

NATRIURETIC PEPTIDE RECEPTORS IN THE KIDNEY AND THE VENTRAL AND DORSAL AORTAE OF THE ATLANTIC HAGFISH *MYXINE GLUTINOSA* (AGNATHA)

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Accepted 12 May 1995

Summary

The character of natriuretic peptide receptors (NPRs) in the kidney and aortae of the Atlantic hagfish *Myxine glutinosa* was determined and compared with that of NPRs in hagfish gills. The relationship of hagfish kidney and aortic NPRs with NPRs from higher vertebrates was also examined. Iodinated atrial and C-type natriuretic peptides (NPs) (^{125}I -ANP, ^{125}I -CNP) were used in tissue section autoradiography, competition studies and guanylate cyclase (GC) assays. Rat atrial and porcine C-type NPs (rANP, pCNP) and rat des[Gln¹⁸, Ser¹⁹, Gly²⁰, Leu²¹ Gly²²]ANP-(4-23)-NH₂ (C-ANF, which binds to the mammalian and teleost 'clearance' receptor, NPR-C), were used as competing ligands. ^{125}I -ANP binding sites were observed on both aortae and on the glomeruli, neck segments and archinephric ducts of the kidney. 4.0 nmol l^{-1} rANP competed for 50% of ^{125}I -ANP glomerular sites.

^{125}I -CNP did not visibly bind to any of the tissues, but 300 nmol l^{-1} pCNP competed for 50% of ^{125}I -ANP glomerular sites. C-ANF failed to compete for ^{125}I -ANP sites. rANP and pCNP stimulated cyclic GMP production in kidney membrane preparations, but C-ANF did not, demonstrating that the hagfish kidney NPR is GC-linked. This study suggests that a predominant population of ANP-like receptors, similar to the mammalian NPR-A, exists in the myxinoid aortae and kidney tissue. However, no detectable population of a receptor that binds all NPs, such as is present in the hagfish gill, nor an NPR similar to the NPR-C of higher vertebrates was discovered.

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

Introduction

The discovery by de Bold *et al.* (1981) of the potent diuretic and natriuretic effects of atrial extracts on the mammalian kidney led to the swift isolation and sequencing of atrial natriuretic peptide (ANP; Atlas *et al.* 1984; Currie *et al.* 1984) and, subsequently, to the identification of the other members of the NP family (BNP, Sudoh *et al.* 1988; CNP, Sudoh *et al.* 1990). ANP enhances glomerular filtration rate (GFR) by affecting a number of haemodynamic and glomerular mechanisms (Zeidel and Brenner, 1987; Brenner *et al.* 1990; Awazu and Ichikawa, 1993). In addition to increasing GFR, ANP directly inhibits Na⁺ reabsorption in the inner medullary collecting duct and directly and indirectly inhibits water reabsorption by altering hydraulic pressure gradients and inhibiting antidiuretic hormone (Zeidel and Brenner, 1987; Brenner *et al.* 1990). ANP also indirectly affects Na⁺ transport by inhibiting the renin-angiotensin system (Brenner *et al.* 1990; Awazu and Ichikawa, 1993). Recently, a kidney-specific NP, named urodilatin, has been isolated from human urine; it has the same structure as ANP but with an additional four

amino acid residues on the NH₂ terminus (Schultz-Knappe *et al.* 1988). Urodilatin is probably the result of post-translational processing of the ANP gene expressed in kidney tissue (Abassi *et al.* 1992; Greenwald *et al.* 1992). Urodilatin binds to the same receptor sites as ANP in the kidney and stimulates cyclic GMP production to the same extent, implying the use of the same receptors for both peptides (Valentin *et al.* 1993).

Natriuretic peptide receptors have been characterized in higher vertebrates as belonging to two distinct types: those linked to guanylate cyclase (GC), the ANP-specific receptor NPR-A (which also binds BNP) and the CNP-specific receptor NPR-B; and the 'clearance' type receptor, NPR-C, that binds all NPs, including ring-opened and ring-deleted versions (Koller and Goeddel, 1992; Maack, 1992). Natriuretic peptide receptors in the various regions of the mammalian kidney have been extensively analyzed (Anand-Srivastava and Trachte, 1993). ANP binding is located in the glomeruli, the proximal tubule, the ascending limb of the loop of Henle and the collecting ducts; the majority of receptors found in the kidney

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are either ANP guanylate-cyclase-linked receptors (NPR-As) or 'clearance' receptors (NPR-Cs). Most studies confirm a predominance of NPR-As in the collecting ducts and papillary regions of the kidney and a predominance of NPR-Cs in the glomeruli and cortical regions (Anand-Srivastava and Trachte, 1993). Recently, a guanylate-cyclase-linked CNP receptor (NPR-B) mRNA has been located using the polymerase chain reaction in the human kidney, suggesting a role for CNP in the kidney (Canaan-Kuhl *et al.* 1992; Zhao *et al.* 1994).

Unlike mammals, in which the chief site of salt and water regulation is the kidney, fish partition their osmoregulatory mechanisms between the gills and the kidney (and in elasmobranchs, the rectal gland). Kidneys are the major site of water regulation in fish, with the gills (or rectal gland) being the major salt-regulating tissue (see Evans, 1993, for a review). However, hagfish are marine osmotic conformers and would apparently have no need for major osmotic regulation, in spite of the presence of glomerular kidneys. Because of the role of NPs in mammalian salt and water homeostasis, it is thought that NPs might be important in fish osmoregulation and NP systems have now been examined and demonstrated in all classes of fish, including hagfish agnathans (Evans and Takei, 1992; Evans, 1993).

The present study extends the characterization of ^{125}I -ANP binding sites in the kidney and aortae of *Myxine glutinosa*. Kloas *et al.* (1988) observed the presence of ANP binding sites in the glomerular hagfish kidney. ANP bound specifically to the glomeruli, particularly to the arterioles, the inner epithelia of the Bowman's capsule and the neck segment. In the archinephric duct, specific binding was confined to the smooth muscle layer surrounding the intraluminal cells of the tubules, but not apparently on these cells themselves. In the same study, ANP-specific binding was located on the ventral aorta, both on the smooth muscle layer and on the endothelium. However, their study did not extend to a characterization of the binding sites. With the current availability of ^{125}I -CNP, as well as non-radioactive CNP and C-ANF, it is now possible to characterize NPRs in the kidney and aortae further by autoradiographic analysis of the displacement of iodinated radioligands with non-radioactive NPs in tissue sections, by determining the presence or absence of ^{125}I -CNP-specific binding, by competition binding studies and by GC assays to assess whether NPRs are linked *via* GC to the cyclic GMP second messenger system in the hagfish kidney. We have recently characterized NPRs in the hagfish gill (Toop *et al.* 1995) and have identified two NPRs: a GC-linked receptor like NPR-A that binds ANP and to a lesser extent CNP, and a 'promiscuous' type of receptor that binds ANP, CNP and C-ANF. It was in order to place the hagfish kidney NP binding sites within this framework that the current study was undertaken.

Materials and methods

Animal maintenance

Hagfish *Myxine glutinosa* L. were collected from the Bay of Fundy and purchased from Huntsman Marine Laboratory, St

Andrews, NB, Canada, and maintained in 10 °C tanks aerated through charcoal/fibre filters at the University of Florida, Gainesville, USA. Animals were allowed to acclimate for at least 3 weeks before experimentation. Hagfish were anaesthetized in MS 222 (1:1000, Sigma, St Louis, MO, USA) before dissection. They were killed by severance of the spinal chord caudad to the brain, which was then removed. Both kidneys were dissected from hagfish together with the dorsal aorta. The ventral aorta was removed from immediately cranial to the ventricle to the end of the gill arches; part of the afferent branchial arches was left attached to the aorta.

Autoradiography

The aortae and short strips of kidney with the archinephric duct, several glomeruli and the dorsal aorta intact were freeze-mounted in Tissue Tek (Miles Inc. Elkhart, IN, USA) in a microtome cryostat (Minotome, IEC, MA, USA). 18 μm sections were cut and mounted on slides coated in gelatin-chromium aluminium before being 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 MgCl₂, 0.1 % bovine serum albumin and 0.05 % bacitracin. The sections were then incubated for 90 min in the same buffer supplemented with 4 $\mu\text{g ml}^{-1}$ leupeptin, 2 $\mu\text{g ml}^{-1}$ chymostatin, 2 $\mu\text{g ml}^{-1}$ pepstatin, 1 $\mu\text{mol l}^{-1}$ phenylmethylsulphonyl fluoride and rat (3-[^{125}I]iodotyrosol²⁸) atrial natriuretic peptide (^{125}I -ANP, 74 TBq mmol⁻¹; Amersham, IL, USA), or human, porcine or rat (^{125}I -[Tyr⁰]) C-type natriuretic peptide-22 (^{125}I -CNP, 55 TBq mmol⁻¹; Peninsula Laboratories, CA, USA). Iodinated peptides were used in a concentration range between 0.1 and 0.2 nmol l⁻¹. Nonspecific binding was determined in adjacent sections in the presence of 1 $\mu\text{mol l}^{-1}$ unlabelled rat 3-28 ANP (rANP, Bachem, CA, USA) for ^{125}I -ANP-incubated sections and 1 $\mu\text{mol l}^{-1}$ porcine CNP (pCNP; Bachem, CA, USA) for ^{125}I -CNP-incubated sections. Displacement of specific binding was also determined in the presence of 1 $\mu\text{mol l}^{-1}$ pCNP (^{125}I -ANP-labelled sections), 1 $\mu\text{mol l}^{-1}$ rANP (^{125}I -CNP-labelled sections) and 1 $\mu\text{mol l}^{-1}$ rat des[Gln¹⁸, Ser¹⁹, Gly²⁰, Leu²¹, Gly²²]ANP-(4-23)-NH₂ (C-ANF; Bachem, CA, USA), 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- β max (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.

Competition binding assays

Three sets of seven slides of kidney serial sections were prepared and preincubated for 15 min, as described above. Each slide had kidney sections from three individual hagfish, with sections containing at least three different glomeruli for each hagfish. Each set of seven slides was then assigned for incubation with 200 pmol⁻¹ ¹²⁵I-ANP in the presence of increasing concentrations (10⁻¹² to 10⁻⁶ mol⁻¹) of rANP, pCNP or C-ANF added to the incubation buffer to give a visual competition curve. The sections were incubated in the appropriate treatment for 90 min. Following incubation, the slides were processed and exposed to X-ray film, as above. Autoradiographs of individual slides were mounted on microscope slides and viewed with an Olympus BH2 light microscope with a ×4 objective. Images of the individual glomeruli were captured on TDK E-HG video film using a Sony DXC-107 video camera with CCD iris and a Sony CMA-D7 camera adapter connected to a Mitsubishi HSU67 video cassette recorder. The glomerular images were then imported onto a Macintosh Quadra and the glomerular area analysed for a mean grayscale value using the NIH Image program (Version 1.54, 1994). A mean for each animal's glomerular grayscale value was computed for every concentration of the competing peptides. The mean ± S.E.M. for the three animals was then calculated and the percentage of the maximum value was determined for each data point. Competition binding curves were then generated for each competing peptide.

Guanylate cyclase assays of kidney membranes

Three kidney membrane preparations were made using three hagfish kidneys per preparation. Kidneys were removed from anaesthetized hagfish and placed in a 50 ml centrifuge tube in 5 ml of ice-cold 50 mmol⁻¹ Tris-HCl and 1 mmol⁻¹ NaHCO₃ (pH 7.4) and quickly homogenised with a Tissue-Tearor (Biospec, Bartlesville, OK, USA). The homogenate was diluted with 5 ml of 50 mmol⁻¹ Tris-HCl, 1 mmol⁻¹ EDTA and 1 mmol⁻¹ MgCl₂ (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⁻¹ Tris-HCl (pH 7.4) and 250 mmol⁻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 bovine serum albumin standards.

To determine guanylate cyclase activity, 50 μg of kidney protein was added to 50 mmol⁻¹ Tris-HCl, 2 mmol⁻¹ isobutyl methylxanthine, 10 mmol⁻¹ creatine phosphate, 1000 i.u. ml⁻¹ creatine phosphokinase, 4 mmol⁻¹ MnCl₂, 1 mmol⁻¹ GTP and increasing concentrations of rANP, pCNP

or 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 24 °C and were terminated by the addition of 4 mmol⁻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, IL, USA). The data are presented as the means ± S.E.M. of the three membrane pools and are plotted as a percentage of basal cyclic GMP production rate (Fig. 6). Regression analyses were carried out on both the rANP and the pCNP cyclic GMP data using SuperANOVA 1.11 (Abacus Concepts Inc., 1989).

Results

Autoradiography

The paired myxinoïd kidney is a persistent mesonephros with very large glomeruli (0.7–1.0 mm in length) arranged segmentally along the body, caudal to the heart. The glomeruli are paired in each muscle segment on either side of the dorsal midline, and each glomerulus is drained by a short neck segment into an archinephric duct (Fig. 1), which traverses the length of the body and drains into the cloaca. The morphology of the glomerulus is similar to that of mammals, being perfused from a single afferent arteriole and drained by paired efferent arterioles (Fels *et al.* 1989). The neck segment and archinephric duct are functionally and structurally similar to proximal tubules. They are lined by epithelial cells with an extensive luminal brush border and they operate in divalent ion regulation and in the reabsorption of glucose and amino acids. Neither salt nor water appears to be reabsorbed, so that the GFR is equal to the urine flow rate (Munz and McFarland, 1964; Alt *et al.* 1981; Fels *et al.* 1989; Evans, 1993).

¹²⁵I-ANP-specific binding was located on the glomeruli, neck segments and the archinephric duct of the kidney (Fig. 2A) and on both the dorsal (Fig. 2A) and ventral aortae (Fig. 2C). Specific binding was displaced by 1 μmol⁻¹ rANP, indicating the level of nonspecific binding (Fig. 2B, kidney and dorsal aorta); the nonspecific background appeared to be quite high in the ventral aorta (Fig. 2D). Specific binding was visibly

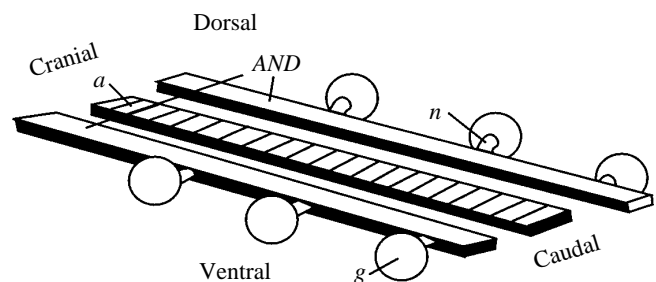


Fig. 1. Schematic diagram of a portion of the hagfish kidneys and dorsal aorta. The sections shown in Figs 2A,B, and Fig. 3 were frontal sections cut at a slightly oblique angle through these tissues. *a*, dorsal aorta; *g*, glomerulus; *n*, neck segment; *AND*, archinephric duct.

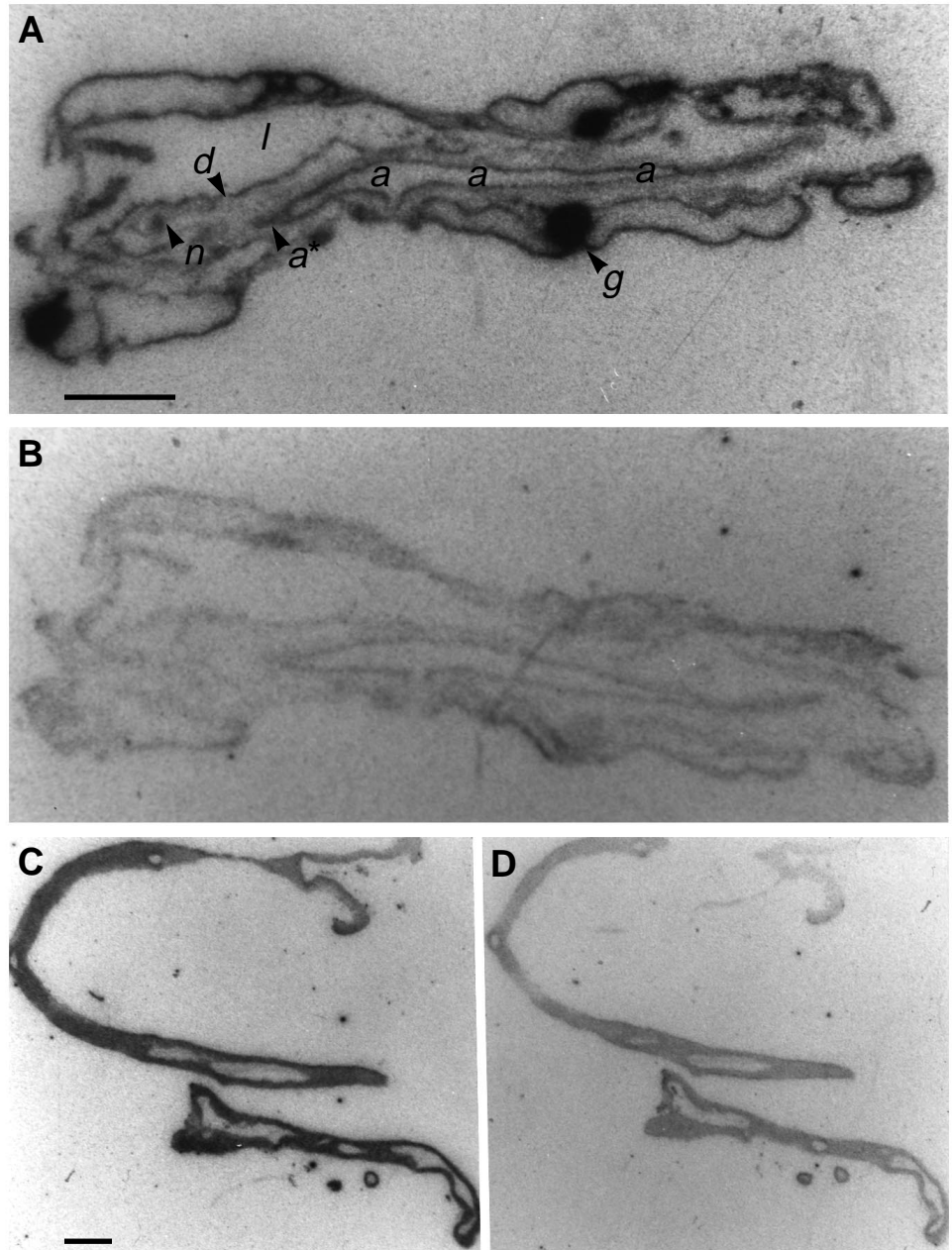


Fig. 2. Autoradiographs of ¹²⁵I-ANP binding in serial frontal oblique sections through both hagfish kidneys, dorsal aorta and the ventral aorta. (A) ¹²⁵I-ANP binding to glomeruli (g), archinephric ducts (d), dorsal aorta (a, a*) and neck segment (n). l, lumen of the archinephric duct. (B) ¹²⁵I-ANP + 1 μmol l⁻¹ rANP showing displacement of specific binding from kidney tissue. (C) ¹²⁵I-ANP binding to ventral aorta. (D) ¹²⁵I-ANP + 1 μmol l⁻¹ rANP showing displacement of specific binding from ventral aorta. Scale bars, 1 mm.

displaced to some extent by 1 μmol l⁻¹ pCNP but not by C-ANF (results not shown). In order to define the areas of ¹²⁵I-ANP-specific binding better in the kidney and dorsal aorta, some slides were dipped in nuclear track emulsion. The areas of particular interest are shown in Fig. 3A. Most of the silver grains were scattered over the endothelium of the dorsal aorta (Fig. 3A), with less pronounced grains over the smooth muscle layer (Fig. 3A). In the kidney, the distribution of silver grains was greatest over the glomeruli (Fig. 3B), but the particular cell types were not able to be resolved. In the neck segment leading from the glomerulus to the archinephric duct, specific binding was predominantly located over the luminal surface of the epithelial cells (Fig. 3C); above the neck segment lumen, some glomerular tissue is shown with silver grains scattered

over the cells (Fig. 3C). Similar to the distribution in the neck segment, the densest distribution of silver grains appeared along the luminal surface of the archinephric duct epithelial cells (Fig. 3D). Most of the silver grains were displaced by 1 μmol l⁻¹ rANP (not shown). There was no specific ¹²⁵I-CNP binding observed on either tissue (Fig. 4A, kidney and dorsal aorta; Fig. 4B, ventral aorta).

Competition binding assays

Competition binding analysis was performed using a grayscale image analysis on autoradiographs of glomeruli. Competition curves were generated from the grayscale values for each competing cold peptide (rANP, pCNP and C-ANF, Fig. 5). 4 nmol l⁻¹ rANP competed for 50% of ¹²⁵I-ANP

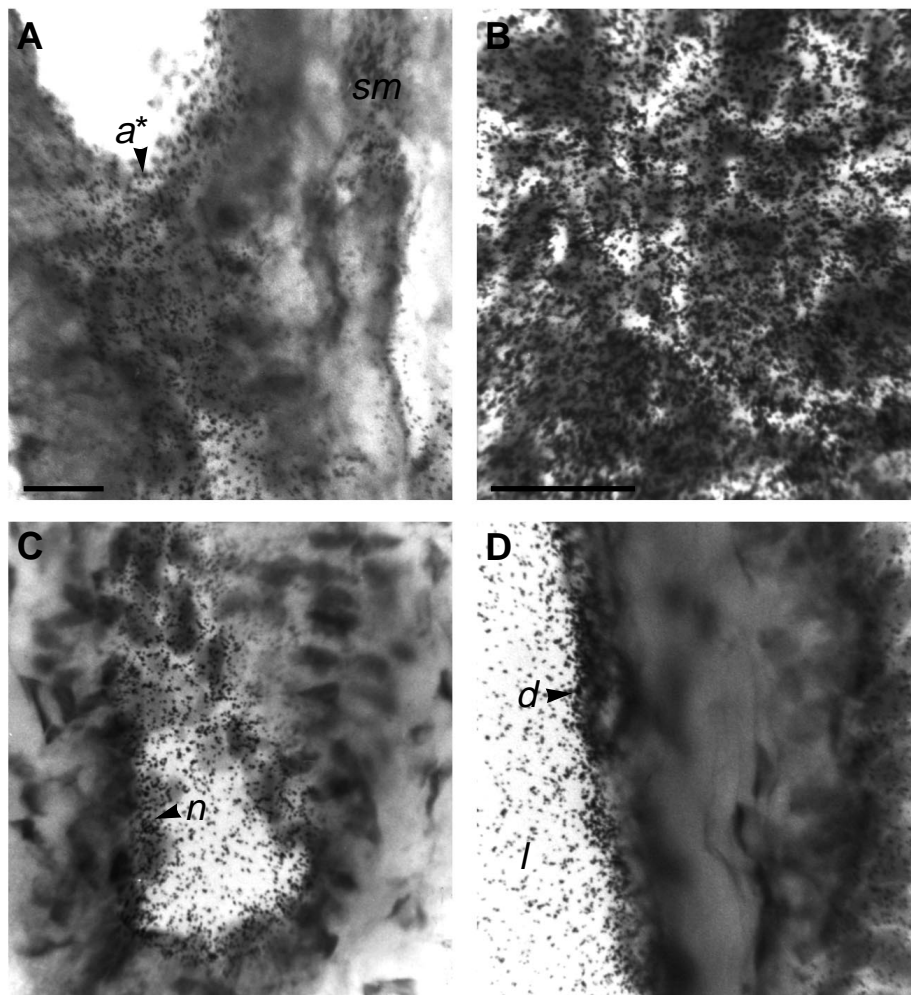


Fig. 3. Light micrograph of longitudinal sections of dorsal aorta (A), glomerulus (B), neck segment (C) and archinephric duct (D) dipped in X-ray-sensitive emulsion showing the distribution of ^{125}I -ANP specific binding. (A) ^{125}I -ANP-specific binding to the dorsal aorta. This section is cut at a slightly oblique angle. Binding to endothelial cells, a^* ; binding on vascular smooth muscle layer out of focus to the endothelial cells, sm . Specific binding is indicated by the density of the silver grains. (B) ^{125}I -ANP-specific binding on the glomerulus. (C) ^{125}I -ANP-specific binding to the neck segment, n . (D) ^{125}I -ANP-specific binding to the luminal (l) side of the epithelial cells (d) of the archinephric duct. Scale bars, $25\ \mu\text{m}$.

binding sites. pCNP did not compete with ^{125}I -specific binding below a concentration of $10\ \text{nmol l}^{-1}$; $300\ \text{nmol l}^{-1}$ pCNP competed for 50% of the ^{125}I -ANP sites. pCNP displaced all but 20% of ^{125}I -ANP-specific binding at the maximum concentration used in this study ($1\ \mu\text{mol l}^{-1}$). C-ANF failed to compete for ^{125}I -ANP-specific binding sites.

Guanylate cyclase assays

The basal cyclic GMP accumulation rate was $6.1 \pm 0.6\ \text{pmol cGMP mg}^{-1}\ \text{protein min}^{-1}$. Regression analyses indicated that both rANP and pCNP stimulated cyclic GMP production above the basal rate ($P < 0.01$, $r = 0.60$ for rANP, and $r = 0.69$ for pCNP). There was no difference between the slopes and intercepts of the rANP and the pCNP regression lines. rANP stimulated cyclic GMP production 40–50% above basal rates at concentrations of $10\ \text{nmol l}^{-1}$ and greater; rANP-stimulated cyclic GMP production rate appeared to plateau at these concentrations (Fig. 6). pCNP elevated cyclic GMP production 45–50% above basal levels between 0.1 and $1\ \mu\text{mol l}^{-1}$; cyclic GMP production also appeared to be reaching a plateau at these levels (Fig. 6). C-ANF failed to stimulate cyclic GMP production above basal levels at any concentration (Fig. 6). The maximum rate of rANP-stimulated

cyclic GMP production was $10.6 \pm 2.0\ \text{pmol cGMP mg}^{-1}\ \text{protein min}^{-1}$, and that of pCNP was $11.1 \pm 3.0\ \text{pmol cGMP mg}^{-1}\ \text{protein min}^{-1}$; both rates were observed at an NP concentration of $1\ \mu\text{mol l}^{-1}$.

Discussion

The specific NP binding profile observed in the hagfish kidney and aortic vasculature is different from that observed in the gill, in which both ^{125}I -ANP- and ^{125}I -CNP-specific binding have been found (Toop *et al.* 1995). The binding sites in the hagfish gill were resolved into two receptors: the first demonstrated similarities to the mammalian NPR-A, binding ANP preferentially; the second bound all NPs including C-ANF. In the kidney and aortae, however, only ^{125}I -ANP binding was observed (Figs 2–4). Additionally, pCNP only partially displaced ^{125}I -ANP binding and C-ANF failed to do so. The competition studies for the glomeruli were consistent with the autoradiographical results (Fig. 5). rANP competed for 50% of ^{125}I -ANP binding in the glomeruli at a concentration comparable with that found in the gill ($4.0\ \text{nmol l}^{-1}$ and $1.0\ \text{nmol l}^{-1}$, respectively; Toop *et al.* 1995). There was an order of magnitude difference between pCNP

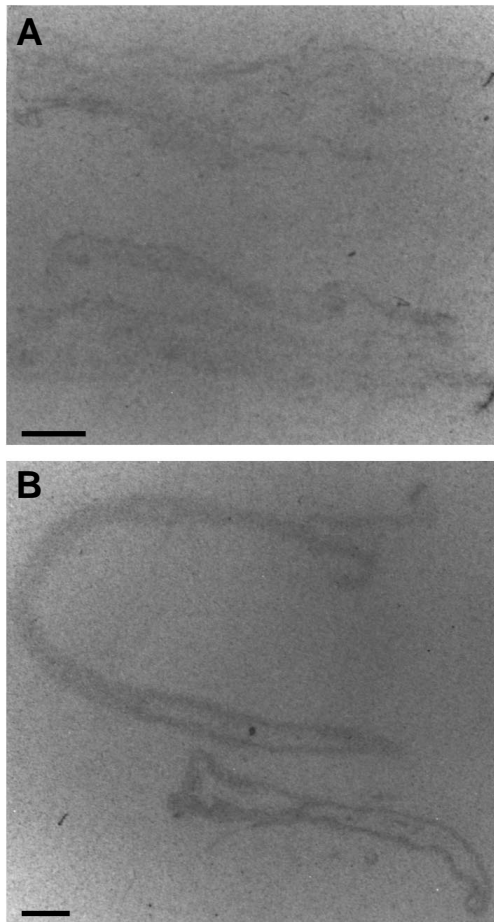


Fig. 4. Frontal sections indicating lack of ^{125}I -CNP binding to the hagfish kidney and dorsal and ventral aortae. (A) Kidney and dorsal aorta. (B) Ventral aorta; scale bars, 1 mm.

competition for 50% of ^{125}I -ANP in the gill (20 nmol l^{-1} ; Toop *et al.* 1995) and in the glomeruli (300 nmol l^{-1}). C-ANF, which competed for ^{125}I -ANP and ^{125}I -CNP binding sites in the gill, failed to compete for ^{125}I -ANP binding sites in the kidney. It is clear from these data that the kidney and aortae lack the 'promiscuous' receptor that is present in the hagfish gill. The stimulation of cyclic GMP production by NPs in the kidney may be interpreted as evidence for a GC-linked NPR in this tissue.

These data suggest that the predominant receptor type in the kidney is of the NPR-A type originally described in gill tissue (Toop *et al.* 1995). The following reasons are offered as arguments: (1) the binding sites appear to be particularly sensitive to ^{125}I -ANP; (2) the displacement capability of rANP for this site is similar in the gill and the glomeruli; (3) there is no observable ^{125}I -CNP binding; pCNP displaces ^{125}I -ANP binding and stimulates cyclic GMP production, but only at concentrations in excess of $0.1\ \mu\text{mol l}^{-1}$ (CNP was shown to have a low affinity for this site in the gill); (4) C-ANF does not appear to bind to this site or to stimulate cyclic GMP production, at the concentrations examined. The observations that ^{125}I -CNP failed to bind to the tissues whereas pCNP

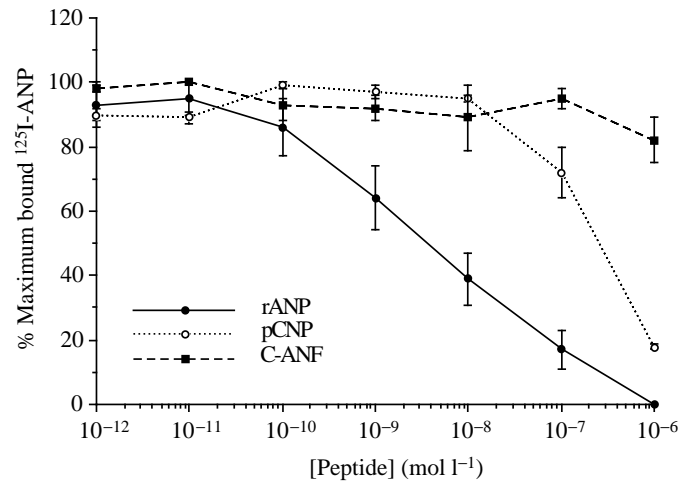


Fig. 5. Competition study indicating the relative abilities of rANP, pCNP and C-ANF at increasing concentrations to compete for ^{125}I -ANP-specific binding sites in hagfish glomeruli. Each point is the mean \pm S.E.M. of a mean grayscale value of the autoradiographs of glomeruli from three hagfish.

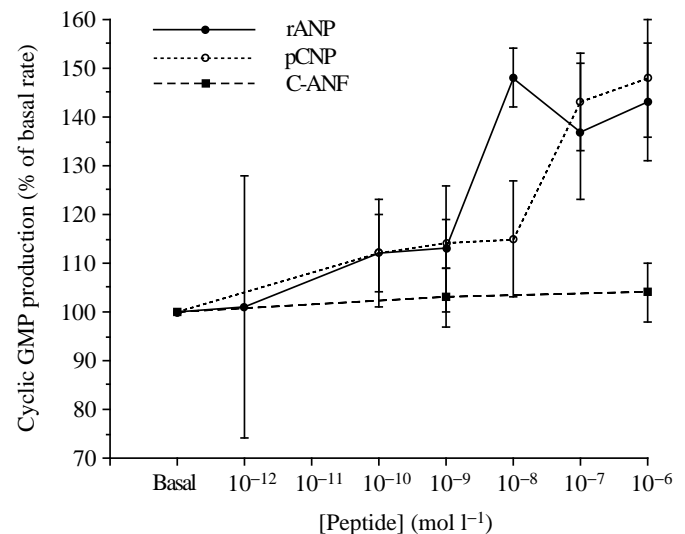


Fig. 6. Relative effects of increasing concentrations of the natriuretic peptides rANP, pCNP and C-ANF on the rate of cyclic GMP production in hagfish kidney membrane preparations. Data points are mean \pm S.E.M. of three pooled membrane preparations each containing kidneys from three individual hagfish.

competed for ^{125}I -ANP binding sites and stimulated cyclic GMP production may be accounted for by the relative concentrations used for each experiment: the iodinated peptide was used in a concentration range between 0.1 and 0.2 nmol l^{-1} , but competition for ANP sites and increased cyclic GMP production was not observed at concentrations below $0.1\ \mu\text{mol l}^{-1}$.

The present study confirms the original autoradiographical findings of Kloas *et al.* (1988), with the exception that Kloas and co-workers found no discernible binding on the luminal

side of the archinephric duct epithelial cells, in direct contrast with this study in which the location of most of the binding was on the luminal cell border (Fig. 3D). The majority of ^{125}I -ANP binding in the earlier study was found on the smooth muscle layer adjacent to the epithelial layer (Kloas *et al.* 1988). Although there was some displaceable binding observed on the smooth muscle cells in this study (not shown), it was not to the extent observed on the archinephric duct and neck segment epithelium. The specific binding of ^{125}I -ANP along the luminal borders is similar to the binding observed on the apical border of the luminal cells in the kidney of antarctic fishes (Uva *et al.* 1993). The luminal positioning of silver grains is also noteworthy in the light of the current hypothesis that urodilatin (and ANP) binds to the luminal border of mammalian collecting duct cells (Goetz, 1991).

Clearly, an extensive population of NPRs is present in the glomerulus (Figs 2A, 3B). NPs possibly have some effect on the filtration dynamics in this structure, as has been reported for mammals (Zeidel and Brenner, 1987; Brenner *et al.* 1990; Awazu and Ichikawa, 1993). Interestingly, the majority of receptors in the mammalian glomerulus are of the NPR-C type, with a smaller population of NPR-A and NPR-B types (Martin *et al.* 1989; Awazu and Ichikawa, 1993; Zhao *et al.* 1994), in contrast to the hagfish glomerulus in which the NPR-A type alone has been identified. NPR-A is reported to be the predominant receptor type in the tubular portions of the mammalian nephron (Martin *et al.* 1989; Awazu and Ichikawa, 1993), a condition which is also apparent in the hagfish, if we assume some homology between the mammalian and hagfish 'A' receptors. However, it is possible that small populations of other NPRs exist in the kidney and aortae that are not detectable using the methods employed in the current study. It is difficult to hypothesize on the effect of NPs in the archinephric duct and neck segment since no salt or water transport has been detected in the hagfish kidney (Fels *et al.* 1989); possibly other transport systems, such as those for acid-base or divalent ion regulation, are affected.

Although no competition or GC assay data exist for the hagfish ventral and dorsal aortae, it is evident from the similarity between the kidney and vascular autoradiographic data that the receptor of the NPR-A type also predominates in these tissues (Figs 2, 3). The presence of NPRs on hagfish vascular smooth muscle supports the vasodilatory function of NPs, not only in other fishes and mammals, but in the hagfish itself (Evans *et al.* 1989, 1993; Evans, 1991; Evans and Takei, 1992). The role of NPs in hagfish vascular endothelial function is unknown.

The systemic NPR system in the hagfish tissues examined to date must operate principally *via* receptors of the NPR-A type, since it is the only NPR identified outside the gills. However, caution must be used in the interpretation of receptor subtypes present in the hagfish until assays using native ligands are performed or the structure of the native receptors themselves are known. Apart from the vasodilatory action of NPs in the hagfish, we have no knowledge of the functions of NPs in the glomerulus, ducts of the kidney or the vascular

endothelium. The hagfish NPR-A appears to be linked with GC activity, and it is presumed, therefore, that any postbranchial systemic effects are mediated through the guanylate cyclase/cyclic GMP second messenger system.

This research was supported by National Science Foundation Grants DCB 8916413 and IBN 9306997 to D.H.E. and by NIH EHS-P30-ESO3828 to the Center for Membrane Toxicity Studies at MDIBL. We thank Dr Larry McEdward for the use of his microscopes and video equipment. Laurie Walz prepared Fig. 1. We also thank Dr Carmine Lanciani for his assistance and advice with data analysis.

References

- ABASSI, Z. A., GOLOMB, E., KLEIN, H. AND KEISER, H. R. (1992). Urodilatin: a natriuretic peptide of renal origin. *Cardiovascular Drug Revs.* **10**, 199–210.
- ALT, J. M., STOLTE, H., EISENBACH, G. M. AND WALVIG, F. (1981). Renal electrolyte and fluid excretion in the Atlantic hagfish, *Myxine glutinosa*. *J. exp. Biol.* **91**, 323–330.
- ANAND-SRIVASTAVA, M. B. AND TRACHTE, G. J. (1993). Atrial natriuretic factor receptors and signal transduction mechanisms. *Pharmac. Rev.* **45**, 455–497.
- ATLAS, S. A., KLEINERT, H. D., CAMARGO, M. J., JANUSZEWICZ, A., SEALEY, J. E., LARAGH, J. H., SCHILLING, J. W., LEWICKI, J. A., JOHNSON, L. K. AND MAACK, T. (1984). Purification, sequencing and synthesis of natriuretic and vasoactive rat atrial peptide. *Nature* **309**, 717–727.
- AWAZU, M. AND ICHIKAWA, I. (1993). Biological significance of atrial natriuretic peptide in the kidney. *Nephron* **63**, 1–14.
- BRENNER, B. M., BALLERMANN, B. J., GUNNING, M. E. AND ZEIDEL, M. L. (1990). Diverse biological actions of atrial natriuretic peptide. *Physiol. Rev.* **70**, 665–699.
- CANAAN-KUHL, S., JAMISON, R. L., MYERS, B. D. AND PRATT, R. E. (1992). Identification of 'B' receptor for natriuretic peptide in human kidney. *Endocrinology* **130**, 550–552.
- CURRIE, M. G., GELLER, D. M., COLE, B. R., SIEGEL, N. R., FOK, K. F., ADAMS, S. P., EUBANKS, S. R., GALLUPPI AND G. R. AND NEEDLEMAN, P. (1984). Purification and sequence analysis of bioactive atrial peptides (atriopeptins). *Science* **223**, 67–69.
- DE BOLD, A. J., BORENSTEIN, H. B., VERESS, A. T. AND SONNENBERG, H. (1981). A rapid and potent natriuretic response to intravenous injection of atrial myocardial extract in rats. *Life Sci.* **28**, 89–94.
- EVANS, D. H. (1991). Rat atriopeptin dilates vascular smooth muscle of the ventral aorta from the shark (*Squalus acanthias*) and the hagfish (*Myxine glutinosa*). *J. exp. Biol.* **157**, 551–555.
- EVANS, D. H. (1993). Osmotic and ionic regulation. In *The Physiology of Fishes* (ed. D. H. Evans), pp. 315–341. Boca Raton, FL: CRC Press.
- EVANS, D. H., CHIPOURAS, E. AND PAYNE, J. A. (1989). Immunoreactive atriopeptin in plasma of fishes: its potential role in gill hemodynamics. *Am. J. Physiol.* **257**, R939–R945.
- EVANS, D. H., DONALD, J. A. AND STIDHAM, J. D. (1993). C-type natriuretic peptides are not particularly potent dilators of hagfish (*Myxine glutinosa*) vascular smooth muscle. *Bull. Mt Desert Isl. Biol. Lab.* **32**, 106.
- EVANS, D. H. AND TAKEI, Y. (1992). A putative role for natriuretic peptides in fish osmoregulation. *News physiol. Sci.* **7**, 15–19.

- FELS, L. M., RAGUSE-DEGENER, G. AND STOLTE, H. (1989). The archinephron of *Myxine glutinosa* L. (Cyclostomata). In *Structure and Function of the Kidney* (ed. R. K. H. Kinne). *Comp. Physiol.* **1**, 73–102. Basel: Karger.
- GOETZ, K. L. (1991). Renal natriuretic peptide (urodilatin?) and atriopeptin: evolving concepts. *Am. J. Physiol.* **261**, F921–F932.
- GREENWALD, J. E., RITTER, D., TETENS, E. AND ROTWEIN, R. S. (1992). Renal expression of the gene for atrial natriuretic factor. *Am. J. Physiol.* **263**, F974–F978.
- KLOAS, W., FLUGGE, G., FUCHS, E. AND STOLTE, H. (1988). Binding sites for atrial natriuretic peptide in the kidney and aorta of the hagfish (*Myxine glutinosa*). *Comp. Biochem. Physiol.* **91A**, 685–688.
- KOLLER, K. J. AND GOEDEL, D. V. (1992). Molecular biology of the natriuretic peptides and their receptors. *Circulation* **86**, 1081–1088.
- MAACK, T. (1992). Receptors of atrial natriuretic factor. *A. Rev. Physiol.* **54**, 11–27.
- MAACK, T., SUZUKI, M., ALMEIDA, F. A., NUSSENZWEIG, D., SCARBOROUGH, R. M., McENROE, G. A. AND LEWICKI, J. A. (1987). Physiological role of silent receptors of atrial natriuretic factor. *Science* **238**, 675–678.
- MARTIN, E. R., LEWICKI, J. A., SCARBOROUGH, R. M. AND BALLERMAN, B. J. (1989). Expression and regulation of ANP receptor subtypes in rat renal glomeruli and papillae. *Am. J. Physiol.* **257**, F649–F657.
- MUNZ, F. W. AND MCFARLAND, W. N. (1964). Regulatory function of a primitive vertebrate kidney. *Comp. Biochem. Physiol.* **13**, 281–400.
- SCHULZ-KNAPPE, P., FORSSMANN, K., HERBST, F., HOCK, D. AND FORSSMANN, W. G. (1988). Isolation and structural analysis of urodilatin, a new peptide of the cardiodilatin-(ANP)-family, extracted from human urine. *Klin. Wochenschr.* **66**, 752–759.
- SUDOH, T., KANGAWA, K., MINAMINO, N. AND MATSUO, H. (1988). A new natriuretic peptide in porcine brain. *Nature* **332**, 78–81.
- SUDOH, T., MINAMINO, N., KANGAWA, N. AND MATSUO, H. (1990). C-type natriuretic peptide (CNP): a new member of natriuretic family identified in porcine brain. *Biochem. biophys. Res. Commun.* **168**, 863–870.
- TOOP, T., DONALD, J. A. AND EVANS, D. H. (1995). Localisation and characteristics of natriuretic peptide receptors in the gills of the Atlantic hagfish, *Myxine glutinosa* (Agnatha). *J. exp. Biol.* **198**, 117–126.
- UVA, M. B., MASINI, M. A., NAPOLI, L. AND DEVECCHI, M. (1993). Immunoreactive atrial natriuretic-like peptide in antarctic teleosts. *Comp. Biochem. Physiol.* **104A**, 291–297.
- VALENTIN, J.-P., SECHI, L. A., QIU, C., SCHAMBELAN, M. AND HUMPHREYS, M. H. (1993). Urodilatin binds to and activates renal receptors for atrial natriuretic peptide. *Hypertension* **21**, 432–438.
- ZEIDEL, M. L. AND BRENNER, B. M. (1987). Actions of atrial natriuretic peptides on the kidney. *Sem. Nephrol.* **7**, 91–97.
- ZHAO, J., ARDAILLOU, N., LU, C.-Y., PLACIER, S., PHAM, P., BADRE, L., CAMBAR, J. AND ARDAILLOU, R. (1994). Characterization of C-type natriuretic peptide receptors in human mesangial cells. *Kidney Int.* **46**, 717–725.