Identification of renal natriuretic peptide receptor subpopulations by use of the non-peptide antagonist, HS-142-1

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1 The renal actions of natriuretic peptides are dictated by the distribution of guanylyl cyclase-linked (NPR_A and NPR_B) and non-guanylyl cyclase-linked (NPR_C) receptors. Natriuretic peptide receptors have previously been distinguished on the basis of their differential affinity for peptide fragments and analogues; however, most of the available ligands are not fully selective. We have used the specific guanylyl cyclase-linked receptor antagonist, HS-142-1, to investigate the differential distribution of natriuretic peptide receptor subtypes in the human, bovine and rat kidney.

2 Specific, high affinity 3-([^{125}I]-iodotyrosyl)-rat-ANP-(1-28)([^{125}I]-rANP1-28) binding sites were identified in all three species, localized to glomeruli, inner medulla, intrarenal arteries and regions in the outer medulla corresponding to vasa recta bundles. Binding sites were also identified in the smooth muscle lining of the hilar region in the bovine and rat kidney.

3 In the rat, [^{125}I]-rANP1-28 binding was inhibited by unlabelled peptide sequences with a rank order of potency (rANP1-28 > pCNP1-22 > C-ANP4-23). The glomeruli exhibited a heterogeneous population of binding sites, C-ANP4-23 and pCNP1-22 producing a significantly better fit to a two component inhibition curve compared to the single component curve for rANP1-28.

4 Competitive inhibition experiments with the receptor selective ligands, C-ANP4-23 and HS-142-1, suggested that, like the rat, human and bovine glomeruli possessed a heterogeneous population of binding sites, whilst those in the inner medulla and intrarenal arteries of all three species represented a homogeneous population. Rat glomeruli exhibited a high proportion (>80%) of the NPR_C receptor subtype whereas in human and bovine glomeruli this receptor represented less than 20% of the total population, the majority of binding sites being HS-142-1-sensitive.

5 C-ANP4-23 exhibited a significantly higher inhibitory potency for binding sites in rat glomeruli compared to those in human and bovine kidney whilst HS-142-1 was significantly more potent in the rat and bovine kidney compared to man. No evidence was found to suggest the presence of a renal NPR_B receptor subtype.

6 The relative density, affinity and proportion of natriuretic receptor subtypes in the kidney exhibit significant species differences. HS-142-1 may be a valuable tool in further elucidating the localization and function of these receptors, but heterogeneity between species should be considered when selecting experimental models.

Keywords: atrial natriuretic peptide; natriuretic peptide receptors; kidney; autoradiography

Introduction

The natriuretic peptide family is considered to have a significant role in maintaining cardiovascular homeostasis and consists of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP) and urodilatin (Wilkins *et al.*, 1993). The biological effects of these peptides are mediated by two classes of receptor, one of which comprises a monomeric transmembrane protein possessing an intracellular guanylyl cyclase domain and an extracellular domain displaying rigorous criteria for ligand binding and activity (De Léan *et al.*, 1985; Chinkers *et al.*, 1989). Two subtypes of this receptor class have been identified, so-called A and B natriuretic peptide receptors (NPR_A and NPR_B), which exhibit different rank orders of potency for guanosine 3':5'-cyclic monophosphate (cyclic GMP) production (Bennett *et al.*, 1991; Suga *et al.*, 1992). The NPR_B subtype is selectively activated by CNP, displaying an affinity 10 to 50 fold greater than either ANP or BNP (Koller *et al.*, 1991; Komatsu *et al.*, 1991).

The other high-affinity receptor class (NPR_C) is a dimeric protein, composed of two disulphide-linked subunits which lacks the guanylyl cyclase domain (Porter *et al.*, 1990). It is

generally thought that these receptors have a clearance role (Maack *et al.*, 1987); however, receptor subtypes may exist which are linked to other second-messenger systems (Hirata *et al.*, 1989; Isales *et al.*, 1992). NPR_C receptors are much less demanding than NPR_A and NPR_B receptors in their structural requirements for ligand binding and display high affinity for D-amino acid substitutions and ring-deleted analogues of ANP1-28 such as des[Gln¹⁸,Ser¹⁹,Gly²⁰,Leu²¹,Gly²²]-rANP4-23 (C-ANP4-23) (Scarborough *et al.*, 1988). This analogue is ineffective in eliciting natriuretic and diuretic responses in the isolated perfused kidney (Maack *et al.*, 1987) and has been used in autoradiographic studies as a selective ligand to distinguish NPR_C receptors in organs such as the kidney and heart (Brown *et al.*, 1990; Rutherford *et al.*, 1991; 1992). In contrast, the investigation of guanylyl cyclase-linked NPR_A and NPR_B receptors has been hampered by the lack of selective antagonists. For example, it is unclear whether both subtypes are present in the kidney and recent binding studies have produced either negative or equivocal findings regarding the possible presence and localization of renal NPR_B receptors (Brown & Zuo, 1992; Konrad *et al.*, 1992). A number of peptide sequences have recently been synthesized which are selective for the guanylyl cyclase-linked ($\text{NPR}_{A/B}$) receptor subtypes (Weber *et al.*, 1991; Delporte *et*

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et al., 1992; Mimeault *et al.*, 1993); however, the best characterized receptor antagonist to date is a novel microbial polysaccharide, HS-142-1, which selectively binds to guanylyl cyclase-linked natriuretic peptide receptors in rat, rabbit, bovine and porcine tissues inhibiting ANP-induced responses in the kidney, cultured neuronal cells, lung and adrenocortical membranes (Morishita *et al.*, 1991; 1992; Toki *et al.*, 1992; Oda *et al.*, 1992; Ohyama *et al.*, 1993). In contrast to other ligands, HS-142-1 exhibits no apparent affinity for non-guanylyl cyclase-linked natriuretic receptors (Morishita *et al.*, 1992; Tanaka *et al.*, 1992) and thus provides a significant new tool with which to determine the differential distribution of natriuretic peptide receptor subtypes in heterogeneous tissues such as the kidney. The majority of binding studies to date have employed experimental animals to examine renal natriuretic peptide receptors. However, their relevance to man is uncertain as there may be significant species differences in the distribution of these receptors (Féthière & De Léan, 1991). We have used quantitative *in vitro* autoradiography and receptor selective ligands to investigate the differential distribution, relative proportion and affinity of natriuretic receptor subtypes in the rat, bovine and human kidney.

Methods

Tissues

Rat ($n = 6$) and bovine ($n = 6$) kidney samples were obtained immediately after death caused by pentobarbitone overdose or exsanguination respectively. Non-involved regions of cortex and medulla were also obtained at nephrectomy from six male patients aged 40–66 years. Tissue blocks were placed on cork mats, surrounded in mounting medium (Tissue-Tek, Miles Inc., Elkhart, Indiana, U.S.A.) and frozen in melting dichlorodifluoromethane (Arcton 12, ICI, Cheshire) suspended in liquid nitrogen.

In vitro autoradiography

Cryostat sections (10 μm) were thaw mounted onto chromalum gelatin coated glass slides and stored at -20°C . Sections were preincubated at 20°C for 15 min in 30 mM phosphate buffer (comprising 20 mM $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$, 10 mM $\text{NaH}_2\text{PO}_4 \cdot (2\text{H}_2\text{O})$, 123 mM NaCl, pH 7.4) and then incubated in fresh buffer containing 0.5% bovine serum albumin, 0.18 mM bacitracin and 250 pM [^{125}I]-rANP1-28 at room temperature for up to 90 min. [^{125}I]-rANP1-28 was used as pilot studies had previously demonstrated an identical distribution and density of binding for ^{125}I -labelled rat and human ANP1-28 on sections of rat, human and bovine kidney. We have also previously shown that the inclusion of 0.18 mM bacitracin in the incubation buffer is sufficient to inhibit significantly degradation of the radioligand over the incubation period used, without having a detrimental effect on the specific binding of [^{125}I]-rANP1-28 (Wharton *et al.*, 1994). Following incubation, the slides were washed twice in buffer at 4°C for 5 min, rinsed in distilled water at 4°C and dried under a stream of cold air. The NPR_C subpopulation was distinguished from the NPR_A and NPR_B subpopulations by co-incubation of 250 pM [^{125}I]-rANP1-28 with an excess of either HS-142-1 (2 mg ml $^{-1}$) or the NPR_C selective sequence, C-ANP4-23 (1 μM). Autoradiographic images were produced by exposing dry, labelled sections to Hyperfilm ^3H (Amersham, Bucks.) for 36 h at 4°C . The resulting autoradiographic film was developed in Kodak D-19 developer for 4–5 min, fixed in Amfix and rinsed in running water.

Microautoradiographs provided a higher degree of anatomical resolution of the binding sites and were obtained following exposure of labelled sections to Hyperfilm ^3H . Sections were fixed in Bouin's solution for 1 h at 20°C , washed

twice in phosphate buffer (2–3 min) at 4°C , rinsed in cold distilled water, dried under a stream of cold air and dipped in liquid emulsion (LM-1, Amersham, Bucks) at 43°C . The dried slides were then stored in the dark at 4°C for up to 6 days, developed in Kodak D-19 for 2.5 min at 20°C , fixed and stained with haematoxylin and eosin.

Quantification and characterization of binding sites

An IBAS 386 Image Analysis System (Kontron Elektronik, Watford) was used to quantify ligand binding to specific tissue structures in autoradiographic film images. Specific binding (amol mm $^{-2}$) was determined by subtracting the non-specific binding from the total binding obtained by incubating adjacent sections with 250 pM [^{125}I]-rANP1-28 either alone or in the presence of 1 μM unlabelled rANP1-28.

Association binding kinetics were examined by incubating sections of rat kidney with 250 pM [^{125}I]-rANP1-28 for up to 90 min at 20°C . Dissociation kinetics were determined following the addition of 1 μM unlabelled rANP1-28 to sections at equilibrium and incubating for a further 150 min. Competitive inhibition of specific [^{125}I]-rANP1-28 (250 pM) binding to human, bovine and rat kidneys was examined in the presence of increasing concentrations (10^{-12} – 10^{-5} M) of unlabelled rANP1-28, pCNP1-22, C-ANP4-23 or HS-142-1 (0.1–2000 $\mu\text{g ml}^{-1}$), with or without concomitant blockade of the NPR_C receptor subpopulation by C-ANP4-23 (10 μM). Further investigation of the guanylyl cyclase-linked subpopulation was afforded by co-incubation of serial sections with [^{125}I]-rANP1-28 and C-ANP4-23 (1 μM), either alone or in the presence of pCNP1-22 (0.1 μM), the selective agonist for the NPR_B receptor subtype. Binding data were analysed by the non-linear curve fitting programme, GraphPad InPlot version 4.0 (GraphPad Software, San Diego, U.S.A.). A four parameter logistic equation was used to describe the relationship between the percentage of [^{125}I]-rANP1-28 bound and the concentration of rANP1-28, pCNP1-22, C-ANP4-23 and HS-142-1 in competitive inhibition experiments. Data from inhibition of [^{125}I]-rANP1-28 was fitted to one or two site models and the 'best-fit' sums of squares compared statistically by an F-test. The concentration of peptide which inhibited 50% of the specific ligand binding (IC_{50}) was calculated for each competing peptide and expressed as a geometric mean with 95% confidence interval (95% CI), being derived from logarithmically transformed data.

Statistics

Binding data were analysed by one way or repeated analysis of variance, differences being considered significant if the P value was less than 0.05. To reduce the probability of significant differences arising by chance, a Bonferroni correction was applied to the data (Wallenstein *et al.*, 1980). The IC_{50} values of competitive inhibition data for glomeruli, inner medulla and intrarenal blood vessels were used as three separate dependent variables and the competing peptides as within subject factors.

Drugs

Drugs used were 3-([^{125}I]-iodotyrosyl)-rat ANP1-28 (~ 2000 Ci mmol $^{-1}$; Amersham, Bucks.), bacitracin, bovine serum albumin (fraction V) (Sigma Chemical Co., UK) and HS-142-1 (Tokyo Research Laboratories, Kyowa Hakko Kogyo Co. Ltd., Japan). Rat ANP1-28, human ANP1-28, porcine CNP1-22 and C-ANP4-23 were obtained from Peninsula Laboratories, St. Helens.

Results

The distribution of [^{125}I]-rANP1-28 binding sites was similar in human, bovine and rat renal tissues. Specific binding sites were localized to glomeruli, non-glomerular cortex, inner

Figure 1 Autoradiographic localization of [125 I]-rANP1-28 binding sites in the rat kidney. Serial cryostat sections were incubated with 250 pM [125 I]-rANP1-28 in the absence (a) or presence of 1 μ M rANP1-28 (b), C-ANP4-23 (c) or 2 mg ml $^{-1}$ HS-142-1 (d). Binding sites are localized to glomeruli in the cortex (C), the inner medulla (IM), vasa recta bundles in the inner stripe of the outer medulla (O), intrarenal arteries (arrows) and smooth muscle in the hilar region of the kidney (arrowheads). Bar = 3 mm.

medulla, intrarenal arteries and regions in the outer medulla corresponding to vasa recta bundles. Binding sites were also observed in the hilar region of the bovine and rat kidney (Figures 1 and 2). Co-incubation of [125 I]-rANP1-28 with an excess (1 μ M) of unlabelled ANP1-28 inhibited specific binding to all renal sites whereas co-incubation with receptor-selective ligands produced a differential inhibition of ligand binding (Figures 1 and 2). Microscopic examination demonstrated a high density of [125 I]-rANP1-28 binding to glomeruli and cortical radial, arcuate and interlobar arteries in all species, as well as non-vascular smooth muscle in the hilar region of rat and bovine kidney (Figure 3). The possibility of distinct binding to vascular endothelium could not be established due to the high density of binding sites on the medial layer and limited resolution of the autoradiographic images obtained with [125 I]-rANP1-28. Silver grains were also diffusely distributed in the inner medulla, non-glomerular cortex and medullary stripes. Of the three species examined, the rat exhibited the highest density of renal natriuretic peptide binding sites in all of the regions examined, the relative binding density of 250 pM [125 I]-rANP1-28 binding being 3–5 fold higher than in bovine and human kidney (Table 1). In the glomeruli and intrarenal arteries of all three species, non-specific binding was low (<10%), however, the human medulla exhibited a relatively high level of non-specific binding corresponding to 40% of the total compared to less than 10% in the bovine and rat medulla.

Specific [125 I]-rANP1-28 binding to rat kidney was high affinity, time-dependent and reversible, reaching apparent steady-state conditions by 30 min at 20°C (Figure 4, Table 2). Binding was inhibited by unlabelled peptide sequences with a rank order of potency rANP1-28 > pCNP1-22 > C-ANP4-

23 (Figure 5, Table 3). The inhibition curve for unlabelled rANP1-28 was best described by a single site model whereas the inhibition curves for pCNP1-22 and C-ANP4-23 were best described by a two site model ($P < 0.01$) (Figure 5, Table 3). Non-linear regression analysis of these two site models suggested that in the rat glomeruli 58.9% (51.4–66.0) of the binding sites corresponded to the clearance receptor subtype (NPR_C). Unlabelled pCNP1-22 and C-ANP4-23 both displayed a high affinity for NPR_C binding sites; however, at high concentrations pCNP1-22 (>0.1 μ M) and C-ANP4-23 (>1 μ M) also appeared to bind, albeit with lower affinity, to another population of [125 I]-rANP1-28 binding sites (Figure 5, Table 3). Unlike the inhibition of binding to glomeruli both rANP1-28 and pCNP1-22 displayed a monophasic inhibition of [125 I]-rANP1-28 binding in the inner medulla and intrarenal arteries whereas C-ANP4-23 had no apparent affinity for these sites at concentrations up to 10 $^{-6}$ M (Figure 5, Table 3).

The differential distribution and relative affinity of natriuretic peptide receptors in the human, rat and bovine kidney were also examined by competitive inhibition studies, using the receptor selective ligands C-ANP4-23 and HS-142-1 (Figures 6 and 7). As in the rat, bovine and human glomeruli exhibited a heterogeneous population of [125 I]-rANP1-28 binding sites with high affinity for both C-ANP4-23 (Figure 6) and HS-142-1 (Figure 7). In the presence of excess (10 μ M) C-ANP4-23, 70.5% (63.8–77.2) of the binding to rat glomeruli was inhibited whereas 58.9% (54.0–63.8) and 61.0% (42.5–79.5) of glomerular binding was inhibited in the bovine and human kidney respectively. C-ANP4-23 exhibited significantly higher affinity ($P < 0.001$) for binding sites in rat glomeruli (IC₅₀ 38.2 nM (19.9–72.4)) compared with those

Figure 2 Autoradiographic localization of [125 I]-rANP1-28 binding sites in the human kidney. Serial cryostat sections were incubated with 250 pM [125 I]-rANP1-28 in the absence (a) or presence of 1 μ M rANP1-28 (b), C-ANP4-23 (c) or 2 mg ml $^{-1}$ HS-142-1 (d). Binding sites are localized to glomeruli in the cortex (C), the medulla (M) and intrarenal arteries (arrows). Bar = 3 mm.

identified in human (IC $_{50}$ 182 nM (96–345)) and bovine glomeruli (IC $_{50}$ 5058 nM (3048–8395)) (Figure 6).

Co-incubation of [125 I]-rANP1-28 (250 pM) with excess HS-142-1 (2 mg ml $^{-1}$) inhibited 17.1% (7.7–33.9) of the specific binding in rat glomeruli; however, in the human and bovine glomeruli, as many as 80.7% (69.9–88.2) and 88.0% (84.6–90.7) of the binding sites were HS-142-1-sensitive respectively. In addition, HS-142-1 inhibited 94–100% of the specific binding to the inner medulla and intrarenal arteries in all three species. Co-incubation of serial sections with [125 I]-rANP1-28 and C-ANP4-23 (1 μ M), either alone or in the presence of pCNP1-22 (0.1 μ M), exhibited no significant differences in the relative ligand binding density between sections of either the rat, bovine or human kidney. Under conditions of concomitant NPR $_C$ receptor blockade with C-ANP4-23 (10 μ M), HS-142-1 exhibited significant inter-species differences in its inhibitory potency of [125 I]-rANP1-28 binding to kidney structures (Table 4). HS-142-1 was significantly more potent in inhibiting ligand binding to sites in the rat and bovine kidney than in man, and exhibited a 2.5–3.6 fold greater inhibitory potency for binding to bovine glomeruli and intrarenal arteries than in the rat kidney (Figure 7, Table 4).

Discussion

[125 I]-rANP1-28 binds to a heterogeneous population of receptors in rat, human and bovine kidney displaying both regional and species differences in their affinity and distribution. The localization and affinity of specific binding sites are consistent with our previous studies in the rat kidney (Rutherford *et al.*, 1991) as well as those of other autoradiographic and immunohistochemical studies which have demonstrated natriuretic peptide receptors in the kidney of all three species studies, localized to glomeruli, medulla, intrarenal arteries and ureter smooth muscle (Mantyh *et al.*, 1986; Ishikawa *et al.*, 1987; Shimonaka *et al.*, 1987; Brucksch *et al.*, 1988; Kawaguchi *et al.*, 1989; Brown *et al.*, 1990). Non-linear regression analysis of the biphasic inhibition curves produced by pCNP1-22 and C-ANP4-23 indicated that 40–60% of the binding to glomeruli in all three species was to the NPR $_C$ receptor subtype and the remainder to the guanylyl cyclase-linked NPR $_{A/B}$ receptor subtypes. This is consistent with our previous estimates of the proportion of guanylyl cyclase-linked and non-guanylyl cyclase-linked natriuretic peptide receptors in the rat kidney, obtained from binding studies in the presence of C-ANP4-23 (Brown *et al.*, 1990; Rutherford

Figure 3 Microautoradiographic localization of [^{125}I]-rANP1-28 binding sites in human kidney. Sections were incubated in the absence (a,c) and presence (b,d) of $1\text{ }\mu\text{M}$ unlabelled rANP1-28. Specific binding sites are demonstrated by silver grains in the emulsion overlying the media of an intrarenal artery (a) and a glomerulus in the cortex (c). Bar = $50\text{ }\mu\text{m}$.

Table 1 Relative binding density of [^{125}I]-rANP1-28 to human, bovine and rat kidney

	$[^{125}\text{I}]\text{-rANP1-28 bound (amol mm}^{-2}\text{)}$		
	Glomeruli	Medulla	Intrarenal arteries
Human	71.0 (45.5–96.5)**	29.8 (1.40–57.2)*	79.8 (46.3–113.3)**
Bovine	113 (86.2–140.2)**	16.7 (13.0–20.4)**	55.2 (37.3–73.1)**
Rat	313.6 (245.1–382.1)	61.0 (44.7–77.3)	184.6 (149.2–220.0)

Specific binding of 250 pM [^{125}I]-rANP1-28 for 30 min at 20°C expressed as the mean and 95% confidence interval for six separate human, bovine and rat kidneys. Significantly lower (* $P < 0.05$; ** $P < 0.001$) binding density compared to the rat.

Table 2 Association and dissociation constants for [^{125}I]-rANP1-28 binding to rat kidney

	$k_{+1} (\times 10^{-8}\text{ M}^{-1}\text{ min}^{-1})$	$k_{-1} (\times 10^{-2}\text{ min}^{-1})$	$K_d (\text{nM})$
Glomeruli	3.88 (3.43–4.30)	2.42 (1.72–3.11)	0.08 (0.03–0.17)
Inner medulla	1.85 (1.20–2.49)	3.54 (0.81–6.28)	0.17 (0.06–0.45)
Intrarenal arteries	1.98 (0.91–3.04)	2.60 (1.14–4.07)	0.12 (0.03–0.47)

Association (k_{+1}), dissociation (k_{-1}) and apparent equilibrium dissociation constant (K_d) values derived from non-linear regression analysis of time course studies of 250 pM [^{125}I]-rANP1-28 binding at 20°C . Values expressed as the mean and 95% confidence interval for six separate kidneys.

et al., 1991). Use of the selective guanylyl cyclase-linked receptor antagonist HS-142-1, however, provided a different estimate of the receptor populations which reflects more closely the results of affinity cross-linking studies in human glomeruli (Féthière & De Léan, 1991) and studies on the rat isolated perfused kidney (Maack *et al.*, 1987). In contrast to pCNP1-22 and C-ANP4-23, which appear not to be selective for glomerular NPR_C receptors when at high concentrations ($\geq 10^{-6}$ M), affinity cross-linking studies have demonstrated that HS-142-1 displays no affinity for the 60 kDa non-guanylyl cyclase-linked ANP receptor and is highly selective for particulate guanylyl cyclase (Morishita *et al.*, 1992; Tanaka *et al.*, 1992). Thus, the use of HS-142-1 may provide a more accurate means of estimating the proportion of natriuretic peptide receptor subtypes.

In contrast to the glomeruli, binding to the inner medulla of all three species was insensitive to inhibition by C-ANP4-

Figure 4 Specific binding of [125 I]-rANP1-28 to inner medullary binding sites in the rat kidney. Association (■) of 250 pM [125 I]-rANP1-28, at 20°C, and dissociation (□) after the addition of 1 μ M unlabelled rANP1-28 at 30 min (arrow). Each point represents the mean $\pm$ s.e.mean derived from six kidneys, expressed as a percentage of maximum specific binding. Similar time course results were observed for glomeruli and intrarenal arteries (data not shown).

23. HS-142-1, on the other hand, completely inhibited the specific binding of [125 I]-rANP1-28 indicating an absence of the NPR_C receptor subtype from renal medullary sites. This

Figure 5 Competitive inhibition curves of specific [125 I]-rANP1-28 binding to glomeruli (a) and intrarenal arteries (b) in rat kidney. Each point represents the mean $\pm$ s.e.mean derived from six kidneys, expressed as a percentage of ligand binding in the presence of increasing concentrations of unlabelled rANP1-28 (■), pCNP1-22 (□) and C-ANP4-23 (●). Competitive inhibition of binding to the inner medulla gave very similar curves to intrarenal arteries (data not shown).

Table 3 Competitive inhibition of specific [125 I]-rANP1-28 binding to rat kidney by unlabelled peptide sequences

	rANP1-28 IC ₅₀ (nM)	nH	pCNP1-22 IC ₅₀ (nM)	nH	C-ANP4-23 IC ₅₀ (nM)
Glomeruli	2.31 (1.87–2.86)	1.15 (0.96–1.34)	3.67 (2.14–6.17) 1514* (912–2512)	– –	38.2* (19.9–72.4) 17179* (4786–60256)
Inner medulla	1.03 (0.77–1.37)	0.99 (0.65–1.34)	676* (407–1122)	0.83 (0.59–1.07)	>> 10 μ M
Intrarenal arteries	1.82 (1.41–2.36)	1.33 (1.05–1.61)	1202* (631–2291)	0.91 (0.52–1.29)	>> 10 μ M

IC₅₀ values and Hill coefficients (nH) derived from non-linear regression analysis of the inhibition of 250 pM [125 I]-rANP1-28 binding to rat kidney. Data are expressed as the geometric mean and 95% confidence interval for six separate kidneys. *Significantly lower ($P < 0.001$) inhibitory potency of pCNP1-22 and C-ANP4-23 compared with rANP1-28.

Table 4 Competitive inhibition of specific renal [125 I]-rANP1-28 binding by the antagonist, HS-142-1

	Human	Bovine	Rat
Glomeruli	57.1 (43.1–75.7)**	0.54 (0.31–0.95)*	1.95 (1.25–2.66)
Medulla	216 (83.0–562.3)**	1.73 (0.71–4.25)	1.54 (1.07–2.22)
Intrarenal arteries	93.8 (54.6–160.7)**	0.73 (0.27–2.06)*	1.82 (1.13–2.92)

IC₅₀ values (μ g ml⁻¹) derived from non-linear regression analysis of [125 I]-rANP1-28 binding to human, bovine and rat kidney. Data are expressed as the geometric mean and 95% confidence interval for six separate kidneys. Significant differences (* $P < 0.05$; ** $P < 0.001$) in the inhibitory potency of the functional antagonist, HS-142-1, for sites in human and bovine kidney compared to those in the rat.

Figure 6 Competitive inhibition curves of specific [125 I]-rANP1-28 binding to glomeruli in the rat (■), human (●) and bovine (□) kidney. Each point represents the mean $\pm$ s.e.mean derived from six kidneys, expressed as a percentage of ligand binding in the presence of increasing concentrations of C-ANP4-23.

is supported by studies demonstrating that papillary membranes, collecting duct cells and interstitial cells express only a 130–140 kDa, guanylyl cyclase-linked receptor subtype (Gunning *et al.*, 1988; Martin *et al.*, 1989; Fontoura *et al.*, 1990). The similarity between the binding profiles in the inner medulla and intrarenal arteries indicates that, like the inner medulla, NPR_{A/B} subtypes also predominate in the interlobar and arcuate arteries of rat, human and bovine kidney. Activation of these receptors by natriuretic peptides has been shown to induce a selective relaxation of rat arcuate arteries, mediated by an increase in guanylyl cyclase activity (Aalkjaer *et al.*, 1985; Ohishi *et al.*, 1988; Veldkamp *et al.*, 1988).

Clearance of ANP from the circulation is achieved both via NPR_C receptors and the enzyme endopeptidase 24.11 (EC.3.4.24.11). This enzyme has been identified on human vascular endothelial cells and, in the kidney, on brush border cells of the proximal tubule (Schulz *et al.*, 1989; Llorens-Cortes *et al.*, 1992). It is likely that endopeptidase 24.11 and NPR_C receptors may act in concert to regulate plasma ANP levels (Barclay *et al.*, 1992); however, species differences in the relative density of renal NPR_C receptors may indicate that this pathway is less influential in the human and bovine kidney than in the rat. The highest density of natriuretic peptide binding sites was found in the rat kidney. Despite the homology which exists between mammalian ANP sequences, it was considered that the lower binding density observed in the human and bovine kidney may be due to the use of species-specific ligands. Studies using both [125 I]-rANP1-28 and [125 I]-hANP1-28, however, demonstrated an identical localization and density of binding on glomeruli, inner medulla and intrarenal arteries in all three species indicating that the differential binding was not due to use of the rat ligand.

In the present study, [125 I]-rANP1-28 binding was inhibited by HS-142-1 in the rat and bovine kidney with an inhibitory potency similar to that previously found in rabbit kidney cortex and bovine adrenocortical membrane preparations (Morishita *et al.*, 1991). *In vivo*, HS-142-1 has been shown to inhibit the natriuresis and diuresis induced by acute volume expansion in rats (Sano *et al.*, 1992b) whilst *in vitro* it inhibits ANP binding to its guanylyl cyclase-linked receptor, ANP-stimulated cyclic GMP accumulation and binding of [125 I]-rANP1-28 to isolated rat glomeruli (Morishita *et al.*, 1991; Sano *et al.*, 1992a), suggesting that the inhibition of natriuresis and diuresis is due in part to the direct antagonism by HS-142-1 of guanylyl cyclase-linked receptors in renal glomeruli. The differences in affinity which HS-142-1 displayed for [125 I]-rANP1-28 binding sites, in rat, bovine and

Figure 7 Competitive inhibition curves of specific [125 I]-rANP1-28 binding to glomeruli (a) and inner medulla (b) in the presence of increasing concentrations of HS-142-1. Incubations were carried out in the presence of excess (10 μ M) C-ANP4-23, blocking the NPR_C receptor subpopulation. Each point represents the mean $\pm$ s.e.mean derived from six kidneys expressed as a percentage of ligand binding in bovine (□), rat (■) and human (●) kidney. Competitive inhibition of binding to intrarenal arteries gave very similar inhibition curves to glomeruli (data not shown).

human kidney suggest a significant species heterogeneity in the guanylyl cyclase-linked receptor subtype, an observation supported by pharmacological investigation in membrane preparations of rat, human and bovine glomeruli and papillae (Féthière & De Léan, 1991).

No evidence was obtained for the presence of a renal NPR_B subtype although recent reports have indicated expression of this receptor in human kidney, following PCR amplification (Canaan-Kühl, 1992), and by the autoradiographic demonstration of low level [125 I]-Tyr⁰CNP1-22 binding to rat kidney sections (Konrad *et al.*, 1992). This apparent discrepancy may reflect the relatively low level expression of the NPR_B receptor in the kidney and the lack of a specific antagonist for binding studies. HS-142-1 does not appear to distinguish between NPR_A and NPR_B receptor subtypes (Toki *et al.*, 1992) while CNP1-22, the proposed endogenous ligand for the NPR_B subtype (Koller *et al.*, 1991; Suga *et al.*, 1992), is non-selective and has a high affinity for both NPR_B and NPR_C subtypes (Brown & Zuo, 1992; 1993). The present findings suggest that although CNP1-22 may produce a vasodilatation *in vivo* via activation of NPR_B receptors (Wong & Garbers, 1992) this subtype is not involved in the renal response to natriuretic peptides.

Studies investigating the functional significance of the natriuretic peptide family have to date been hindered by the lack of specific receptor antagonists. Although a number of peptide sequences have been synthesized which are selective for the NPR_C receptor (Maack *et al.*, 1987; Koepke *et al.*, 1989) and more recently for the guanylyl cyclase-linked NPR_A

receptor (Delporte *et al.*, 1992; Mimeault *et al.*, 1993), the discovery and characterization of the non-peptide guanylyl cyclase-linked receptor antagonist, HS-142-1, may help to elucidate further the physiological and pathophysiological roles of the natriuretic peptides. The apparent differences displayed in the affinity for competing sequences implies the existence of a significant interspecies heterogeneity in natriuretic peptide receptors which may be of importance in the interpretation of results obtained using animal models to

study peptide receptor-ligand interactions and their relevance to man.

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