The Journal of Experimental Biology 201, 1799–1808 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
JEB1476

NATRIURETIC PEPTIDE BINDING SITES IN THE GILLS OF THE POUCHED LAMPREY *GEOTRIA AUSTRALIS*

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Accepted 5 March; published on WWW 12 May 1998

Summary

Iodinated atrial natriuretic peptide (ANP) binding sites were examined in the gills and ventral aorta of the adult upstream-migrating lamprey *Geotria australis* using tissue section autoradiography, *in vitro* competition analysis and affinity cross-linking, while guanylate cyclase assays were performed on gill membranes of both adult and juvenile lampreys. A partial natriuretic peptide (NP) receptor sequence was amplified using reverse transcription/polymerase chain reaction (RT-PCR). The results indicated that there was specific NP binding to the aortic endothelium and to pillar cell regions in the axial plate and secondary lamellae. In competition studies, 50 % of NP binding was abolished by 4 nmol l^{-1} rat ANP, 35 nmol l^{-1} porcine C-type NP (CNP) and 45 nmol l^{-1} C-ANF (a truncated ANP). Affinity cross-linking followed by SDS–PAGE demonstrated two binding sites at 205 and 65 kDa under non-reducing conditions and at 85 and 65 kDa under reducing conditions. Guanylate cyclase assays demonstrated that, while no NP-stimulated GC activity occurred in adult lampreys, NP-stimulated enhancement of cyclic GMP accumulation was found in juveniles in fresh water and more particularly in salt water. RT-PCR amplified a 471 base pair fragment with 68 % amino acid sequence homology to the eel natriuretic peptide receptor D (NPR-D). This study suggests that NP binding sites in the adult gill and aorta are of an NPR-C/D type, whereas an additional GC-coupled site exists in juveniles.

Key words: lamprey, *Geotria australis*, Agnatha, natriuretic peptide, natriuretic peptide receptor.

Introduction

Early research on the natriuretic peptide (NP) system in mammals gave rise to the view that the main function of NPs, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP), was to control salt and water balance *via* primary and secondary effects on the cardiovascular and renal systems (Brenner *et al.* 1990). The nomenclature of this peptide family reflects its ability to stimulate renal salt excretion, a feature which, in combination with diuretic and vasodilatatory effects, reduces the systemic blood volume and hence blood pressure. However, the most recent research on the mammalian system indicates that the functions of the above peptide family are wide-ranging and complex, and include roles in the function of the central nervous system, vascular remodelling and antiproliferation (see, for example, Anand-Srivastava and Trachte, 1993; Espiner *et al.* 1995).

In common with other peptide hormone systems, NPs require a plasma-membrane-bound receptor on target cells to mediate their effects *via* intracellular second messenger systems. There are two NP membrane-bound receptor (NPR) types, namely the guanylate-cyclase-coupled receptors (molecular mass approximately 130 kDa) that activate the guanosine 3′,5′-cyclic monophosphate (cGMP) intracellular second messenger system, and the ‘clearance’ receptor (NPR-C, a homodimer of a 65 kDa protein), which is not coupled to guanylate cyclase (GC) activity (Rosensweig and Seidman, 1991). NPR-C was originally thus named because it was believed to modulate the circulating concentrations of NPs by removing them from the blood (Maack, 1992). However, subsequent research has linked NPR-C with other second messenger systems (Anand-Srivastava and Trachte, 1993). At least two GC-linked receptors have been identified, namely NPR-A and NPR-B. Although NPR-A binds ANP preferentially, it will also bind BNP and to a lesser extent CNP, whereas NPR-B binds CNP almost exclusively.

The discovery that a mammalian peptide system was implicated in salt and water balance suggested that NPs might play a role in fish osmoregulation (Evans, 1990). Ensuing studies quickly identified NP immunoreactivity in the heart, plasma and brain of both agnathan and gnathostome fishes and demonstrated vasodilatatory and various iono- and osmoregulatory effects (Evans and Takei, 1992). The precise actions of NPs in fish still await clarification (for general reviews, see Takei and Balment, 1993; Hagiwara *et al.* 1995). The

traditional view that NPs act as saltwater hormones, because the circulating NP concentrations increase during saltwater adaptation (Evans and Takei, 1992), may need to be reappraised in the light of recent data indicating that an upregulation of gill NP receptors occurs in fresh water (Katafuchi *et al.* 1994; Takashima *et al.* 1995; Sakaguchi *et al.* 1996). Such findings become particularly relevant as there is now evidence that NPR-C may be linked with second messenger systems, as opposed to being a 'silent' clearance receptor present only to modulate circulating concentrations of NPs (Maack, 1992; Anand-Srivastava and Trachte, 1993). However, there is no doubt that the gills are a location for the clearance of NPs from the blood. Olson and Duff (1993) demonstrated that 60 % of injected ANP is removed from the circulation in a single pass through the gills of the rainbow trout *Oncorhynchus mykiss*; furthermore, when the gills were treated with an NPR-C inhibitor, the extracted proportion fell to 18 %. The influence of receptor regulation on NP function in fish has only just begun to be explored, although binding sites have been identified in the gills, kidneys, heart and vasculature of all fishes examined (Donald *et al.* 1994, 1997; Duff and Olson, 1992; Kloas *et al.* 1988; Toop *et al.* 1995*a*,*b*). Several NPRs have been cloned and sequenced from the Japanese eel *Anguilla japonica*: NPR-B (Katafuchi *et al.* 1994), NPR-C (Takashimi *et al.* 1995) and a novel NPR, NPR-D, which is related to NPR-C (Kashiwagi *et al.* 1995). Recently, a fragment of the gill NPR-C has been cloned from the dogfish *Squalus acanthias* (Donald *et al.* 1997).

Lampreys, together with the hagfishes, are the sole survivors of the jawless (agnathan) stage in vertebrate evolution (Hardisty, 1979). Lampreys, such as *Geotria australis*, undergo an anadromous migration, i.e. they breed in fresh water and feed (grow) mainly in the sea, and therefore require sophisticated mechanisms of osmotic control. The lampreys can thus be contrasted with the stenohaline hagfishes, which are essentially iso-osmotic to sea water and thus apparently have no osmotic problems in the marine environment (Hardisty, 1979) and possess a well-developed NP system (Reinecke *et al.* 1987; Evans *et al.* 1989; Kloas *et al.* 1988; Donald *et al.* 1992; Toop *et al.* 1995*a*,*b*). To date, the only study on the NP system of lampreys is that of Freeman and Bernard (1990), which indicated that the plasma ANP immunoreactivity of *Petromyzon marinus* increased following acclimation to sea water.

The following study was undertaken to characterise NP binding sites in the gills and ventral aorta of the pouched lamprey *Geotria australis* and to compare them with the NP binding site profiles that have been observed in the hagfishes, on the one hand, and in the gnathostome fishes, on the other. The juveniles (young adults) of *Geotria australis*, both prior to and after 'entry' into sea water, and the fully grown adults of this species caught following their re-entry into fresh water on their spawning run were used in order to determine whether changes occurred during the life cycle and, if so, whether they were attributable to the lamprey having been in salt water. We have used mammalian NPs as pharmacological tools to demonstrate NP binding characteristics in these tissues because the native NP is not available. In addition, we have examined NP stimulation of guanylate cyclase activity and the apparent molecular mass of putative lamprey NPRs in the gills using the heterologous NPs. We have also used polymerase chain reaction (PCR) on lamprey gill cDNA with primers designed to conserved regions of NPR-C and NPR-D to identify a partial sequence of a lamprey NPR.

Materials and methods

Animal maintenance

Adult pouched lampreys *Geotrila australis* Gray were trapped in eel traps on the Derwent River, Tasmania, as they were migrating upstream, following the completion of the marine trophic phase. They were held in running stream water at the Salmon Ponds Hatchery, Plenty, Tasmania, before either dissection at the site or shipment to Deakin University, Geelong, Australia, where they were held at 7 °C in aerated recirculating fresh water before dissection. Juvenile (young adult) lampreys were obtained just prior to their downstream migration to the sea by electrofishing in the Donnelly River, Western Australia. They were transported to Murdoch University, Perth. All fish were held in appropriate aerated or running water for at least a week prior to experiments. Half of the juveniles were progressively acclimated to full-strength sea water (35 ‰) over 3 weeks and maintained in full-strength sea water for at least a further week before use. All animals were anaesthetised in either MS222 (0.1 %) or benzocaine (0.01 %) before being killed by either decapitation or spinal cord severance caudad to the brain. The gills and ventral aorta were dissected out, snap-frozen in liquid nitrogen and stored at −70 °C until use. Adult upstream-migrating lampreys were used in all parts of this study. The freshwater (FW) and saltwater (SW) juveniles were used for guanylate cyclase assay studies only.

Autoradiography

The gills and ventral aorta of three upstream-migrating adult lampreys were freeze-mounted in Tissue Tek (Miles Inc. Elkhart, Indiana, USA) and sectioned on a microtome cryostat (Cryocut E, Reichert-Jung). Sections (18 μm) were mounted on gelatine–chromium aluminium-coated slides and dried at 45 °C for 1 h. The sections were stored in sealed boxes at −20 °C.

Autoradiography was performed according to previously established methods (Toop *et al.* 1995*a*). Briefly, sections were preincubated for 15 min at room temperature (20 °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 2 h 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} phenylmethylsulphonylfluoride (PMFS) and 0.2 nmol l^{-1} rat (3-[^{125}I]iodotyrosol^{28}) atrial natriuretic peptide ([^{125}I]ANP, 74 TBq $mmol^{-1}$; Amersham, UK). Nonspecific binding was determined in adjacent sections in the presence of 1 μmol l^{-1}

unlabelled rat 3-28 ANP (rANP, Auspep, Victoria). The ability of 1 μmol l^{-1} porcine CNP (pCNP, Auspep, Victoria) and 1 μmol l^{-1} rat des[Gln^{18},Ser^{19},Gly^{20},Leu^{21},Gly^{22}]ANP-(4-23)-NH_2 (C-ANF; Peninsula Laboratories, California, USA) to compete for specific radiolabelled NP binding sites was also determined in adjacent sections. C-ANF is a truncated ANP that binds only to NPR-C in mammals and to putative NPR-C in a range of fish tissues (Maack *et al.* 1987; Donald *et al.* 1997). An incubation time of 2 h was chosen because pilot studies had indicated that maximum binding was achieved in this time. We consider that mammalian NPs are appropriate for use as a tool because many piscine studies now confirm that the most important consideration for the specificity of ligands for NPR is the type of NP assayed (i.e. ANP, BNP, CNP or C-ANF), rather than the native ligand itself (Evans *et al.* 1993; Kashiwagi *et al.* 1995; Donald *et al.* 1997).

Slides were subsequently washed (twice for 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 for several hours at 60 °C. Sections were apposed to Hyperfilm-βmax (Amersham, UK) for 5–14 days at room temperature (20 °C). The film was processed using Kodak GBX developer (4 min), rinsed in water (2 min) and fixed with Kodak GBX fixer (5 min). To examine binding sites using light microscopy, some sections were dipped in nuclear track emulsion (Hypercoat, Amersham, UK) according to the manufacturer's instructions. Sections were stored for 2 weeks at 4 °C, before development as above. Following development, the sections were stained with haematoxylin and eosin and then photographed.

Membrane preparation

Membrane preparations from the gills of individual adult upstream-migrant lampreys were used for competition binding studies and affinity cross-linking and sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS–PAGE). For guanylate cyclase assays, membrane preparations were obtained from the gills of upstream-migrant adults and FW- and SW-acclimated juveniles. Frozen gills were homogenised in 5 ml of ice-cold 50 mmol l^{-1} Tris–HCl and 1 mmol l^{-1} $NaHCO_3$ (pH 7.4). 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 through gauze and subsequently 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 using a BCA protein assay kit (Pierce) calibrated against bovine serum albumin standards. Membranes were stored at −20 °C and used within 1 month of preparation.

Radioligand binding assays

For competition binding studies, 50 μg of gill membrane protein was incubated in 250 μl of the same incubation buffer that was used for autoradiography plus 25 pmol l^{-1} [^{125}I]ANP with the unlabelled peptides rANP, C-ANF and pCNP present in increasing concentrations (10^{-12} to 10^{-6} mol l^{-1}). Mixtures were incubated for 2 h and the reaction was stopped with the addition of 2 ml of ice-cold 50 mmol l^{-1} Tris–HCl (pH 7.4). The bound ligand was separated from the free ligand by vacuum-filtering through Whatman GF/C filters treated with 1 % polyethylenimine. Filters were rinsed with 5 ml of the ice-cold stop solution, and radioactivity was counted on a Minax gamma Auto-gamma 5000 series (United Technologies, Packard Instrument Company, Illinois, USA) at 75 % efficiency.

Affinity cross-linking

Gill membrane protein (75 μg) was incubated in incubation buffer with 0.25 nmol l^{-1} iodinated peptide in the presence or absence of excess unlabelled rANP or C-ANF. The final incubation volume was 250 μl. Affinity cross-linking was performed using the method described by 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 left to react for 20 min at 20 °C. The activity of the cross-linker was arrested 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 a benchtop microcentrifuge at 13,800 ***g*** for 20 min to pellet the membranes and bound hormone, which were resuspended in 30 μl of either nonreducing or reducing buffer for SDS–PAGE (62.5 mmol l^{-1} Tris–HCl, pH 6.8, 2 % SDS, 5 % glycerol, 0.01 % Bromophenol Blue; 2 % β-mercaptoethanol was added for reducing gels) and then boiled for 4 min. Samples, including one of molecular mass markers (30 000–200 000 kDa), were loaded onto a 7.5 % unidimensional polyacrylamide gel and electrophoresed at 200 V. Gels were stained with Coomassie Brilliant Blue (Bio-Rad), dried, and apposed to Hyperfilm MP (Amersham, UK) for 2 weeks at 20 °C. Films were developed as described above. 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, and the apparent molecular masses are reported as averaged values from three gels.

Guanylate cyclase assays

Potential GC activity associated with the binding of NPs to gill membranes was assayed according to previously published protocols (Toop *et al.* 1995*a*). Gill protein (50 μg) was assayed in GC buffer (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 cGMP generation was determined in tubes without ligand. Following a 15 min incubation at 20 °C, the reaction

was 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 cGMP content was determined by radioimmunoassay (cGMP RIA kit, Amersham, UK). Radioactivity was counted on a 2000 CA Tri-Carb liquid scintillation analyser (United Technologies, Packard Instrument Company, Illinois, USA). Adult upstream-migrating and FW and SW juveniles were used in GC assays. Data were analysed by analysis of variance (ANOVA) using Statview SE.

Molecular cloning

Gill total RNA was isolated by the acid-phenol technique (Chomczynski and Sacchi, 1987). Total RNA was transcribed into first-strand cDNA using SuperScript II RT reverse transcriptase according to the manufacturer's (Life Technologies) protocol. Degenerate primers, based on conserved regions from aligned bovine, human and eel NPR-C cDNA sequences, were used for PCR, and computer analysis predicted amplification of a 470 base pair (bp) product. PCR was carried out using the following 50 µl reaction mixture: 1×PCR buffer, dNTPs, 0.8 µmol l^{-1} forward and reverse primers, *Taq*Bead Hot Start Polymerase (Promega), 6 mmol l^{-1} $MgCl_2$ and 50 ng of gill cDNA. An initial cycle of 5 min at 94 °C, 2 min at 60 °C and 2 min at 75 °C was followed by 34 cycles of 1 min at 94 °C and 2 min at 60 °C, dropping to 50 °C by the seventh cycle, and 2 min at 75 °C. 10 µl of the PCR reaction was run on a 1 % agarose gel to confirm that a band was present at approximately 470 bp. The remainder of the PCR reaction was precipitated in isopropanol and 4 mol l^{-1} ammonium acetate. The precipitated DNA was cloned into a pCR2.1 plasmid (Invitrogen), and five positive clones were isolated and sequenced on an Applied Biosystems automated sequencer (Westmead Hospital, Sydney, Australia) in both directions. The sequence was analysed using BLAST (National Centre for Biotechnology Information).

Results

Autoradiography and competition binding

Specific [^{125}I]ANP binding was observed on the endothelial layer of the ventral aorta of the adult upstream-migrant lampreys (Fig. 1A, arrowhead), this binding being displaced by the addition of 1 µmol l^{-1} rANP (Fig. 1B), pCNP and C-ANF (not shown). In order to orient the reader to the cryostat sections of the gill used in this study, a formalin-fixed, paraffin-wax-embedded section of a gill filament in a transverse-oblique plane was cut and stained with haematoxylin and eosin and labelled to illustrate the regions of interest (Fig. 2). Emulsion-dipped cryostat sections showed specific [^{125}I]ANP binding in the adult upstream migrant gill (Fig. 3A,C,E), which was completely displaced by cold rANP (Fig. 3B,D,F, pCNP and C-ANF (not shown). Binding in the gill appeared to be confined to the endothelial area of the secondary lamellae (Fig. 3C,E), the marginal channels (Fig. 3A) and axial plate (Fig. 3C,E). From the globular binding pattern in the secondary lamellae, it appears likely that binding occurred over regions of pillar cells (Nakao and Uchinomiya, 1978). Pillar cells are also found in the axial

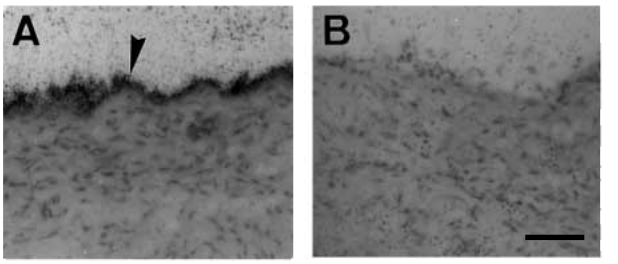

Fig. 1. Photomicrographs of X-ray-sensitive emulsion-dipped longitudinal sections through the ventral aorta of an adult upstream-migrant *Geotria australis*. (A) Silver grains indicate the specific binding of [^{125}I]ANP to the vascular endothelium (arrowhead). (B) Specific binding is abolished in the presence of 1 µmol l^{-1} rANP. pCNP and C-ANF also abolished specific binding in the same manner as shown in B. Scale bar, 50 µm.

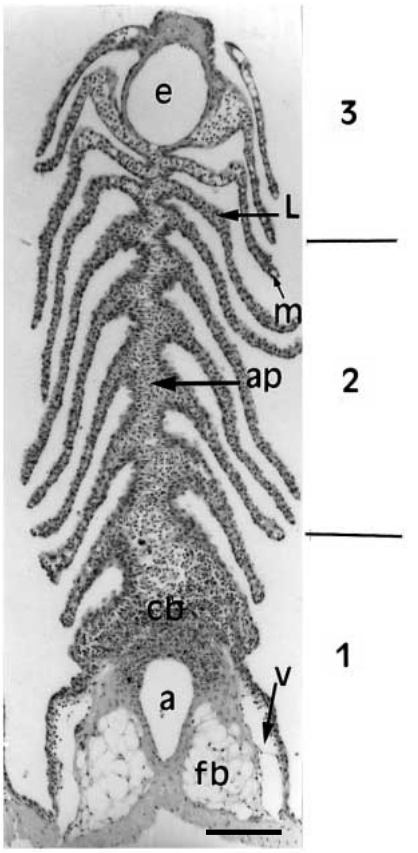

Fig. 2. Photomicrograph of an oblique-transverse section through a gill filament of an adult upstream-migrant *Geotria australis*, stained with haematoxylin and eosin. Regions 1, 2, and 3 refer to regions of gill shown in Fig. 3; e, efferent filamental artery; L, secondary lamella; m, marginal channel; ap, axial plate; cb, cavernous body; fb, fat body; v, filament vein; a, afferent filamental artery. Scale bar, 100 µm.

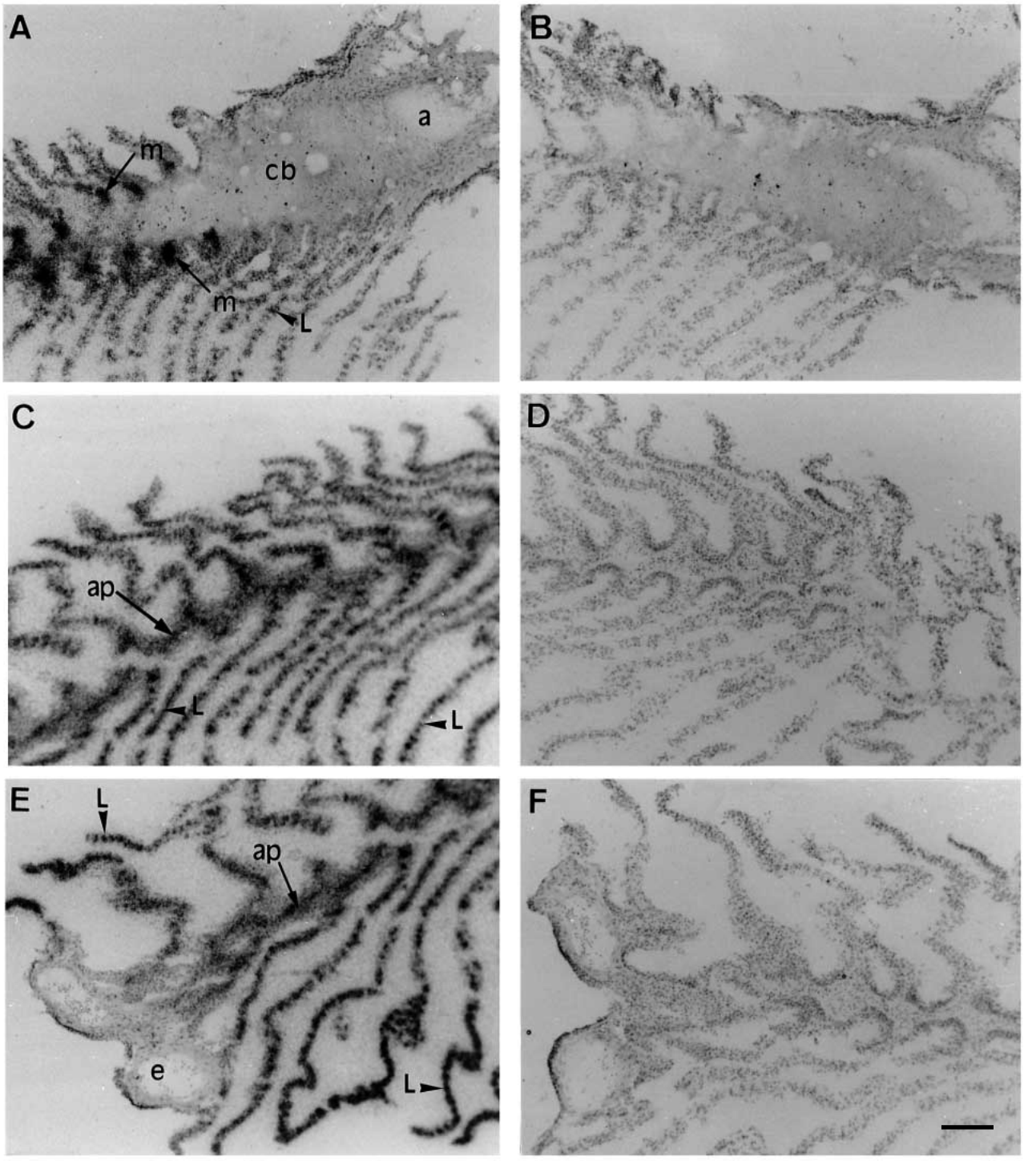

Fig. 3. Photomicrographs of X-ray-sensitive emulsion-dipped serial oblique-transverse sections through a gill filament of an adult upstream-migrant *Geotria australis*. A and B correspond to region 1 in Fig. 2, C and D to region 2 in Fig. 2, and E and F to region 3 in Fig. 2. (A) Specific [^{125}I]ANP binding occurs in the afferent region of the filament. Binding is observed on the marginal channels (m) and secondary lamellae (L). No binding is observed on cavernous body (cb) or on the afferent filamental artery (a). (C) Specific [^{125}I]ANP binding in the central region of the filament to the axial plate (ap) and the secondary lamellae (L). (E) Specific [^{125}I]ANP binding in the efferent region of the gill. Specific binding occurs on the axial plate (ap) and the secondary lamellae (L), but not on the efferent filamental artery (e). (B,D,F) All specific binding is abolished by the addition of 1 μmol l^{-1} rANP. Results from the addition of 1 μmol l^{-1} pCNP and C-ANF were identical. Scale bar, 100 μm.

plate, but whether binding occurs over pillar cells in this area cannot be established. No specific binding was observed on the afferent or efferent filament arteries or on epithelial tissue (Fig. 3A,E).

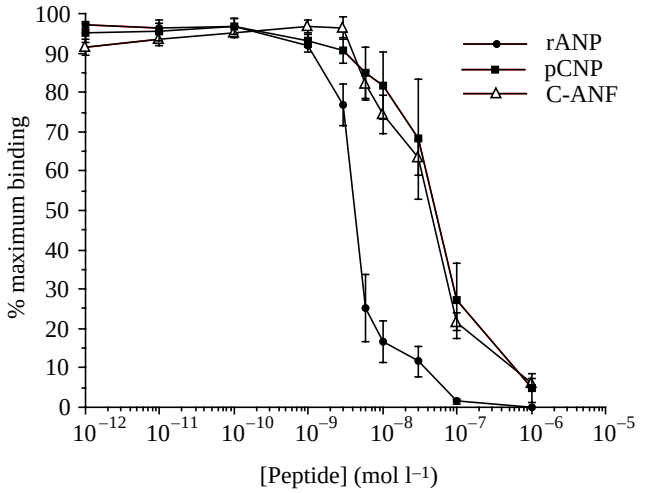

Fig. 4. Competition curves, reflecting the relative abilities of rANP, pCNP and C-ANF at increasing concentrations to compete for [^{125}I]ANP-specific binding sites. Values are means ± S.E.M. for eight individual gill membrane preparations from adult upstream-migrating *Geotria australis*.

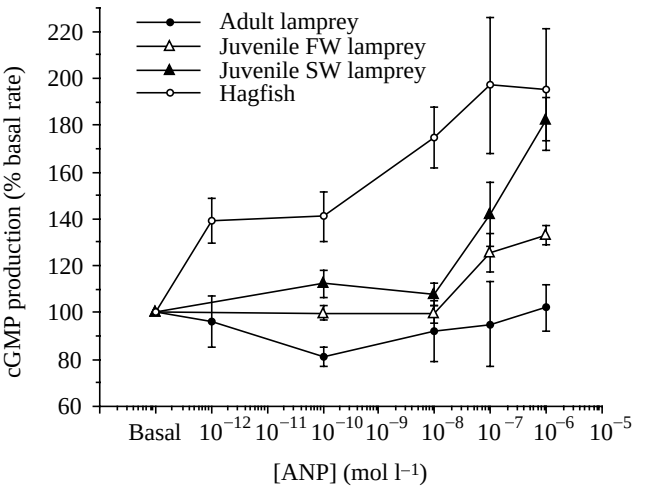

Fig. 5. Relative effects of increasing concentrations of rANP on cGMP production in gill membrane preparations from adult upstream-migrating lampreys (*Geotria australis*), juvenile lampreys in fresh water (FW) and salt water (SW) and Atlantic hagfish *Myxine glutinosa* (data from Toop *et al.* 1995*a*). Value are means ± S.E.M. for membrane preparations from five individuals in each group.

Competition studies of the adult upstream-migrant gill membranes showed a dose-dependent decrease in [^{125}I]ANP binding with increasing concentrations of cold peptides (Fig. 4). rANP competed for 50 % of iodinated binding sites at 4 nmol l^{-1}, whereas pCNP and C-ANF competed for 50 % of the sites at 35 and 45 nmol l^{-1} respectively.

Guanylate cyclase assays

Rat ANP failed to stimulate guanylate cyclase activity above basal rates in isolated gill membrane preparations from the adult upstream-migrating lamprey (Fig. 5). Porcine CNP and C-ANF also failed to stimulate cGMP production in these adults (not shown). However, 0.1 µmol l^{-1} and 1 µmol l^{-1} rANP stimulated cGMP production above basal rates in both FW- and SW-acclimated juvenile lampreys (P=0.0001, Fig. 5). In FW juveniles, the rates increased to 126±8 % and 133±4 % (means ± S.E.M.) of basal rates at 0.1 µmol l^{-1} and 1 µmol l^{-1} rANP, respectively. In SW juveniles, the rates increased above basal rates to 142±14 % at 0.1 µmol l^{-1} rANP and to 183±9 % (means ± S.E.M.) at 1 µmol l^{-1} rANP. There was no significant difference between the rates in FW and SW juveniles, except that the rate for SW juveniles at 1 µmol l^{-1} rANP was significantly different from all other lamprey values (P=0.002). Neither pCNP nor C-ANF was assayed for guanylate cyclase stimulation in juveniles in this study. Stimulation of guanylate cyclase activity by rANP in gill membranes isolated from the Atlantic hagfish *Myxine glutinosa*, previously described by Toop *et al.* (1995*a*), is also shown for comparison (Fig. 5).

Affinity cross-linking and SDS–PAGE

Affinity cross-linking of [^{125}I]ANP to gill membranes followed by SDS–PAGE under non-reducing conditions revealed two binding sites with the apparent molecular masses of 205 kDa and 65 kDa (Fig. 6A, lane 1). The 65 kDa band was of greater density than the 205 kDa band. Both bands were

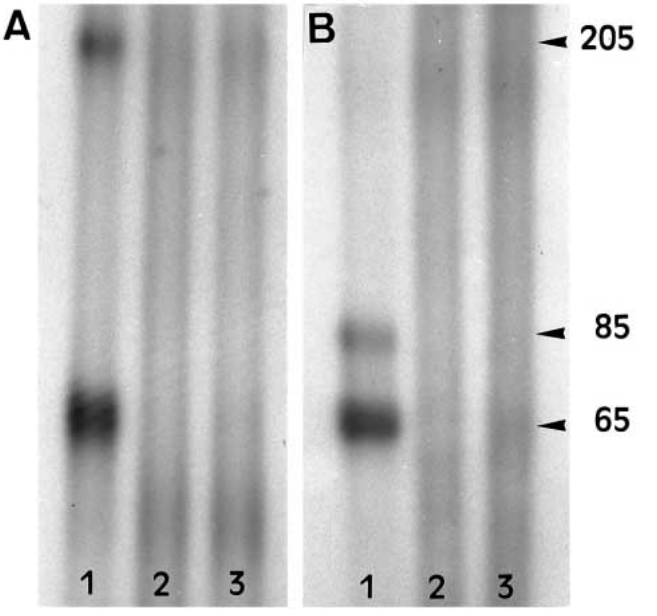

Fig. 6. Autoradiograph of SDS–polyacrylamide gel electrophoresis of affinity cross-linked [^{125}I]ANP to gill membrane binding sites from adult upstream-migrant *Geotria australis* under non-reducing (A) and reducing (B) conditions. Specifically labelled bands indicate apparent molecular masses of 205 and 65 kDa under non-reducing conditions (A, lane 1), and 85 and 65 kDa under reducing conditions (B, lane 1). Cross-linking of radiolabelled ligand was inhibited by the presence of 1 µmol l^{-1} rANP (A and B, lanes 2) and 1 µmol l^{-1} C-ANF (A and B, lanes 3).

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GAGCGGAATTGTTATTTCACAATGGAAGGGGTGCACCTCAAATTCATGGAGGAATATCAG  60
E  R  N  C  Y  F  T  M  E  G  V  H  L  K  F  M  E  E  Y  Q

ACTGTCACCTATGCCATCAACTCTAAAGATGAGCGCCTGAACACAGACGAAATCATCAAA 120
T  V  T  Y  A  I  N  S  K  D  E  R  L  N  T  D  E  I  I  K

TATATCTACGAGAGTGAAGTTGTTATCATGTGTGCGGGAGCTGACATGGTCCGAGACATC 180
Y  I  Y  E  S  E  V  V  I  M  C  A  G  A  D  M  V  R  D  I

ATGCTAGCTGCTCATCGACGGAGGCTCACTAATGGCAGCTACATCTTCTTCAACATCGAA 240
M  L  A  A  H  R  R  R  L  T  N  G  S  Y  I  F  F  N  I  E

CTCTTCAACTCCACATCTTATGGCAACGGCTCGTGGAAGCGAGGGGATAAGCACGACAGT 300
L  F  N  S  T  S  Y  G  N  G  S  W  K  R  G  D  K  H  D  S

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

GAATTTGAGCAATTCTCTCTAGAGGTCAAGAGGTCCATTCAGAAGGCTGGCCTACCGGAC 420
E  F  E  Q  F  S  L  E  V  K  R  S  I  Q  K  A  G  L  P  D

TGTGACGACTGTGACAACATCAACATGTTCATCGAAGGCTTTCACGATGCT          471
C  D  D  C  D  N  I  N  M  F  I  E  G  F  H  D  A
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Fig. 7. Nucleotide sequence and deduced amino acid sequence of a PCR-amplified 471 base pair cDNA fragment of putative NPR-D from lamprey *Geotria australis* gill. Primer sites are underlined. Cysteine residues appear in bold underlined type. Numbers to the right indicate the base pair number.

displaced by the addition of 1 μmol l^{-1} rANP and C-ANF (Fig. 6A, lanes 2 and 3, respectively). Under reducing conditions, the 65 kDa band remained the dominant band, whereas the 205 kDa band was replaced by a band with an apparent molecular mass of 85 kDa (Fig. 6B, lane 1). Both bands were again displaced by 1 μmol l^{-1} C-ANF and rANP (Fig. 6B, lanes 2 and 3, respectively).

Molecular cloning

PCR of cDNA isolated from the lamprey gill, using degenerate primers specific for NPR-C, resulted in the amplification of a 471 bp product that was subsequently cloned and sequenced; the amino acid sequence was deduced from the resulting nucleotide sequence (Fig. 7). Computer comparison of the amino acid sequence with known NPR-C and NPR-D protein sequences demonstrated the closest homology with the eel NPR-D (68 %) and a 63 % homology with the eel NPR-C. The cysteines at amino acid positions 4 (part of the forward primer sequence), 51 and 141 were conserved between the eel NPR-D and the lamprey sequence (all cysteines are indicated in bold and numbered according to the lamprey sequence in Fig. 8); the cysteines at positions 4 and 51 are also conserved in vertebrate NPR-C sequences. An additional cysteine is present in the lamprey sequence (position 144) that appears to be unique (Fig. 8).

Discussion

The location of [^{125}I]ANP binding sites in the adult upstream migrants of *Geotria australis* demonstrates that specific binding occurred in the endothelium of the ventral aorta (Fig. 1) and in large areas of the gill endothelium and particularly the pillar cell regions (Fig. 3). Binding was also greater in the efferent than in the afferent parts of the gill (Fig. 3). The fact that specific binding of [^{125}I]ANP to the ventral aorta was displaced by 1 μmol l^{-1} rANP, pCNP and C-

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laNPR     1  ERNCYFTMEGVH--LKFMEEYQTVTYAINSKDERLNTDEIIKYIYESE-VVIMCAGADMVR
eNPR-D  176  QRNCYFTLEGVHNILK-TENVHIDALNIHSKDNKVDSDEIIKLIYDSE-VIIMCAGADIIR
              ++++++ ++++  ++  +        + +++     +++++ ++ ++ + +++++++  +
eNPR-C  175  ERNCYFTIEGVHSSLH-VEGYKVDSVVIH-KDHRVETDEIIKDIYKTEAVVVMCAGGDTVR
sNPR        ERNCYFTVEGVHMAFVGQDYSMHSISI-YDEEDQVD--HIVQEIQDNARIVIMCASSHTIR
bNPR-C  206  ERNCFFTLEGVHEVF--QEEGLHTSAYNFDETKDLDLEDIVRHIQASERVVIMCASSDTIR
              *** ** ****                          *   *       ***     *

laNPR    59  VRDIMLAAHRRRLTNGSYIFFNIELFNSTSYGNGSWKRGDKHDSEARQEYSALNTVTLLRT
eNPR-D  233  IRDIMLAAHRRRLTNGSYIFFNIELFNSSSYGNGSWKRGDKFDMDAKQAYATLNTVTLLRT
              ++++++++++++++++++++++++++ ++++++++++ +  + + +  +++++++++
eNPR-C  232  VRDIMLAAHRRRLTSGGYIFFNIELFNSSSYGDGSWRRGDKYDAEAKLAYSALNVVTLMRT
sNPR        IRKIMLAAHRQGMTNGDYVFFNIELFNSSLYGNGSWKQGDKFDPEAKQAYQSLQTVTLLRT
bNPR-C  263  IRGIMLAAHRHGMTSGDYAFFNIELFNSSFYGDGSWKRGDKFDPEAKQAYQSLQTVTLLRT
              * *******   * * * *********  ** ***  *** *  *   *  *   ** **

laNPR   120  VKPEFEQFSLEVKRSIQKAGLPDCDDCDNINMFIEGFHDA
eNPR-D  294  VKPEFEDFSMEVKKSLQKAGIRHCDS-DNINVFVEGFHDA
             ++++++ ++ +++ + ++++   ++  ++++ + ++++++
eNPR-C  293  AKAEFETFTTEVKKSIQRAGIGP--DSANVNMFMEGFHDA
sNPR        AKPEFHKFAADMRNKSRHQ-YDLTSEGDNVNMFVEGFHDA
bNPR-C  324  AKPEFEKFSMEVKSSVEKQ--GL-SEEDYVNMFVEGFHDA
              * **  *                    * * ******
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Fig. 8. Amino acid sequence of the putative NPR-D fragment from lamprey *Geotria australis* gill (laNPR) compared with the same region of the eel NPR-D (eNPR-D) sequence and various NPR-C sequences. + indicates homology between eel NPR-D and the lamprey sequence; * indicates homology with eNPR-D, shark NPR (sNPR) and bovine NPR-C (bNPR-C). Cysteines are in underlined bold type. Numbers to the left correspond to the respective amino acid positions in the lamprey fragment, the eel NPR-D and the eel and bovine NPR-C. Sequences for comparison were obtained from BLAST (National Centre for Biotechnology Information).

ANF contrasts with the situation regarding binding to the vascular endothelium which occurs in the hagfish *Myxine glutinosa*, in which specific [^{125}I]ANP binding was only displaced by rANP and [^{125}I]CNP failed to bind (Toop *et al.* 1995*b*). The displacement of binding in the lamprey aorta strongly suggests that the major binding site is of the NPR-C/D type, which is consistent with the fact that the predominant NPR in the mammalian vasculature is NPR-C (Anand-Srivastava and Trachte, 1993). The distribution of NP binding sites in the lamprey gill is similar to that of [^{125}I]CNP binding sites in the gill of the dogfish *Squalus acanthias* (Donald *et al.* 1997) and to some extent that of NP binding sites in the hagfish *Myxine glutinosa* (Toop *et al.* 1995*a*), although it is not known whether binding occurred over epithelial or endothelial tissue in the respiratory lamellae of the latter agnathan. Specific [^{125}I]ANP binding sites in the teleost gill, however, seem to vary according to species. In the toadfish *Opsanus beta*, NP binding occurs to a greater extent on the gill vasculature than on the secondary lamellae but, as in the lamprey, appears to be more concentrated in the efferent than in the afferent regions of the gill (Donald *et al.* 1994). In the eel *Anguilla japonica*, strong specific binding was observed in the chondrocytes of the gill cartilage, whereas only weak binding occurred in the secondary lamellae and interlamellar cells (Sakaguchi *et al.* 1993). In the antarctic fishes *Chionodraco hamatus* and *Pagothenia bernacchii*, NP binding was densest on the interlamellar chloride cells, implying that, in these cells, NPs play a role in ion transport (Uva *et al.* 1993). Specific binding in the trout is located on the efferent vasculature and also on the chondrocytes in gill cartilage (M. Powell, personal communication).

It is clear from binding studies in the gills of all fishes studied, and now also in the lamprey, that the majority of sites are probably of the NPR-C/D type. In the present study, displacement of specific binding in both autoradiography and competition assays indicates that CNP and C-ANF compete for [^{125}I]ANP binding sites and displace virtually all of the specific binding at 1 μmol l^{-1}, which suggests that a 'clearance'-type receptor is present. The lack of NP-stimulated cGMP production in upstream-migrant lampreys indicates that a GC-type binding site is absent at the level of sensitivity of this assay (Fig. 5). This finding contrasts with the fact that NP-stimulated GC activity occurs in the gills of the hagfish, dogfish and toadfish in salt water, as determined by GC assay (Donald *et al.* 1994, 1997; Toop *et al.* 1995*a*). Additionally, in upstream-migrant adult lampreys, CNP and C-ANF failed to stimulate GC activity. In the case of lampreys, ANP GC stimulation would appear to be linked to developmental stage as well as to environmental salinity, since the NP-stimulated GC activity in FW-acclimated juveniles was not as pronounced as that observed in SW-acclimated juveniles (Fig. 5). This observation suggests that a developmental trigger initiates the process of GC receptor upregulation before the juveniles have reached salt water, but that the stimulation of GC activity by NP is not fully developed until the animals are in 100 % salt water. It would be interesting to determine whether the reverse situation is true for adult upstream migrants, in which NP-stimulated GC activity might be progressively arrested as the adults enter the estuaries. Future studies will also determine whether CNP stimulates guanylate cyclase activity in juveniles. Interestingly, Takei and Balment (1993) report that stimulation of GC activity by ANP occurred in saltwater but not freshwater trout *Oncorhynchus mykiss*, thereby mirroring the results observed here in juvenile and adult lampreys (Fig. 5).

The apparent molecular mass of binding sites, as shown by affinity cross-linking and SDS–PAGE (Fig. 6), differs from the apparent molecular mass of hagfish binding sites, in which a single site was revealed under reducing conditions at 150 kDa (Toop *et al.* 1995*a*). This binding site was displaced by rANP, but only partially by C-ANF. In the toadfish, NP binding sites were observed at 140 and 75 kDa under reducing conditions (Donald *et al.* 1994). In a two-receptor system, the GC receptors would be expected to migrate at approximately 130–140 kDa under reducing conditions, whereas the monomer of the dimeric NPR-C receptor would be expected to migrate at approximately 65–75 kDa. The size of the binding site of the hagfish therefore appears to be close to that of an expected GC receptor, whereas the toadfish appears to have two sites with the approximate molecular masses observed for both GC and NPR-C receptors. In contrast, a band at 65 kDa appears under reducing conditions in upstream-migrant lampreys, which is consistent with an NPR-C type, but an additional band at 85 kDa appears, with no apparent band at the size predicted for a GC receptor (Fig. 6B). In view of the lack of GC activation by NPs in the FW upstream migrant, it is not surprising that there is no evidence of a GC receptor from affinity cross-linking. However, the additional 85 kDa band still needs to be accounted for. Under non-reducing conditions (Fig. 6A), the 65 kDa band remains, but the 85 kDa band is lost and a 205 kDa band appears, suggesting that the 85 kDa site is an oligomeric species in the membrane. The presence of a strong band at 65 kDa is surprising since NPR-C is dimeric under non-reducing conditions and migrates at approximately 130–140 kDa, together with the GC-type NPR. Whether this finding may be interpreted as a monomeric form of NPR-C in the membrane is not known. Analysis of NP affinity cross-linking and SDS–PAGE of membranes from cultured COS-7 cells expressing the novel eel receptor NPR-D revealed a binding pattern similar to that observed in the lamprey (Kashiwagi *et al.* 1995). The eel NPR-D is reported to be a tetramer of approximately 240 kDa. However, instead of being reduced to an 85 kDa protein, as is the case in the lamprey, it forms a 65 kDa monomer on reduction (Kashiwagi *et al.* 1995).

Additional evidence from our lamprey study that a major binding site in the lamprey gill is NPR-D is provided by the fact that the PCR product of a fragment of NPR shows considerable homology to the eel NPR-D (Figs 7, 8). PCR primers were designed to amplify NPR-C, and coincidently NPR-D, fragments from the extracellular binding domains of

these NPRs. Five separate clones from the resultant PCR band were sequenced, each producing the same sequence, which is shown in Figs 7 and 8. Apart from showing the greatest sequence homology to the eel NPR-D (68 %), the sequence also contained a cysteine residue at position 141, which was hitherto unique to the eel NPR-D and is not shared with the NPR-C from any other species examined (Fig. 8). Additionally, the lamprey sequence contains a novel cysteine residue at position 144, which is not observed in either the NPR-C or the NPR-D, indicating that the tertiary and/or quaternary structure may be different in the lamprey. The exact identification and structure of this lamprey NPR will be revealed by complete sequencing and molecular analysis.

In summary, the NP binding sites in the gill and ventral aorta of the lamprey *Geotria australis* are not similar to those of the hagfish *Myxine glutinosa* (Toop *et al.* 1995*a*,*b*). This lack of similarity between these representatives of the two extant agnathan groups is consistent with the current view that lampreys and hagfishes are not closely related (Forey and Janvier, 1993). Indeed, since lampreys are now regarded as more closely related to gnathostomes than the hagfishes, it is relevant that NP binding sites in the lampreys appear to be mainly of a non-GC type (i.e. NPR-C/D-like), as is the case in gnathostomous fishes. The location of binding sites in the region of pillar cells suggests either that the binding site in question has a clearance function to remove NPs from the circulation or that the binding site is linked to second messenger systems other than cGMP, as has been suggested to be the case with the mammalian NPR-C (Anand-Srivastava and Trachte, 1993). The clearance of NPs at the gills from the lamprey circulation is indeed a possibility because the majority of the NPRs are located on the pillar cells, which are in direct contact with the perfused blood. Clearance of NPs at the gills has already been observed in trout (Olson and Duff, 1993) and is therefore also likely in the lamprey, although this must await confirmatory studies. In addition, since pillar cells may have contractile properties, enabling blood flow through the lamellar spaces to be regulated (Farrell *et al.* 1980), it is possible that NPs regulate blood flow through the lamellae by binding and stimulating non-cGMP second messenger systems in pillar cells. While the gills of FW adult migrants clearly lack NP-stimulated GC activity, indicative of GC-type receptors, ANP-stimulated GC activity is present in FW juveniles and to an even greater extent in SW juveniles. This suggests that there is a developmental/environmental switch that results in an upregulation of a putative GC-type NPR in the saltwater phase of the life-cycle.

Our gratitude is expressed to David Morgan for collecting juvenile *Geotria australis* and to Andrew Sanger and Kevin Langey of the Inland Fisheries Commission, Hobart, Tasmania, for the provision of the adults. John Donald is thanked for his photographic assistance and comments on the manuscript. Financial support was provided to I.C.P. by the Australian Research Grants Committee and to T.T. by the School of Biological and Chemical Sciences, Deakin University.

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