4 mmol l^{-1} $MgCl_2$ in a final volume of 100 μl. In a separate set of experiments, the reaction mixture also contained 0.25 mmol l^{-1} ATP. The reaction mixtures were incubated at room temperature for 15 min after the addition of either 0.1 nmol l^{-1} or 1 μmol l^{-1} rANP, pCNP or C-ANF. Basal guanylyl cyclase activity was determined in the absence of natriuretic peptide ligand, and toad (*Bufo marinus*) kidney membrane preparations were used as positive controls. Following incubation, 20 μl of 40 mmol l^{-1} EDTA was added to each reaction mixture, and the sample tubes were boiled for 3 min. The tubes were then centrifuged at 8000 ***g*** for 10 min (Biofuge 13, Heraeus Instruments), after which the supernatant was collected and frozen at −20 °C. Guanylyl cyclase activity was indirectly measured *via* a cGMP radioimmunoassay (Biotrak cyclic GMP [^{3}H] assay system, Amersham) according to the manufacturer's protocol, and samples were counted on a 2000 CA Tri-Carb liquid scintillation analyser (United Technologies, Packard Instrument Company) with 63 % efficiency.

Molecular cloning

Total RNA was obtained by the acid-phenol extraction technique (Chomczynski and Sacchi, 1987). First-strand cDNA was synthesised using the reverse transcriptase Superscript II (Life Technologies, Australia). A hot-start polymerase chain reaction (PCR) was performed using degenerate sense and antisense oligonucleotide primers based on conserved regions of the guanylyl cyclase domain from human, bovine, rat and eel receptors. Primer sequences were: 5′-ATGGAA/GCAA/GTAC/TGC*N*AA-3′ (sense) and 5′-CCC/TTTICCC/TTTCATC/TTCIACA/GTC*N*CC-3′ (antisense) (I is deoxyinosine; *N* is any base). Amplification of a 673-base-pair product was predicted, including primer sequences. PCR was performed with 1 μl of first-strand cDNA in a 50 μl reaction mixture consisting of 20 mmol l^{-1} Tris, 50 mmol l^{-1} KCl, 0.2 mmol l^{-1} of each dNTP, 0.8 μmol l^{-1} of each primer, 3 mmol l^{-1} $MgCl_2$ and 2.5 units of Platinum Taq (Life Technologies, Australia) at pH 8.4. Reactions were overlaid with 30 μl of mineral oil. An initial cycle of 300 s at 94.5 °C, 70 s at 60 °C and 90 s at 72 °C was followed by two cycles of 60 s at 94.5 °C, 70 s at 60 °C and 90 s at 72 °C. Cycles 4–34 consisted of 60 s at 95 °C, 60 s at 55 °C and 90 s at 72 °C. The elongation step of the thirty-fifth cycle was allowed to proceed for 290 s. The PCR products were separated on a 1 % TBE (45 mmol l^{-1} Tris-borate, 1 mmol l^{-1} EDTA, pH 8.0)–agarose gel to confirm amplification of the predicted product. The remainder of the PCR reaction was precipitated in 2 vols of isopropanol and 1 vol of 4 mol l^{-1} ammonium acetate (pH 5.2) and was subsequently cloned into the pCR 2.1 plasmid vector according to the manufacturer's protocol (Invitrogen Original TA cloning kit, The Netherlands). Transformed cells were grown on agar plates containing 50 μg ml^{-1} ampicillin with the addition of 50 μl of 40 mg ml^{-1} X-gal. White colonies with potential inserts were grown up overnight in 5 ml of LB medium, and plasmids were subsequently isolated using the Concert Rapid Plasmid Miniprep System (Life Technologies, Australia). The presence of inserts was confirmed by restriction digest of the plasmids with *Eco*RI followed by electrophoresis on a 1 % TBE–agarose gel. Inserts were sequenced on an Applied Biosystems automated sequencer (Westmead Hospital, Sydney, Australia) (*N*=3) and analysed using BLAST (National Center for Biotechnology Information).

Data analysis

Data are expressed as mean ±1 S.E.M and were calculated using Microsoft Excel (version 7.0). Regression analysis and analysis of variance (ANOVA) were performed on guanylyl cyclase assay data using Statview SE (Abacus Concepts). Graphs were prepared using CricketGraph (version 1.3.2, Cricket Software). Independent samples *t*-tests were performed on guanylyl cyclase assay data using SPSS for MS Windows (Release 6.0).

Results

Autoradiography

The morphology of hagfish gills differs from that of other fishes in that the lamellae are contained in ovoid muscular pouches. Water enters the gill pouch from the pharynx and flows countercurrent to the blood before exiting *via* the branchial duct. In *Eptatretus cirrhatus*, seven gill pouches are present on either side of the oesophagus and, in contrast to the gill anatomy of the genus *Myxine*, each gill pouch possesses its own efferent branchial duct (Elger, 1987; Fernholm, 1998). The lamellar region is devoid of smooth muscle and lies between the afferent and efferent portions of the gill. The lamellar folds are arranged parallel to the water current (Elger, 1987). The present study demonstrated specific ^{125}I-rANP and ^{125}I-pCNP binding throughout the gill. The majority of specific binding was to the efferent portion of the gill, with some binding to the endothelial cells of the efferent cavernous tissue and the densest binding located on the lamellar folds (Fig. 1A,C). Some binding was apparent on free cells that were present within the lumen of the efferent cavernous tissue (Fig. 1E,F). These cells appear to belong to the endothelial layer and may be artefactually present in the lumen because of cryosectioning. Because there was no obvious difference between the binding of the radiolabelled ligands, only the results for ^{125}I-rANP are shown. Sparse but specific radioligand binding was also apparent on the afferent cavernous tissue (not shown), while a generalised scattering of radioligand over the rest of the gill was only partially displaced by unlabelled ligand. ^{125}I-rANP and ^{125}I-pCNP binding was equally displaced by 1 μmol l^{-1} rANP (Fig. 1B,D), 1 μmol l^{-1} pCNP (not shown) and 1 μmol l^{-1} C-ANF (not shown).

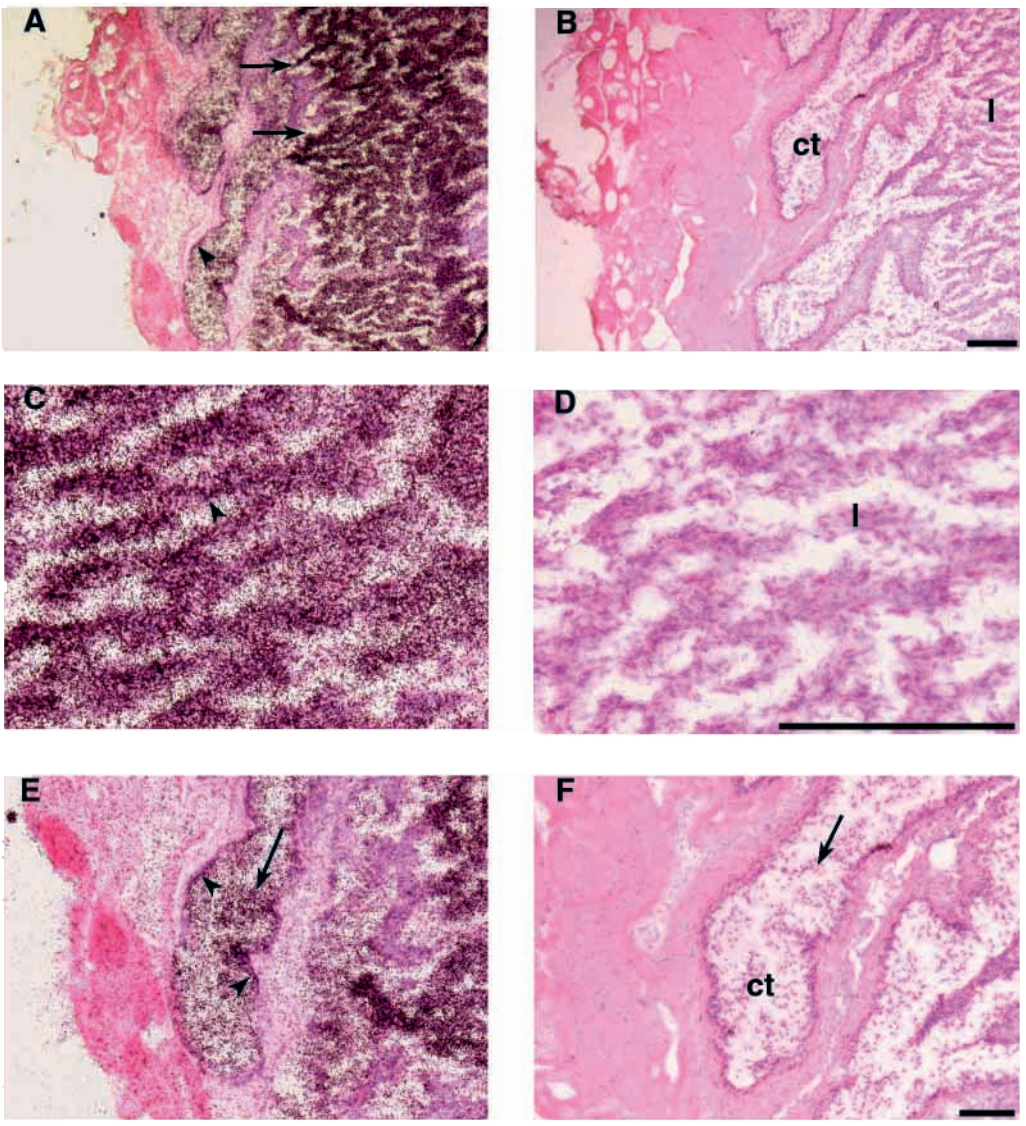

Fig. 1. Photomicrographs of X-ray-sensitive emulsion-dipped serial transverse sections through the gill pouch of *Eptatretus cirrhatus*. (A) Specific binding of ^{125}I-labelled rat atrial natriuretic peptide (^{125}I-rANP) to the efferent cavernous tissue (arrowhead) and lamellar folds, particularly at the efferent edge (arrows). (B) Specific ^{125}I-rANP binding to the cavernous tissue (ct) and lamellar folds (l) is displaced by the addition of 1 μmol l^{-1} unlabelled rANP. Scale bar for A and B, 100 μm. (C) Specific binding of ^{125}I-rANP to the lamellar folds of the gill (arrowhead). It is not resolved whether binding is to the vascular or epithelial tissue. (D) Specific ^{125}I-rANP binding to the lamellae (l) is displaced by the addition of 1 μmol l^{-1} unlabelled rANP. Scale bar for C and D, 100 μm. (E) Specific binding of ^{125}I-rANP to endothelial cells of the efferent cavernous tissue (arrowheads) and to apparently free cells (arrow). (F) Specific ^{125}I-rANP binding to the endothelial cells of the efferent cavernous tissue (ct) and to free cells within the lumen of the efferent cavernous tissue (arrow) is displaced by the addition of 1 μmol l^{-1} unlabelled rANP. Scale bar for E and F, 50 μm.

Competition binding assays

The unlabelled natriuretic peptides competed for ^{125}I-rANP binding sites in a dose-dependent manner. Rat ANP displaced 50 % of ^{125}I-rANP binding at a concentration of 0.3 nmol l^{-1}, and 10 nmol l^{-1} rANP successfully competed for all ^{125}I-rANP binding sites (Fig. 2). Porcine CNP displaced 50 % of ^{125}I-rANP binding at a concentration of 2 nmol l^{-1} and successfully competed for all binding sites at 30 nmol l^{-1} (Fig. 2). C-ANF competed for 50 % of ^{125}I-rANP binding at 3 nmol l^{-1} and for all of the specific ^{125}I-rANP binding at 0.3 μmol l^{-1} (Fig. 2).

Affinity cross-linking and SDS–PAGE

Affinity cross-linking of ^{125}I-rANP to gill membranes followed by SDS–PAGE under reducing conditions indicated a single binding site with an apparent molecular mass of 150 kDa. The cross-linking of radioligand was inhibited in the presence of 1 μmol l^{-1} rANP, pCNP and C-ANF (Fig. 3).

Guanylyl cyclase assays

The basal rate of cGMP accumulation (in the absence of ATP and natriuretic peptides) was 11.78± 1.04 pmol cGMP mg^{-1} protein min^{-1} in the presence of Mn^{2+} (Fig. 4A). Cyclic GMP levels decreased in response to the lower (0.1 nmol l^{-1}) concentration of rANP ($P \leqslant 0.05$). A rate of cGMP production of 8.88±0.70 pmol cGMP mg^{-1} protein min^{-1} was observed in the presence of 0.1 nmol l^{-1} rANP. At the higher rANP concentration (1 μmol l^{-1}), cGMP production was elevated ($P \leqslant 0.05$). In the presence of 1 μmol l^{-1} rANP, the rate of cGMP production was 18.36±3.05 pmol cGMP mg^{-1} protein min^{-1}. In response to 1 μmol l^{-1} pCNP, cGMP levels rose to 17.23±1.86 pmol cGMP mg^{-1} protein min^{-1} ($P \leqslant 0.05$) (Fig. 4C). However, no decrease in cGMP levels was observed at low pCNP concentrations. C-ANF had no effect on guanylyl cyclase activity (not shown).

When the Mn^{2+} incubation mixture was supplemented with 0.25 mmol l^{-1} ATP, basal guanylyl cyclase activity was depressed to 6.35±0.64 pmol cGMP mg^{-1} protein min^{-1}

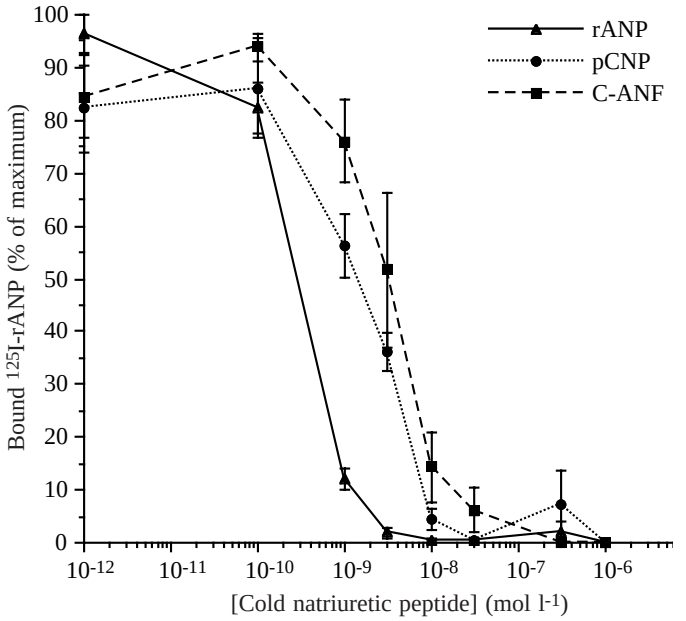

Fig. 2. Competition curves reflecting the relative abilities of rat atrial natriuretic peptide (rANP), C-type natriuretic peptide (pCNP) and C-ANF (a truncated ANP that in mammals binds only to natriuretic peptide receptor C) at increasing concentrations to compete for ^{125}I-rANP-specific binding sites in *Eptatretus cirrhatus* gill membranes. Values are expressed as means ± S.E.M. from five individual hagfish.

(Fig. 4A). This value was significantly lower than the basal guanylyl cyclase activity observed in the absence of ATP ($P \leqslant 0.05$). Although guanylyl cyclase activities due to 0.1 nmol l^{-1} rANP and 1 μmol l^{-1} rANP were significantly lower in the presence of ATP and Mn^{2+} than in the presence of Mn^{2+} alone ($P \leqslant 0.05$), guanylyl cyclase activity in the presence of 1 μmol l^{-1} rANP, ATP and Mn^{2+} was greater than basal guanylyl cyclase activity in the presence of ATP and Mn^{2+} ($P \leqslant 0.05$). The effect of ATP on pCNP-stimulated guanylyl cyclase activity was similar to its effect on rANP-stimulated guanylyl cyclase activity (Fig. 4C). Guanylyl

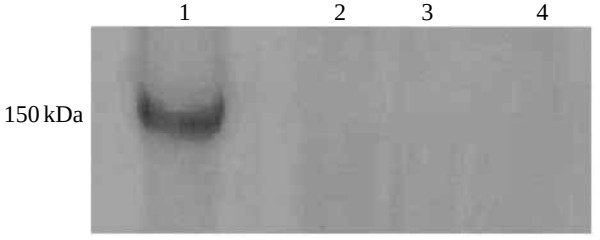

Fig. 3. Autoradiograph of SDS–PAGE under reducing conditions of affinity cross-linked ^{125}I-labelled rat atrial natriuretic peptide (^{125}I-rANP) to *Eptatretus cirrhatus* gill membranes. Lane 1, ^{125}I-rANP alone; lane 2, ^{125}I-rANP plus 1 μmol l^{-1} rANP; lane 3, ^{125}I-rANP plus 1 μmol l^{-1} C-type natriuretic peptide (pCNP); lane 4, ^{125}I-rANP plus 1 μmol l^{-1} C-ANF (a truncated ANP that in mammals binds only to natriuretic peptide receptor C). Lane 1 shows a single band at approximately 150 kDa, which is displaced in lanes 2–4 by the addition of the unlabelled peptides.

cyclase activities due to 0.1 nmol l^{-1} pCNP and 1 μmol l^{-1} pCNP were significantly lower in the presence of ATP and Mn^{2+} than in the presence of Mn^{2+} alone ($P \leqslant 0.05$). Guanylyl cyclase activity in the presence of 1 μmol l^{-1} pCNP, ATP and Mn^{2+} was greater than basal guanylyl cyclase activity in the presence of ATP and Mn^{2+} ($P \leqslant 0.05$).

The basal rate of cGMP accumulation (in the absence of ATP and natriuretic peptides) was 0.73±0.13 pmol cGMP mg^{-1} protein min^{-1} in the presence of Mg^{2+} (Fig. 4B). This is 6.2 % of the basal cGMP accumulation rate in the presence of Mn^{2+}. In the presence of 1 μmol l^{-1} rANP, guanylyl cyclase activity was elevated to 1.93±0.33 pmol cGMP mg^{-1} protein min^{-1} ($P \leqslant 0.05$). When the Mg^{2+} incubation mixture was supplemented with 0.25 mmol l^{-1} ATP, basal guanylyl cyclase activity was not significantly different from basal guanylyl cyclase activity in the absence of ATP. However, the guanylyl cyclase activity due to 1 μmol l^{-1} rANP was 5.82± 1.13 pmol cGMP mg^{-1} protein min^{-1}, which was significantly greater than basal guanylyl cyclase activity and guanylyl cyclase activity stimulated by 1 μmol l^{-1} rANP in the absence of ATP ($P \leqslant 0.05$). With Mg^{2+} present, both 0.1 nmol l^{-1} pCNP and 1 μmol l^{-1} pCNP failed to stimulate guanylyl cyclase activity above basal levels either in the presence or in the absence of ATP (Fig. 4D). However, in these experiments, guanylyl cyclase activity in the presence of 1 μmol l^{-1} pCNP was significantly greater with 0.25 mmol l^{-1} ATP than without ($P \leqslant 0.05$) (Fig. 4D).

Molecular cloning

A 630-base-pair product (excluding primer regions) was produced by PCR amplification of *E. cirrhatus* gill cDNA using degenerate natriuretic-peptide-receptor-specific primers to the conserved guanylyl cyclase domain (GenBank accession number AF231034). Alignment of the deduced amino acid sequence with published guanylyl cyclase natriuretic peptide receptor amino acid sequences from other species indicated 91 % homology with dogfish (*Squalus acanthias*) NPR-B, 88 % homology with rat NPR-A and 87 % homology with eel NPR-A. In the 17-residue catalytic domain (green lettering, Fig. 5), the hagfish amino acid sequence showed 94 % homology with dogfish NPR-B and rat NPR-A, and in the 32-residue GTP binding domain (blue lettering in Fig. 5), the hagfish sequence showed 97 % homology with dogfish NPR-B and rat NPR-A. Three of the six cysteines (red lettering in Fig. 5) were novel to the *E. cirrhatus* sequence.

Discussion

The current study demonstrates that natriuretic peptide binding sites are present in the gills of the New Zealand hagfish *E. cirrhatus* and show considerable similarity with those found in the gills of *Myxine glutinosa* (Toop et al., 1995a). In both species, natriuretic peptide receptors are linked to guanylyl cyclase activity and, when covalently bound to ^{125}I-rANP, have an apparent molecular mass of 150 kDa. This latter observation is unique to hagfishes and is in contrast to similar

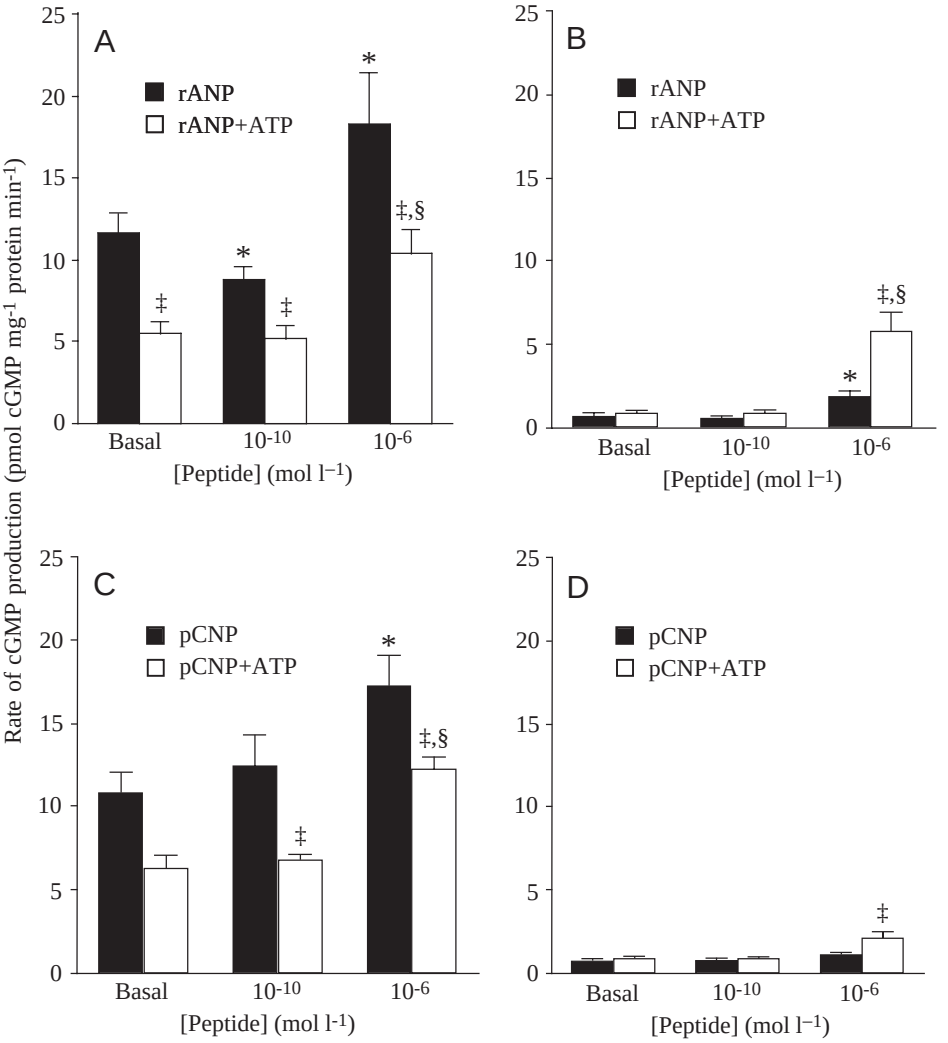

Fig. 4. Effects of rat atrial natriuretic peptide (rANP) and C-type natriuretic peptide (pCNP) on guanylyl cyclase activity in membrane preparations of *Eptatretus cirrhatus* gills. (A) Effects of rANP; the experiment was performed with 4 mmol l^{-1} Mn^{2+} in the presence and absence of 0.25 mmol l^{-1} ATP. (B) Effects of rANP; the experiment was performed with 4 mmol l^{-1} Mg^{2+} in the presence and absence of 0.25 mmol l^{-1} ATP. (C) Effects of pCNP; the experiment was performed with 4 mmol l^{-1} Mn^{2+} in the presence and absence of 0.25 mmol l^{-1} ATP. (D) Effects of pCNP; the experiment was performed with 4 mmol l^{-1} Mg^{2+} in the presence and absence of 0.25 mmol l^{-1} ATP. Data are expressed as the mean + S.E.M. of gill membranes from six individual hagfish. Asterisks (*) indicate cGMP levels that are significantly different from the basal level measured in the absence of 0.25 mmol l^{-1} ATP ($P \leqslant 0.05$). Double daggers (‡) indicate cGMP levels measured in the presence of 0.25 mmol l^{-1} ATP that are significantly different from corresponding measurements made in the absence of 0.25 mmol l^{-1} ATP ($P \leqslant 0.05$). An § indicates cGMP levels that are significantly different from the basal level measured in the presence of 0.25 mmol l^{-1} ATP ($P \leqslant 0.05$).

cross-linking experiments that, in all other species, show two binding sites on SDS–PAGE. Both *E. cirrhatus* and *M. glutinosa* have the highest density of specific binding sites for ^{125}I-rANP and ^{125}I-pCNP on the lamellar folds. The lamellar location of hagfish natriuretic peptide binding sites is consistent with observations made in other fishes (Donald et al., 1994, 1997; Toop et al., 1998). Although natriuretic peptide binding sites were not identified on branchial blood vessels, it is possible that natriuretic peptide binding over the respiratory lamellae may regulate blood flow by influencing pillar cell contraction. Evidence for the ability of endothelin-1 to regulate pillar cell contraction and branchial microcirculation has recently been provided in teleost fishes (Stenslokken et al., 1999). The presence of binding sites on the efferent cavernous tissue, which is part of the arterio-arterial vasculature in hagfishes (Elger, 1987), also suggests a regulatory role for natriuretic peptides in the gill vascular compartment.

The existence of a guanylyl cyclase receptor population in the gills of *E. cirrhatus* is demonstrated by the generation of a partial cDNA using primers designed against conserved regions of known guanylyl cyclase natriuretic peptide receptor sequences. This partial sequence shows considerable homology with dogfish NPR-B and rat and eel NPR-A (Fig. 5). Affinity cross-linking and SDS–PAGE under reducing conditions demonstrate the presence of a single receptor protein of approximately 150 kDa (Fig. 3). The apparent molecular mass is similar to that of mammalian guanylyl cyclase natriuretic peptide receptors (130–160 kDa; Brenner et al., 1990). Assays for guanylyl cyclase activity indicate that both rANP and pCNP stimulate cGMP accumulation in gill membrane preparations (Fig. 4).

In addition to the ability of 1 μmol l^{-1} rANP to stimulate cGMP production in *E. cirrhatus* gill membrane preparations, guanylyl cyclase activity is significantly lower than basal levels in the presence of 0.1 nmol l^{-1} rANP with Mn^{2+} (Fig. 4A). To our knowledge, this is the first such demonstration of a concentration-dependent bidirectional change in cGMP production elicited by a natriuretic peptide receptor. It may contribute a cellular explanation of the biphasic vasoactive responses to natriuretic peptide infusion that have been observed in *E. cirrhatus* (Glover, 1996), rainbow trout *Oncorhynchus mykiss* (Olson and Duff, 1992; Olson et al., 1997; McKendry et al., 1999), the spiny dogfish *Squalus acanthias* (McKendry et al., 1999), the toadfish *Opsanus tau*

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Ept-GC       1 NLEELVEERTQAYLEEKRKAEALLYQILPHSVAEQLKRGGTVQAEA
S.a NPR-B  826 NLEKLVEERTQAYLEEKRKAENLLYQILPHSVAEQLKWGETVQAEA
rat NPR-A  824 NLEELVEERTQAYLEEKRKAEALLYQILPHSVAEQLKRGETVQAEA
rat NPR-B  811 NLEKLVEERTQAYLEEKRKAEALLYQILPHSVAEQLKRGETVQAEA
eel NPR-A  837 NLEELVEERTQAYLEEKRKAETLLYQILPHSVAEQLKRGETVQAEA
eel NPR-B  815 NLENLVEERTQAYLEEKRKAENLLYQILPHSVAEQLKRGETVQAEA
               *** ***************** ***************.* ******

Ept-GC      47 FDSVTIYFSDIVGFTAMSAECTPMQVVTLLNDLYTCFDAIIDNFDV
S.a NPR-B  872 FDSVTIYFSDIVGFTSMSAESTPLQVVTLLNDLYTCFDAIIDNFDV
rat NPR-A  868 FDSVTIYFSDIVGFTALSAESTPMQVVTLLNDLYTCFDAVIDNFDV
rat NPR-B  857 FDSVTIYFSDIVGFTALSAESTPMQVVTLLNDLYTCFDAIIDNFDV
eel NPR-A  883 FDSVTIYFSDIVGFTAISAESTPMQVVTLLNDLYTCFDAIIDNFDV
eel NPR-B  861 FDSVTIYFSDIVGFTSMSAESTPLQVVTLLNDLYTCFDAIIDNFDV
               ***************..*** **.***************.******

Ept-GC      95 YKVETIGDAYTVVSGLPVHNGNVHAREIARMSLALLDAEHTFTIRH
S.a NPR-B  920 YKVETIGDAYMVVSGLPVRNGKLHARETARMSLALLEAVRSFIIRH
rat NPR-A  916 YKVETIGDAYMVVSGLPVRNGQLHAREVARMALALLDAVRSFRIRH
rat NPR-B  905 YKVETIGDAYMVVSGLPGRNGQRHAPEIARMALALLDAVSSFRIRH
eel NPR-A  931 YKVETIGDAYMVVSGLPVRNGKLHGREIARMSLALLDAVRSFKIRH
eel NPR-B  910 YKVETIGDAYMVVSDSQSRNGKLHAREIAGMSLALLEQVKTFKIRH
               ********** ***.   .**. *. * * *.****.   .* ***

Ept-GC     139 CPEDQLKLRIGIHTGDVCAGVGGLKMPRYCLFGDTVNTASRMESNG
S.a NPR-B  964 RPNDQLKLRIGIHTGPVCAGVVGLKMPRYCLFGDTVNTASRMESTG
rat NPR-A  960 RPQEQLRLRIGIHTGPVCAGVVGLKMPRYCLFGDTVNTASRMESNG
rat NPR-B  949 RPHDQLRLRIGVHTGPVCAGVVGLKMPRYCLFGDTVNTASRMESNG
eel NPR-A  966 RPDQQLKLRIGIHSGPVCAGVVGLKMPRYCLFGDTVNTSSRLESTG
eel NPR-B  953 RPNDQLRLRIGIHTGPVCAGVVGLKMPRYCLFGDTVNTASRMESNG
                *.**.****.*.* ***** ****************.**.** *

Ept-GC     191 EALRIHVSSATREVLEEFCSFELELR
S.a NPR-B 1010 EALKVHVSSTTKNVLDEFGSFELELR
rat NPR-A 1007 EALKIHLSSETKAVLEEFDGFELELR
rat NPR-B  995 QALKIHVSSTTKDALDELGCFQLELR
eel NPR-A 1013 EALKIHVSAATRDVLQEFNCFQLELR
eel NPR-B  999 EALKIHLSSATKEVLDEFGYFDLQLR
               .**..*.*. *.  *.*.  *.*.**
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Fig. 5. Deduced amino acid sequence of the putative *Eptatretus cirrhatus* natriuretic peptide receptor (NPR) guanylyl cyclase domain (Ept-GC) aligned with guanylyl cyclase domains from natriuretic peptide guanylyl cyclase receptors for other species: S.a, *Squalus acanthias*; rat, *Rattus norweigicus*; eel, *Anguilla japonica*. Cysteine residues are coloured red. The catalytic region is coloured green, and the GTP binding domain is coloured blue. An asterisk (*) indicates homology within all sequences; • indicates conservative amino acid changes.

(Baustian and Beyenbach, 1999) and the vasoconstriction observed in *E. cirrhatus* at low concentrations of natriuretic peptides (Glover, 1996). Just as elevated concentrations of intracellular cGMP stimulate vasodilation (Rosenzweig and Seidman, 1991), diminished concentrations presumably lead to an increase in smooth muscle tone and a concomitant vasoconstriction. Natriuretic peptides may therefore be capable of eliciting either vasodilatory or vasoconstrictive effects depending upon the local concentration of the peptides. Interestingly, guanylyl cyclase assays conducted on membrane preparations from the gills of *M. glutinosa* do not demonstrate this apparent natriuretic-peptide-mediated inhibition of guanylyl cyclase activity (Toop et al., 1995a).

We examined the effects of ATP, Mg^{2+} and Mn^{2+} on guanylyl cyclase activity in hagfish gill membranes to assess whether the regulation of hagfish gill natriuretic peptide guanylyl cyclase is similar to the regulation of mammalian natriuretic peptide guanylyl cyclases. The effect of ATP on guanylyl cyclase activity may be stimulatory or inhibitory in mammals, depending on whether Mg^{2+} or Mn^{2+} is present (Duda et al., 1996). Although ATP inhibits basal guanylyl cyclase activity in the presence of Mn^{2+}, it does does not affect the extent to which natriuretic peptides stimulate the enzyme (Duda et al., 1996). More total guanylyl cyclase activity is observed in the presence of Mn^{2+}, either alone or with ATP, than when Mg^{2+} is the cofactor. When Mg^{2+} is the cofactor, basal and natriuretic-peptide-stimulated activities are low, and ATP has no effect on basal enzyme activity. However, the percentage increase in natriuretic-peptide-stimulated guanylyl cyclase activity is much greater in the presence of Mg^{2+} than in the presence of Mn^{2+}, despite the absolute quantities of cGMP generated being smaller (Duda et al., 1996). Duda et al. (1996) concluded that the stimulatory and inhibitory domains of the natriuretic peptide guanylyl cyclase are different and separate. Interestingly, the current study reflects the observations made in mammals and indicates that the same regulatory processes for natriuretic peptide guanylyl cyclase activity have been present since the inception of the vertebrate natriuretic peptide system.

This study, like that of *M. glutinosa*, is ambiguous about the presence of non-guanylyl-cyclase-linked receptors in the hagfish gill. The existence of such a receptor population in the gills of *E. cirrhatus* is suggested by the ability of the NPR-C-specific ligand C-ANF to displace ^{125}I-rANP and ^{125}I-pCNP binding in tissue section autoradiography and its ability to displace ^{125}I-rANP in *in vitro* competition binding assays (Fig. 2) and affinity cross-linking (Fig. 3). However, in the gills of both *M. glutinosa* and *E. cirrhatus*, affinity cross-linking and SDS–PAGE under reducing conditions demonstrate the presence of a single band of approximately 150 kDa (Fig. 3). In contrast, mammalian NPR-C and eel, toadfish and lamprey NPR-C/NPR-D receptors dissociate into the monomeric species under reducing conditions and have a molecular mass of 65–75 kDa (Brenner et al., 1990; Sakaguchi et al., 1993; Donald et al., 1994; Toop et al., 1998). If a non-guanylyl-cyclase receptor population exists in the hagfishes, it is not homologous in structure to the NPR-C of mammals or the NPR-C and NPR-D of gnathostome fishes. Although the current study and that of Toop et al. (1995a) have utilised C-ANF to gain insights into the natriuretic peptide systems of hagfishes, it must be remembered that C-ANF is designed to bind specifically to mammalian NPR-C. The lack of structural homology between mammalian NPR-C and putative hagfish non-guanylyl-cyclase receptors and the demonstration that C-ANF loses specificity for mammalian NPR-C at high concentrations (Redondo et al., 1998) indicate that the ability of C-ANF to displace ^{125}I-rANP binding at high concentrations may not necessarily be indicative of non-guanylyl-cyclase receptors. However, since C-ANF at a concentration of 1 μmol l^{-1} failed to stimulate guanylyl cyclase activity in both species of hagfishes, it is clearly not an agonist of hagfish natriuretic peptide guanylyl cyclases, even if it has binding capability.

It is worth noting that the current study does not distinguish whether natriuretic peptide binding and guanylyl cyclase activity are limited to the gill plasma membranes. Such localisation in fish gills awaits clarification. Interestingly, *in*

situ examination of iodinated natriuretic peptide binding to the rat adrenal gland has identified plasma membrane and intracellular binding of ANP (Morel et al., 1988, 1989). Some studies, using ultracytochemical techniques, have localized natriuretic-peptide-stimulated particulate guanylyl cyclase activity to both the plasma membrane and some intracellular compartments (Rambotti et al., 1993, 1994, 1997). However, in other studies, such guanylyl cyclase activity was limited to plasma membranes (Rambotti et al., 1997; Tei et al., 2000).

In summary, the natriuretic peptide binding sites in the gills of *E. cirrhatus* appear to be similar to those of *M. glutinosa*. The gills of both species contain guanylyl-cyclase-linked receptors of similar molecular mass to mammalian guanylyl cyclase receptors. Because a single binding site is observed with affinity cross-linking and SDS–PAGE in both species, the lack of a second binding site discriminated by SDS–PAGE appears to be common to the hagfish lineage. In contrast to teleosts, in which large branchial populations of non-guanylyl-cyclase binding sites clear natriuretic peptides from the circulation (Olson and Duff, 1993), and lampreys and elasmobranchs, in which non-guanylyl-cyclase binding sites also predominate, the presence of non-guanylyl-cyclase receptors in the hagfishes remains unproven. The concentration-dependent bidirectional changes in guanylyl cyclase activity elicited by natriuretic peptides observed in this study may provide a basis for the bidirectional vascular actions of natriuretic peptides that have been observed in higher fishes (Olson and Duff, 1992; Olson et al., 1997; McKendry et al., 1999; Baustian and Beyenbach, 1999) and in *E. cirrhatus* itself (Glover, 1996). The similarity between the regulatory role of ATP and the divalent cofactors in the mammalian and hagfish systems, together with the sequence homology between the hagfish natriuretic peptide guanylyl cyclase and those of the gnathostomes, indicates a long evolutionary history for the coupling of natriuretic peptide receptors with guanylyl cyclase in vertebrates.

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