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Pharmacological Evidence for the Heterogeneity of Atrial Natriuretic Factor-R_1 Receptor Subtype

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SUMMARY

The atrial natriuretic factor-R_1 (ANF-R_1) receptor is known to mediate the biological effects of diverse natriuretic/diuretic/vasorelaxant peptides. In order to investigate the differential selectivity of this class of receptor, we have compared its pharmacological profile for various natriuretic peptides in rat, bovine, and human kidney. In contrast to bovine and rat, human kidney glomeruli do not express significant amounts of ANF-R_2 receptor. In addition, the binding of ^{125}I-labeled rat ANF-(99–126) to the ANF-R_1 receptor in human and bovine kidney glomeruli is not blocked by rat ANF-(103–123) ($pK < 6$), whereas in rat kidney glomeruli this peptide displays high affinity for the ANF-R_1 receptor ($pK = 8.5$). This observation reveals a species heterogeneity between the rat and the human and bovine kidney receptors. In addition, we have observed striking differences in the pharmacological profiles of rat papillary, bovine papillary, and human kidney glomerular receptors, which contain only the 130-kDa ANF-R_1 monomer. The bovine and human profiles were similar but diverged from that of the rat. In addition to the species heterogeneity of the ANF-R_1 class, we could detect a significant intraspecies heterogeneity. Two distinct profiles could be disclosed, one having high affinity for both ANF and brain natriuretic peptide (BNP) and being identified in all three species studied and the other displaying lower affinity for BNP and being found in rat and human kidneys. We also demonstrate that rat and bovine papillary ANF-R_1 receptors are coupled to guanylate cyclase and that ANF and BNP could activate the enzyme with potency similar to their potency in competing for ^{125}I-labeled rat ANF-(99–126) binding. The results presented demonstrate that the ANF-R_1 receptor class can be subclassified, based on distinct pharmacological profiles, and indicate a wide diversity within the ANF-R_1 receptor family.

Atrial cardiocytes secrete a family of natriuretic peptides that play an important role in the regulation of cardiovascular homeostasis (1). ANF, which was the first factor identified, relaxes vascular smooth muscle, elicits an important diuresis and natriuresis, and inhibits aldosterone secretion from the adrenal cortex (2, 3). These effects of the hormone are mediated by high affinity membrane receptors, which were identified in all target organs of ANF (4). A multiplicity of structural and biological properties have permitted the classification of the ANF receptor into different populations, i.e., ANF-R_1, a monomeric 130-kDa form including a guanylate cyclase moiety, and ANF-R_2, a dimer of 64-kDa subunits for which a direct association with the enzyme has not been shown. The properties of both receptor populations were recently studied independently in model cell lines (5).

More recently, three other members of this growing family of natriuretic/diuretic/vasoactive peptides were discovered. The first to be identified and sequenced was a 26-amino acid peptide from porcine brain, which was termed BNP (6). Later it was found that rat atrium contains iso-rANF, a 45-amino acid peptide (7) that is as distinct from rANF as it is from pBNP, although it might be the BNP equivalent in the rat. Recently, the third member of this family was isolated from porcine brain (8). pCNP-22 shows striking amino acid sequence homology with the other peptides, especially within the disulfide-bridged loop, and has a distinctive feature in that it lacks the carboxyl-terminal extension so important for full expression of the biological effects, which led the authors to suggest that it is likely to be a specific ANF-R_2 ligand. However, it was shown to stimulate a particulate guanylate cyclase in cultured vascular smooth muscle cells more potently than ANF, which suggests that it might be specific for another type of ANF-R_1 receptor (9).

BNP and iso-rANF exert biological effects similar to those

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ABBREVIATIONS: ANF, atrial natriuretic factor; BNP, brain natriuretic peptide; bASIF, bovine aldosterone secretion inhibitory factor; CNP, C-type natriuretic peptide; C-ANF, [Cys^{116}]-atrial natriuretic factor-(102–116)-NH_2; ScGMP-TME, 2′-*O*-monosuccinyl-cGMP tyrosyl methyl ester; SDS, sodium dodecyl sulfate; Met-O-ANF, [Met^{110}-*O*]-atrial natriuretic factor-(99–126); rANF, rat atrial natriuretic factor; pBNP, porcine brain natriuretic peptide; ANP, atrial natriuretic peptide.

of ANF. In addition, it has been shown that pBNP interacts with ANF receptors on bovine aortic smooth muscle cells (10), cultured rat vascular smooth muscle cells (11), rat kidney (12), and bovine adrenal cortex (13). At present, there is little evidence that these peptides bind to distinct receptor subpopulations. Molecular cloning techniques have permitted the identification of two guanylate cyclase-linked ANF receptor-coding sequences, the ANP-A (14, 15) and the ANP-B (16, 17) clones. It was reported that the ANP-B receptor clone was more sensitive to activation by pBNP than by ANF, whereas the sensitivity of the ANP-A receptor clone was similar for both peptides or slightly better for ANF (16, 17). Very recently, Koller *et al.* (18) reported that the ANP-B receptor clone was selectively activated by CNP. Taking in consideration these reports, we have investigated the apparent heterogeneity of the ANF-R_1 receptor class. In this report, we document the pharmacological and biochemical characteristics of the ANF-R_1 receptors from rat, bovine, and human kidneys. Affinity labeling of the receptor, competition binding curves with corresponding affinities of the different peptides, and guanylate cyclase assays with corresponding potencies are reported. The differential selectivity of the peptides for different classes of sites demonstrates the heterogeneous nature of the ANF-R_1 receptor in the rat and in the human species. Comparison of the data also permits classification of the ANF receptors from different species into different families.

Experimental Procedures

Materials. rANF-(99–126) and rANF-(103–123) were purchased from Institut Armand-Frappier (Laval, Canada). C-ANF was obtained from Hukabel (Longueil, Canada). The different peptides from the BNP family, i.e., pBNP-(75–106), pBNP-(81–106), iso-rANF-(64–95) (rBNP-32), and bASIF-(69–103) were bought from Peninsula Laboratories. Male Sprague-Dawley rats were from Charles River (St. Constant, Québec). Bovine kidney was obtained from a local slaughterhouse, and intact parts of human glomeruli from cancerous patients were generously provided by the Pathology Department of the Hotel-Dieu Hospital of Montreal. Carrier-free $Na^{125}I$ was purchased from Amersham Corp. ^{125}I-rANF-(99–126) and ^{125}I-pBNP-(81–106) were prepared by radioiodination of rANF-(99–126) and pBNP-(81–106), using the solid-phase IODO-BEADS method (19). The specific activity of the monoiodinated peptides was 2000 Ci/mmol. cGMP, ScGMP-TME, bovine serum albumin, leupeptin, aprotinin, pepstatin A, amiloride, and phosphoramidon were from Sigma Chemical Co.

Isolation of glomeruli. Rat, bovine, and human glomeruli were isolated according to previously reported modifications (5) of the graded sieving method of Misra (20). The pure glomeruli were then subjected to homogenization for the crude membrane preparation.

Preparation of membranes. Rat and bovine papillae as well as human, rat, and bovine glomeruli were suspended in 20–40 ml of ice-cold homogenizing buffer (20 mM $NaHCO_3$, 1 mM EDTA, 10^{-7} M aprotinin, 10^{-6} M leupeptin, 10^{-7} M pepstatin A) and homogenized with a Polytron homogenizer (Brinkman Instruments) (three times for 10 sec at setting 6), followed by a Potter homogenizer (Heidolph). The papillae homogenate was centrifuged 10 min at 250 × *g*, and then the supernatant together with the homogenate of the glomeruli were centrifuged at 30,000 × *g* for 15 min. The pellet containing the particulate fraction was washed twice with the homogenizing buffer and finally resuspended by gentle homogenization in freezing buffer containing 50 mM Tris·HCl, 250 mM sucrose, 0.1 mM EDTA, and 1 mM $MgCl_2$, pH 7.4. The membranes were then frozen in liquid nitrogen and stored at −70° until used. Protein concentration was measured with the bicinchoninic acid protein assay kit from Pierce.

Receptor binding assay. Binding studies with intact membranes were essentially performed as previously described (5), with minor modifications. Briefly, membranes from rat papillae (70 μg/ml), bovine papillae (54 μg/ml), rat glomeruli (30 μg/ml), bovine glomeruli (10 μg/ml), and human glomeruli (30 μg/ml) were incubated with 10 pM ^{125}I-rANF-(99–126) or ^{125}I-pBNP-(81–106) and varying concentrations of the unlabeled peptides. Binding assay was performed in 1 ml of binding buffer (50 mM Tris·HCl, 0.1 mM EDTA, 0.5% bovine serum albumin, 5 mM $MnCl_2$, pH 7.4) overnight at 4°. Binding with human glomeruli was done in the presence of 10^{-6} M phosphoramidon. Membrane-bound radiolabeled ligand was separated from free ligand by filtration through 1% polyethyleneimine-treated GF/C filters (Whatman, Clifton NJ), followed by extensive washing with ice-cold 50 mM phosphate buffer (pH 7.4). Bound radioactivity remaining on the filters was counted in an LKB 1272 CliniGamma counter, with 80% efficiency. The data presented are from single experiments, representative of two to six separate experiments.

Affinity labeling protocol. Chemical cross-linking of the ANF receptor was performed as previously described (21). Briefly, rat and bovine papillae membranes, as well as rat, bovine, and human glomeruli membranes, were incubated for 16 hr at 10° with 10 pM ^{125}I-rANF-(99–126) in binding buffer, in the presence or absence of the indicated agents. Phosphoramidon (10^{-6} M) was added to the glomeruli membranes. After incubation, the membranes were washed in ice-cold 50 mM phosphate buffer (pH 7.4) and incubated for 30 min at 4° with 0.1 mM bis(sulfosuccinimidyl)suberate, at a final concentration of 1 mg/ml. The reaction was then quenched with ammonium acetate and the membranes were centrifuged. The washed pellet was resuspended in sample buffer and heated at 100° for 3 min before electrophoresis.

SDS-polyacrylamide gel electrophoresis. An equivalent amount of 100–200 μg/ml solubilized membrane proteins was applied to each lane of a slab gel, and electrophoresis was carried out using the discontinuous buffer system of Laemmli (22), with a 7.5% acrylamide separating gel. After electrophoresis, the gels were fixed, stained with Coomassie blue, destained, and dried before autoradiography on Kodak X-Omat Rp film, with exposure at −70° with a DuPont enhancing screen (Wilmington, DE).

Guanylate cyclase assay. Particulate guanylate cyclase activity was measured according to the procedure of Garbers and Murad (23). Particulate fraction containing the enzyme was incubated for 10 min at 37° in 100 μl of the reaction mixture, consisting of 50 mM Tris·HCl, pH 7.6, 10 mM theophylline, 2 mM 3-isobutyl-1-methylxanthine, 10 mM creatine phosphate, 10 units of creatine phosphokinase, 1 mM GTP, 4 mM $MnCl_2$, and varying concentration of the peptides to be tested. The reaction was initiated with the addition of rat or bovine papillae membranes and terminated with the addition of 100 μl of 120 mM EDTA, followed by immersion in boiling water for 3 min. After the addition of 1 ml of 50 mM ammonium acetate, the assay mixture was centrifuged. cGMP in the supernatant was then separated on an alumina column (24), evaporated to dryness in a Speedvac concentrator (Savant), and resuspended in 1 ml of 50 mM sodium acetate, pH 6.2. Aliquots of 100 μl were then assayed for cGMP as previously described (5).

Iodination of ScGMP-TME. Iodination and subsequent purification of ScGMP-TME were carried out according to the method of Patel and Linden (25), with some modifications. Stocks of the cGMP derivative were kept at −20° in 50 mM sodium acetate, pH 4.75. For iodination, 5 μl of the product, corresponding to 5 nmol, were dissolved in 95 μl of 0.1 M phosphate buffer, pH 7.0, together with 1 mCi of $Na^{125}I$. The reaction was triggered by the addition of two IODO-BEADS, which served as the oxidizing agent. After a 15-min incubation period in the ice, the beads were removed and the iodination reaction was diluted with 1.9 ml of 30 mM phosphate buffer, pH 3.5. The product was then directly injected into a C18 Vydac column (Chromatographic Specialties) and purified by high performance liquid chromatography, under isocratic conditions, with an elution buffer consisting of 30 mM phosphate, pH 3.5/acetonitrile, in a ratio of 60:40, at a flow rate of 1

ml/min. Iodinated ScGMP-TME generated in this way was then used in radioimmunoassay for cGMP.

Data analysis. Competition binding curves were analyzed by weighted nonlinear least-squares curve-fitting, based on a four-parameter logistic equation (26), to obtain initial estimates of the ED_{50} and slope factor. Then the curves were analyzed according to a model for the binding of competing ligands to one or several independent classes of sites (27). A model for two classes of sites was retained only when the fit was statistically improved, as evaluated by a partial F test. Binding equilibrium constants are reported as pK or $-\log K_d$, and ED_{50} values are reported as pD_2 or $-\log ED_{50}$.

Results

We have investigated the potential heterogeneity of ANF-R_1 receptors in different species by comparing their selectivity for different natriuretic peptides. The first indication for the subclassification of ANF-R_1 receptors was obtained from binding studies with kidney glomerular membranes from rat, bovine, and human. When rANF-(103–123) was used as the competing peptide against ^{125}I-rANF-(99–126), some species differences were observed. As previously reported (5) and as shown in Fig. 1, the receptor populations in bovine and rat kidney glomeruli do not have the same affinity for rANF-(103–123). When the curves were analyzed with a model for two distinct classes of sites (27), the affinity of the ANF-R_2 receptor for this peptide was relatively the same for rat and bovine tissues, with pK values of 9.8 and 9.4, respectively. In rat, rANF-(103–123) bound with relatively high affinity (pK = 8.5) to the ANF-R_1 receptor, contrasting with its low affinity for the bovine ANR-R_1 in the kidney glomeruli (pK = 6). In both species, C-ANF competed for ANF binding only to the ANF-R_2 receptor (data not shown).

The affinity of the ANF-R_1 for pBNP-(81–106) also differed between these species. In the absence of C-ANF, pBNP-(81–106) competed for ^{125}I-rANF-(99–126) binding with an overall higher potency in bovine than in rat kidney glomeruli. When C-ANF was added to block the ANF-R_2 receptors, in order to investigate exclusively the ANF-R_1 population, the competition curve of pBNP-(81–106) was shifted to the right by about 10-fold in rat glomeruli only (data not shown). Competition curves with and without C-ANF both displayed slope factors smaller than unity, which is also indicative of receptor heterogeneity. The observation that, even under conditions where the ANF-R_2 sites were completely blocked by saturating concentrations of C-ANF, this apparent heterogeneity was still detectable strongly suggested some multiplicity of ANF-R_1 binding sites in rat kidney. These results were then compared with those obtained with human kidney glomeruli. The competition curves for ^{125}I-rANF-(99–126) binding by rANF-(103–123) revealed a low potency (Fig. 2), the pK measured (5.0) was much more similar to that for bovine ANF-R_1 than for rat ANF-R_1, and no intermediate plateau was detectable. In addition, as shown in Fig. 2, C-ANF did not compete for the binding of ^{125}I-rANF-(99–126), indicating that the ANF-R_2 receptor in human kidney glomeruli is absent or is present in very small amounts. This was confirmed by chemical cross-linking of ^{125}I-rANF-(99–126) to human kidney glomerular membranes. Fig. 3 shows the specific labeling of a single band of M_r 130,000, which was potentiated by amiloride and observed in the presence of rANF-(103–123) or C-ANF.

In order to further investigate this apparent heterogeneity of ANF-R_1 receptors, we documented their pharmacological profile in kidney papillae. This tissue expresses solely the ANF-R_1 receptor in bovine and rat, as shown by the specific labeling of a 130-kDa band on SDS-polyacrylamide gel electrophoresis after affinity cross-linking of ^{125}I-rANF-(99–126) (Fig. 4). In both species, labeling of this band was sensitive to amiloride but insensitive to C-ANF. The human kidney glomerulus, being

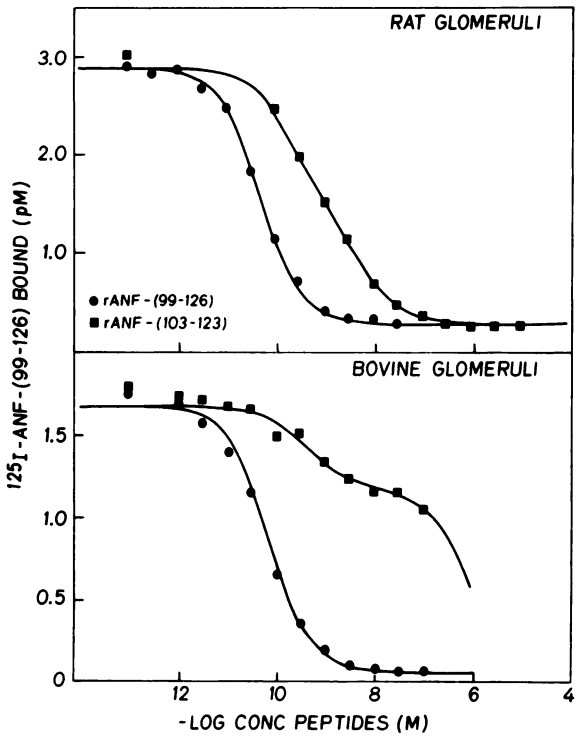

Fig. 1. Competition binding curves of rANF-(99–126) and rANF-(103–123) for the binding of ^{125}I-rANF-(99–126) to rat and bovine kidney glomeruli. Membrane proteins from rat glomeruli (30 μg/ml) and bovine glomeruli (10 μg/ml) were incubated with 8–10 pM ^{125}I-rANF-(99–126) and varying concentrations of the competing unlabeled peptides. Bound was separated from free radioligand by rapid filtration through GF/C filters. The curves were simultaneously analyzed by computer, according to a model for one or two classes of sites, based on the law of mass action (27).

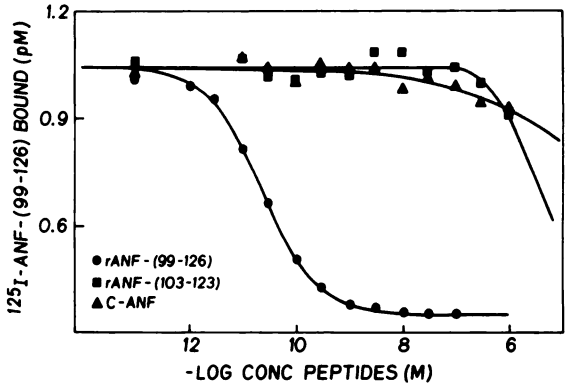

Fig. 2. Competition curves of rANF-(99–126) analogs for the binding of ^{125}I-rANF-(99–126) to human kidney glomeruli membranes. Kidney glomeruli membranes (30 μg/ml) were incubated overnight at 4° with 8–10 pM ^{125}I-rANF-(99–126) and varying concentrations of the unlabeled competing peptides. Phosphoramidon was added to each tubes at a final concentration of 10^{-6} M. Separation of free from bound radioligand was performed as mentioned in the legend to Fig. 1.

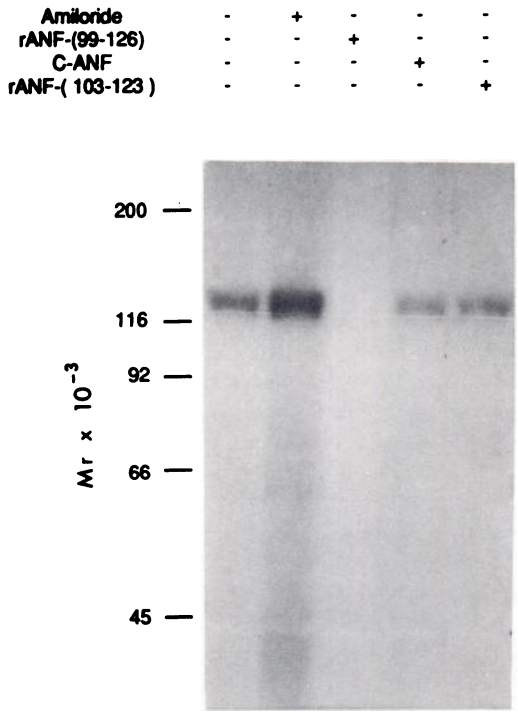

Fig. 3. Affinity cross-linking of ^{125}I-rANF-(99–126) to human kidney glomeruli membranes in the absence or presence of 10^{-4} M amiloride, 10^{-7} M rANF-(99–126), 10^{-6} M C-ANF$_1$-or 10^{-6} M rANF-(103–123). Cross-linking was performed with 0.1 mM bis(sulfosuccinimidyl)suberate at 4° for 30 min. The reaction was quenched and membranes were washed. For electrophoresis, the washed membranes were solubilized in sample buffer containing 5% 2-mercaptoethanol. SDS-gel electrophoresis was performed on a 7.5% acrylamide gel. Autoradiograms of the fixed dried gels are shown after 5-days exposure. Molecular weight standards are myosin (200,000), β-galactosidase (116,250), phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), and carbonic anhydrase (31,000). Similar results were obtained in two separate experiments.

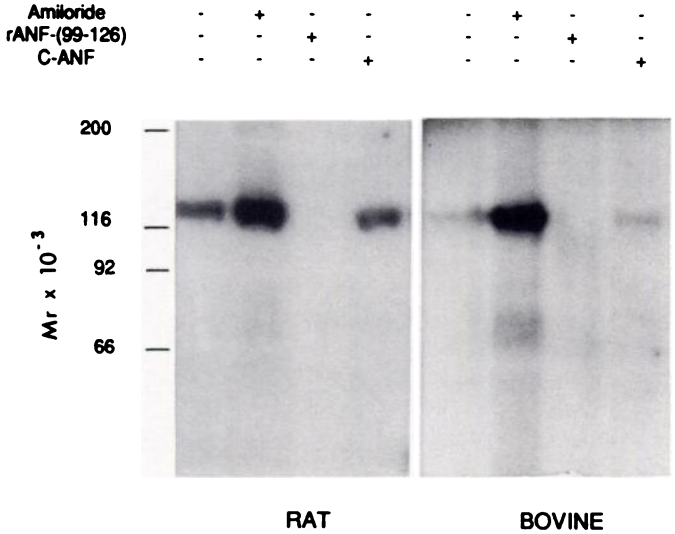

Fig. 4. Affinity labeling of the ANF receptor from rat and bovine papillae with ^{125}I-rANF-(99–126), in the presence or absence of the indicated agents. Cross-linking and SDS-gel electrophoresis were performed as described for Fig. 3. An autoradiogram of the fixed dried gel is shown after 2-weeks exposure. Similar results were obtained in five separate experiments.

homogenous in ANF-R_1 receptors, was selected for comparative studies.

In order to disclose the heterogeneity of ANF-R_1 receptors in the three preparations, various natriuretic peptides were used as competitors for the binding of ^{125}I-rANF-(99–126), i.e., rANF-(99–126), pBNP-(81–106), iso-rANF-(64–95), and bASIF-(69–103). Table 1 shows that, in the rat papillae, rANF-(99–126) and iso-rANF-(64–95) were the best competitors, whereas pBNP-(81–106) and bASIF-(69–103) were less potent (Fig. 5, *upper*). An almost perfect mirror image of this potency order was observed in bovine papillae (Fig. 5, *lower*), where bASIF-(69–103) was the most potent competitor, followed by rANF-(99–126), pBNP-(81–106), and iso-rANF-(64–95). This order was very similar to that observed for human kidney glomeruli (Fig. 6). In bovine papillae, the competition curves displayed slope factors close to unity, strongly suggesting homogeneity of ANF-R_1 sites in that species. This was not the case in the rat and human species. When rANF-(103–123), pBNP-(81–106), or its amino-terminal-elongated form pBNP-(75–106) were used, the competition curves were very shallow (slope factor = 0.6) and displayed an intermediate plateau, revealing ANF-R_1 site heterogeneity. The three competition curves were best fitted to a model involving two classes of sites, with a very high level of statistical significance ($p < 0.01$). There was a minority of sites with higher affinity for these competing peptides, corresponding to approximately 20–30% of the total, whereas a majority of sites displayed high affinity only for rANF-(99–126). Whereas pBNP-(81–106) and rANF-(103–123) displayed a 40–50-fold selectivity ratio between the two sites, pBNP-(75–106) was the most selective peptide, with a 100–150-fold ratio (Fig. 7).

The high affinity sites of rat papillary membranes could be labeled with ^{125}I-pBNP-(81–106). Fig. 8 shows that the specific labeling documents high affinity binding sites ($pK = 9.8$), which correspond to approximately 10% of those labeled with ^{125}I-rANF-(99–126). This corresponds to the affinity and proportion of high affinity binding sites calculated from competition binding curves using ANF as the labeled ligand, indicating that at the concentration used ^{125}I-pBNP-(81–106) is able to bind specifically to high affinity sites. In addition, specific high affinity sites for ^{125}I-pBNP-(81–106) were dramatically enhanced in the presence of amiloride, a property specific to the ANF-R_1 class of receptor (5). These results confirm by direct labeling with ^{125}I-pBNP-(81–106) that only a minor proportion of ^{125}I-rANF-(99–126)-labeled receptors displays high affinity for BNP.

Comparison of binding selectivity of the ANF-R_1 receptor from the three species indicates an almost perfect correlation between the human and bovine species, with an r value of 0.98, whereas no correlation was seen between the rat and bovine species. These results indicate that the human ANF-R_1 pharmacological profile is much more similar to that from bovine than from rat kidney.

Guanylate cyclase activity in renal papillary membrane was also measured, in order to correlate the receptor binding data with catalytic activity. Table 1 summarizes the potency of the different peptides. The results obtained parallel the binding data fairly well, because the pD_2 values are commensurate with the pK for the different peptides. In addition, the rank potency order is the same as that obtained in the binding studies. At maximal concentration, all the peptides stimulated guanylate

TABLE 1

Characterization of the ANF-R_1 receptor from rat and bovine papillae and human kidney glomeruli

Values are the mean of two to six experiments. Standard error for each value was <5%.

Peptides	Slope factor[a]			pD_2[b]		pK[c]			R[d]		
	Rat	Bovine	Human	Rat	Bovine	Rat	Bovine	Human	Rat	Bovine	Human
										%	
rANF-(99–126)	0.9	0.9	0.9	9.2	10.1	10.1	10.5	10.8	100	100	100
rANF-(103–123)	0.6			7.9	6	9.2 7.7	<6	5	28 72		
bASIF-(69–103)	1.4	1.1	0.6	8.0	9.7	7.8	10.8	10.4 8.9	100	100	76 24
pBNP-(75–106)	0.6	1.0	0.8	7.6		9.4 7.3	10.6	10.4 8.7	32 68	100	27 73
pBNP-(81–106)	0.6	0.9	0.5	8.6	9.8	9.7 8	10.2	10.3 8.7	31 69	100	35 65
Iso-rANF-(64–95)	0.9	0.9	0.9	9.3		9.5	9.4	8.9	100	100	100

[a] Slope factor of the corresponding peptides in competition binding curves with ^{125}I-rANF-(99–126).
[b] pD_2 (−log ED_{50}) of the corresponding peptides for the stimulation of particulate guanylate cyclase activity.
[c] pK (−log K_d) of the corresponding peptides in competition binding curves with ^{125}I-rANF-(99–126).
[d] Binding capacity.

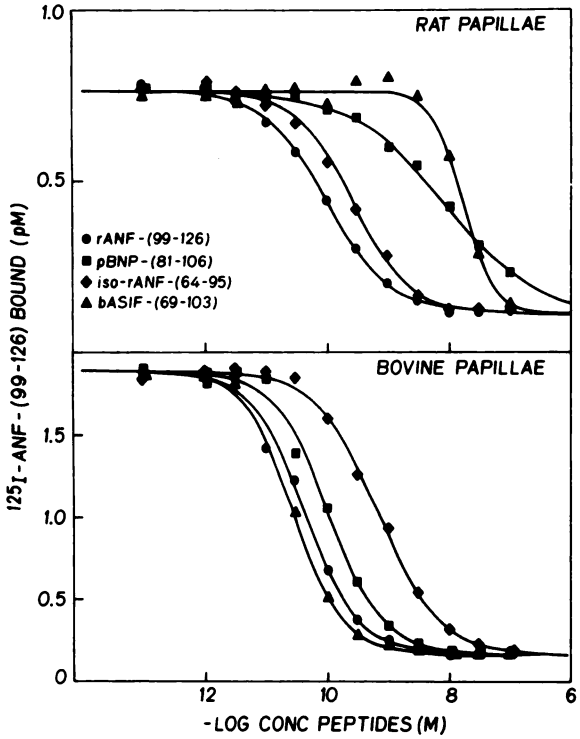

Fig. 5. Competition binding curves of different natriuretic peptides for the binding of ^{125}I-rANF-(99–126) to rat and bovine papillary membranes. Binding conditions were as in Fig. 1. The final concentration of membrane proteins was 70 μg/ml and 54 μg/ml for rat and bovine membranes, respectively.

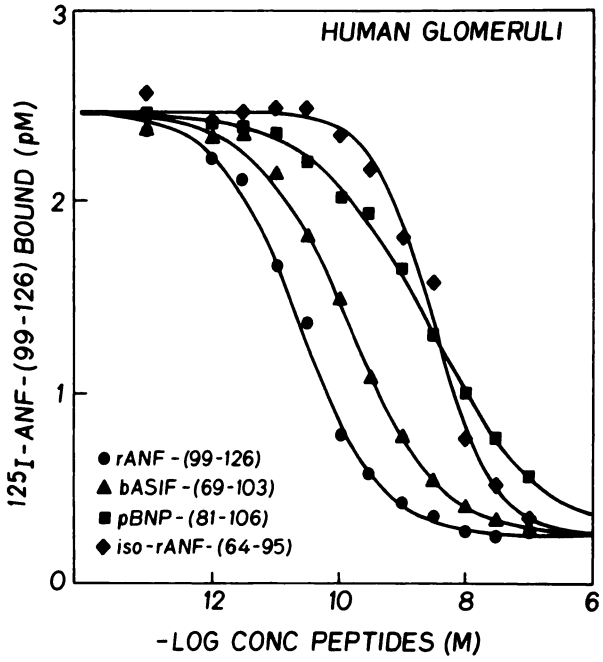

Fig. 6. Competition for ^{125}I-rANF-(99–126) binding to human kidney glomeruli membranes by structurally related peptides. Binding conditions were as described for Fig. 1, and the curves were analyzed with the four-parameter logistic equation (26).

cyclase activity to the same extent, indicating that the total receptor population is coupled to the enzyme.

Discussion

We have further documented the pharmacological diversity of the ANF-R_1 receptor and confirmed its species variability. Previous structure-activity relationship studies suggested species differences in the structural requirements for the relaxant effect of ANF. It was shown that rANF-(103–123) could relax rat aortae with high potency, similar to rANF-(103–125), while presenting very low potency in rabbit aortae (28). It was then proposed that this variation in vasodilatory profiles of ANF observed between species was due to the existence of different ANF receptor subtypes. Our results based on receptor binding studies confirm this species variability of the ANF-R_1 receptors, in agreement with the divergent vasodilatory profiles.

Autoradiographic studies have shown that human kidney also expresses ANF binding sites and their distribution is nearly identical to that seen in rat and guinea pig kidney, i.e., glomerular apparatus, outer medulla, and small renal arteries (29). In addition, specific high affinity receptors for ANF were identified by radioreceptor assay in human renal cortical membranes (30). On the other hand, these data did not assess the heterogeneity of ANF binding sites, and no comparison with extensively studied animal models was reported. Our study shows that ^{125}I-ANF-(99–126) binding in human glomeruli is insensi-

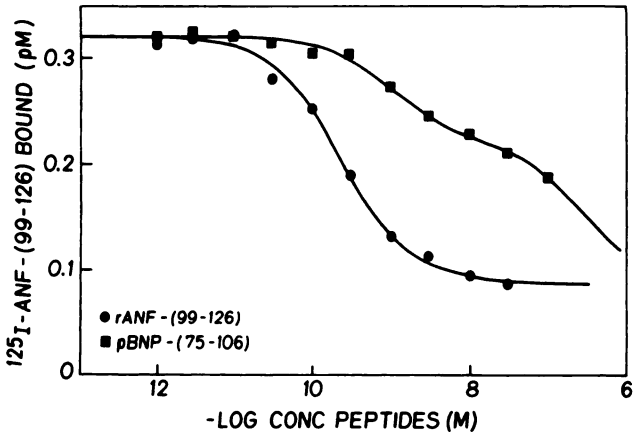

Fig. 7. Competition for ^{125}I-rANF-(99–126) binding to the ANF receptor in rat papillary membranes by rANF-(99–126) and pBNP-(75–106). The competition curves were analyzed by computer as in Fig. 1.

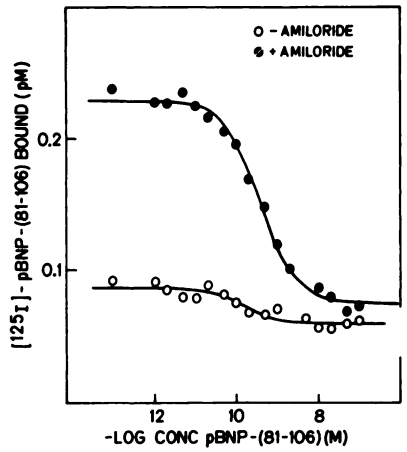

Fig. 8. Competition binding curve of pBNP-(81–106), in the presence or absence of amiloride (10^{-4} M), for the binding of ^{125}I-pBNP-(81–106) to rat kidney papillary membranes. Binding conditions and data analysis were as in Fig. 1.

tive to C-ANF, indicating the lack of significant amounts of ANF-R_2 receptors. This contrasts with the apparent ubiquity of ANF-R_2 sites in kidney glomeruli from other species. The demonstration of the labeling of a single 130-kDa band on SDS-polyacrylamide gel electrophoresis corroborates this hypothesis. If present in human glomeruli, ANF-R_2 sites are in extremely low amounts, because overexposure of the gel failed to document their presence. In addition, the low potency of rANF-(103–123) to compete for the binding of the radioligand in human kidney indicates that the ANF-R_1 expressed in human kidney glomeruli is more homologous to the bovine than to the rat receptor. Actually, the ineffectiveness of rANF-(103–123) in eliciting cGMP production was recently reported in a human adrenal tumor cell line (31).

In a preliminary report, Gunning *et al.* (32) indicated that the ANF receptors of glomeruli and collecting ducts might not be identical. This assumption raises the prospect of the intrinsic heterogeneity of ANF-R_1 receptors. In fact, we have confirmed and extended the initial findings of Gunning *et al.*, by showing that, even in the presence of concentrations of C-ANF saturating ANF-R_2 sites, the competing peptides used, i.e., pBNP-(81–106) and rANF-(103–123), still discriminate between two classes of sites in rat kidney glomeruli. This was more clearly demonstrated in renal papillae, which express only the ANF-R_1 receptor. The observation that rat and bovine renal papillae express only the ANF-R_1 subtype is in agreement with previously published results showing that ANF binds specifically to interstitial (33) and medullary collecting duct cells (34), inhibits sodium-dependent oxygen consumption by rabbit inner medullary collecting duct cells as well as luminal sodium entry in these cells (35), and in addition stimulates cGMP accumulation by these cells (36). Furthermore, our findings are consistent with those of Martin *et al.* (37), who demonstrated that rat renal papillary membranes expressed only the 130-kDa ANF-R_1 receptor, as confirmed by the failure of C-ANF to compete for ^{125}I-ANF binding and by the labeling of a single high molecular weight band with iodinated ANF. In addition, we demonstrate that the receptor in bovine and rat papillae is coupled to the guanylate cyclase enzyme.

The heterogeneity of the guanylate cyclase-coupled ANF-R_1 receptor that was previously suggested by Gunning *et al.* (32) appears to be due, at least in part, to species differences. Receptors from rabbit inner medullary collecting duct cells have similar affinities for pBNP and rANF, in contrast to rat glomeruli, which exhibited a markedly lower affinity for pBNP (38). In terms of species differences, the same statement could be applied to ANF metabolites. rANF-(103–123) showed low potency in rabbit aortae relaxation assays, while being as potent as ANF in rat aortae relaxation assays (28) and competition binding assays in rat glomerular membranes (5). Our results agree well with the hitherto published observations. In addition, we were able to detect species differences in the rank potency order of BNP-related peptides. Rat papillae recognize iso-rANF-(64–95) with higher affinity than pBNP-(81–106) or bASIF-(69–103) and, conversely, the ANF-R_1 receptor from bovine papillae has slightly higher affinity for bASIF-(69–103) than for rANF-(99–126) or pBNP-(81–106). By comparing the natriuretic peptides from different species, we have conspicuously identified intraspecies heterogeneity of ANF-R_1 sites. Our results show that, apart from differing between species, the ANF-R_1 subtype could be pharmacologically subclassified even within a single species. The ANF-R_{1A} subtype displays high affinity for both rANF-(99–126) and pBNP-(81–106). This subtype is predominant in bovine but minor in rat and human kidney (Table 1). In terms of selectivity, it corresponds to the previously characterized natriuretic peptide receptors. The ANF-R_{1B} subtype, which displays high affinity for rANF-(99–126) but low affinity for pBNP-(81–106), is the major subtype in rat and human kidney. The existence of such a subtype, which discriminates so well rANF-(99–126) from pBNP-(81–106), was recently suggested in the human adrenal tumor cell line SW-13 (31). Interestingly, NMR studies have shown that pBNP, which discriminates between the two papillary ANF-R_1 subtypes, displays less conformational averaging than ANF (39). Whether this apparently better conformational stability of pBNP might contribute to its receptor subtype selectivity remains to be ascertained.

The recently documented selectivity of guanylate cyclase receptor clone B for CNP (18) would indicate that it might correspond to a third, ANF-R_{1C}, subtype, with a pharmacological profile of CNP > BNP > ANF, distinct from the two others. This third ANF-R_1 subtype would display low affinity for ANF and, therefore, would not be documented by [^{125}I]-ANF binding but only with [^{125}I]-CNP.

These isoforms of ANF-R_1 might have different distribution, different selectivity towards endogenous ligands, and possibly different biological functions. It was reported that the natriuretic/diuretic effects of ANF in the rat could be dissociated with the oxidized form of human ANF, Met-O-ANF. Met-O-ANF elicits significant increase in diuresis without natriuresis or an increase in urinary cGMP (40). This dissociation was dose dependent, because higher doses of Met-O-ANF produce a slight increase in natriuresis and cGMP excretion. In connection with our results, we could postulate that the diuretic effect of Met-O-ANF could be associated with the high affinity ANF-R_{1A} site, whereas the low affinity ANF-R_{1B} site would be responsible for the natriuretic effect of the hormone. Investigation of the binding properties of this oxidized peptide would be needed to verify such a hypothesis. Most recently, Jennings and Flynn (41) showed that iso-rANF had differential renal effects in the rat, depending on the dose administered. At low doses a lesser effect on natriuresis was noted, whereas its effect on water excretion was comparable to that of rANF-(99–126). As for Met-O-ANF, this differential effect was dose dependent. Although our data did not show a strong selectivity of iso-rANF-(64–95) for the different classes of sites proposed, the same postulate as for Met-O-ANF could apply to these peptides. The use of iso-rANF-(51–95) or iso-rANF-(67–95) in competition binding studies such as those documented in this report might reveal a better discrimination of the receptor subtypes that could correlate with the physiological data.

The present study reports pharmacological evidence for the existence of different subpopulations of ANF-R_1 receptors. In general, subclassification of receptors has relied mainly upon rank order of potencies of different agonists or antagonists for a receptor site and on the discovery of selective drugs. Indeed, most recently multiple receptor subtypes were identified for angiotensin II (42), and the κ-opioid receptor was subclassified into multiple subclasses (43). For this purpose, the discovery of more selective analogs of natriuretic peptides that would better discriminate these subtypes will permit definition of their precise biological roles.

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