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Development of Natriuretic Peptide Analogs Selective for the Atrial Natriuretic Factor-R_{1A} Receptor Subtype

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SUMMARY

A pharmacological characterization of subtypes of the atrial natriuretic factor (ANF) receptor ANF-R_1, found in bovine adrenal cortex and rat papillary membrane preparations, has been carried out using various chimeric analogs based on rat ANF(99–126) [rANF(99–126)] and porcine brain natriuretic peptide 32 (pBNP32). Receptor binding and cGMP production assays in bovine adrenal cortex indicate that replacement of the amino-terminal segment of pBNP32 with that of rANF(99–126) enhances the affinity of the peptide for the ANF-R_{1A} receptor subtype and its stimulation of associated guanylate cyclase activity. In rat kidney papillae, the substitution of amino- and/or carboxyl-terminal portions of pBNP32 with those of rANF(99–126) also results in a large increase in the affinity and agonistic potency for the ANF-R_{1A} subtype but in only modest changes in those for the ANF-R_{1B} receptor subtype. Interestingly, in this preparation the chimeric analogs could discriminate by their differential affinities and cGMP production potencies between the two receptor subtypes. In particular, pBNP1, obtained by combining the ring structure of pBNP32 with the amino- and carboxyl-terminal portions of rANF(99–126), is the most selective analog. pBNP1 displays higher affinity and agonistic potency for ANF-R_{1A} receptor than for ANF-R_{1B} receptor. with selectivity ratios between these two subtypes of 632- and 504-fold, respectively. Moreover, an excellent correlation is observed between the affinity of the peptides for the ANF-R_{1A} receptor and their stimulation of particulate guanylate cyclase activity in bovine adrenal cortex ($r = 0.99$, $p < 0.01$) and rat papillary ($r = 0.97$, $p < 0.01$) membrane preparations. In addition, all the chimeric analogs in this study show affinities similar to those of rANF(99–126) and pBNP32 for the ANF-R_2 receptor in NIH-3T3 membrane preparations. Importantly, the chimeric analogs pBNP1 and pBNP3, which contain the core of pBNP32 and the amino-terminal segment of rANF(99–126), display higher affinities for the ANF-R_{1A} receptor type than for the ANF-R_2 receptor type. These results indicate that the analogs combining the ring structure of pBNP32 with the amino- and/or carboxyl-terminal segments of rANF(99–126) are more selective for the ANF-R_{1A} receptor subtype than are the natural peptides rANF(99–126) and pBNP32.

ANF and BNP are involved in the control of various physiological functions such as natriuresis, diuresis, blood pressure homeostasis, and inhibition of aldosterone secretion (1–7). The existence of ANF(99–126), a 28-amino acid cyclic peptide, was first shown in rat and human atria; BNP32, a 32-amino acid cyclic peptide, was subsequently isolated from porcine brain (8–10).

A high sequence similarity exists between the two 17-residue ring structures of ANF(99–126) and BNP32. pBNP32 differs from ANF by only four residues within this ring portion. However, their carboxyl- and amino-terminal segments show little homology (Fig. 1). Structure-activity relationship studies revealed that the disulfide-bridged loop of these peptides is important for receptor affinity and biological activity (11, 12). Moreover, the pharmacological characterization of numerous analogs and fragments of ANF(99–126) indicated that the carboxyl-terminal segment is required for the full expression of biological activity, whereas the amino-terminal residues could be involved in receptor recognition (13–17).

Immunohistochemical and autoradiographic studies revealed the presence of specific binding sites for ANF and BNP within various tissues including the kidney, adrenal gland, and vascular smooth muscle (18–21). Furthermore, the pharmacological profile of ANF binding sites established by affinity cross-linking and photoaffinity labeling studies with various truncated ANF analogs suggested the existence of at least two classes of ANF receptors, referred to as ANF-R_1 and ANF-R_2 (22–28). The ANF-R_1 type, with a molecular mass of 130 kDa, exhibited high affinity only for the ANF native molecule and was functionally coupled to the production of cGMP, whereas

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ABBREVIATIONS: ANF, atrial natriuretic factor; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; C-ANF, [Cys^{116}]-atrial natriuretic factor-(102–116)-NH_2; BOP, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; pBNP, porcine brain natriuretic peptide; hANF, human atrial natriuretic factor; rANF, rat atrial natriuretic factor; HPLC, high performance liquid chromatography.

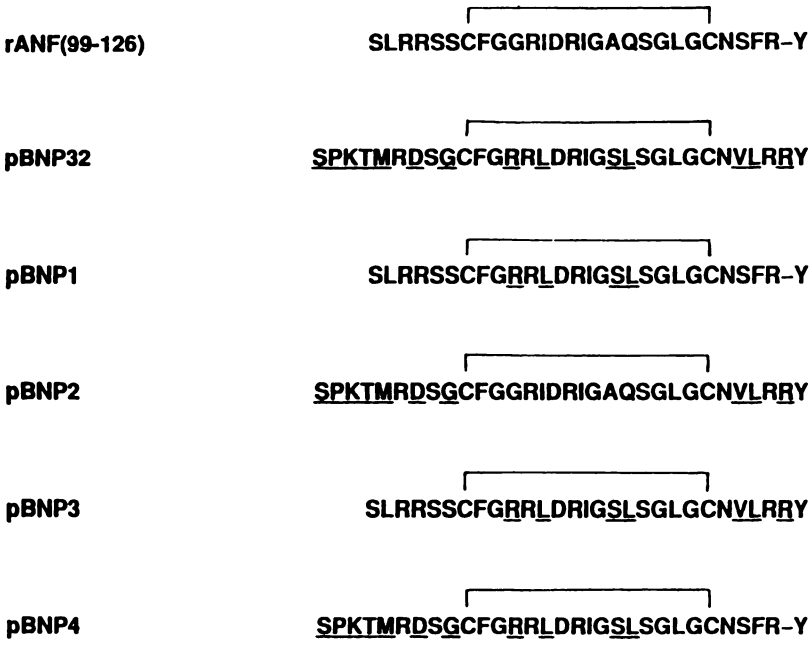

Fig. 1. Primary structures of rANF(99–126), pBNP32, and their related analogs. Substituted amino acid residues found in pBNP32 and chimeric analogs, in comparison with rANF(99–126), are *underlined*.

the ANF-R_2 type, with a molecular mass of 65 kDa, was able to bind a variety of short linear fragments of ANF without associated production of cGMP. Subsequent studies using molecular cloning techniques confirmed the existence of at least three structurally distinct ANF receptor proteins (29–36). The ANF-R_{1A} (GC-A or ANF-A) and ANF-R_{1C} (GC-B or ANF-B) receptors are glycoproteins containing an extracellular peptide binding domain, a single transmembrane portion, and an intracellular region possessing a protein kinase-like region and a particulate guanylate cyclase catalytic domain. The ANF-R_2 receptor, also designated ANF-C, consists of a dimer composed of two disulfide-linked subunits that possess an extracellular peptide binding domain and a short intracellular region devoid of guanylate cyclase activity.

Although important progress has been made in the determination of the pharmacological properties of different ANF receptors, little is known about the molecular mechanisms associated with the various biological actions of natriuretic peptides. The ANF-R_1 receptor class has been proposed to mediate the physiological responses of ANF and BNP, whereas the ANF-R_2 class could play a role in the clearance of peptides (17, 37, 38). Nevertheless, ANF and BNP showed low affinity for the ANF-R_{1C} receptor subtype. More recently, a 22-amino acid peptide designated CNP (or CNP22), which possesses the same 17-residue ring structure as do ANF and BNP but without the extra-loop carboxyl-terminal segment, has been identified as the natural ligand of the ANF-R_{1C} receptor (39–42). CNP22, which shows high affinity for only the ANF-R_{1C} and ANF-R_2 receptors, mediates most of its biological actions, including the diuretic and vasorelaxant effects, through ANF-R_{1C} receptor. However, in contrast to ANF and BNP, CNP has been reported to exhibit an antinatriuretic effect *in vivo*, but the precise signaling function of this response is still unclear (42). Functional evidence for a heterogeneity of ANF receptors between species and within a single species has also been revealed in several studies (43–47). Notably, binding assays carried out in human glomeruli and rat papillary membrane preparations, which contain only the ANF-R_1 receptor class, suggested on the basis of differential affinities for various natriuretic peptides the existence of two classes of specific binding sites, i.e., ANF-R_{1A} and ANF-R_{1B} (46). rANF(99–126) possessed high affinity for the two receptor subtypes, whereas pBNP32 showed high affinity for ANF-R_{1A} receptor and low affinity for ANF-R_{1B} receptor. The specific physiological functions of these receptor subtypes have not been determined.

The present investigation was undertaken to determine the structural requirements of these two ANF-R_1 receptor sybtypes and to develop peptidic analogs more selective for one subtype of ANF-R_1 receptor. Therefore, chimeric peptides based on rANF(99–126) and pBNP32 were designed by substituting alternatively the amino- and/or carboxyl-terminal segments of pBNP32 with those of rANF(99–126) (Fig. 1). Receptor binding and cGMP production assays were carried out in rat papillary membranes, a preparation containing the ANF-R_{1A} and ANF-R_{1B} subtypes, and in bovine adrenal cortex, a preparation with only the ANF-R_{1A} subtype (46). In addition, the affinities of the peptides for ANF-R_2 were evaluated in NIH-3T3 membrane preparations (28). The results indicate that some synthetic peptide derivatives are more selective for the ANF-R_{1A} subtype than is the natural peptide pBNP32. These chimeric analogs can now be used to assess the physiological functions mediated through these receptor subtypes.

Experimental Procedures

Materials. The peptide rANF(99–126) was purchased from Institut Armand-Frappier (Laval, Canada), whereas pBNP32 and C-ANF were from Peninsula Laboratories, Inc. (Belmont, CA). Male Spague-Dawley rats (300–350 g) were obtained from Charles River Canada (St. Constant, Canada). Bovine kidneys were obtained from a local slaughterhouse. ^{125}I-rANF(99–126) was prepared in our laboratory by iodination of rANF(99–126) using the solid-phase Iodo-Beads method (46) and purification by Vydac C_{18} reverse phase HPLC. The specific activity of the monoiodinated peptide was about 2000 Ci/mmol. ^{125}I-cGMP was prepared by radioiodination of cGMP with Iodo-Beads (Pierce Chemical Co., Rockford, IL). Carrier-free Na^{125}I was from Amersham Corp. Cyclic methyl ester (2′-*O*-monosuccinyl-cGMP tyrosyl methyl ester),

bovine serum albumin, leupeptin, aprotinin, and pepstatin A were purchased from Sigma Chemical Co. (St. Louis, MO). Antiserum to cGMP was provided by Dr. A. Belanger (University of Laval, Canada). The embryonic fibroblast cell line NIH-3T3 was a gift from Dr. P. Jolicoeur (Institut de Recherches Cliniques de Montréal, Montreal, Canada). Cell culture media were obtained from GIBCO (Grand Island, NY).

Peptide synthesis. All analogs were synthesized by the solid-phase peptide synthesis methodology (48, 49) using a homemade manual multireactor synthesizer. Peptides were assembled on phenylacetamidomethyl resin, and BOP reagent was used as coupling agent. Deprotection of side chains and cleavage of peptides from the resin were achieved by treatment with liquid hydrofluoric acid in the presence of *m*-cresol. After precipitation with anhydrous diethyl ether, the crude peptides were extracted with pure trifluoroacetic acid, followed by evaporation and lyophilization. The formation of the disulfide bridge was carried out in a degassed 80% acetic acid solution containing iodine as oxidant. The crude preparations were purified by preparative HPLC. The purity of all peptides was estimated to be ≥98%. The structures of the purified peptides were confirmed by analytical HPLC, capillary electrophoresis, and amino acid analysis. A more detailed description of the synthesis of the chimeric analogs will be reported elsewhere.

Preparation of membranes. The membranes were prepared as described previously (27, 28, 46). Briefly, confluent NIH-3T3 cells were washed three times with 10 ml of ice-cold saline and were detached with a rubber policeman. The cells were centrifuged at 600 × *g* for 10 min and the supernatant was aspirated. The cell pellet was then resuspended in 10 volumes of buffer containing 1 mM $NaHCO_3$, 1 mM EDTA, 10^{-6} M aprotinin, 10^{-6} M leupeptin, and 10^{-7} M pepstatin A. The cells were allowed to swell for 10 min at 4° and were homogenized using a Kinemata Polytron (twice, setting 6, for 10 sec), followed by homogenization with a Potter homogenizer. The homogenate was diluted with 1 volume of 50 mM Tris·HCl buffer, pH 7.4, containing 0.1 mM EDTA, 1 mM $MgCl_2$, 10^{-7} M aprotinin, 10^{-6} M leupeptin, and 10^{-7} M pepstatin A, and was centrifuged at 250 × *g* for 10 min. The pellet was discarded and the supernatant was centrifuged at 30,000 × *g* for 15 min. Rat papillary and bovine adrenal cortex tissues were suspended in ice-cold homogenizing buffer containing 20 mM $NaHCO_3$, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 10^{-7} M aprotinin, 10^{-6} M leupeptin, and 10^{-7} M pepstatin A and were homogenized with a Polytron (three times, setting 6, for 10 sec) and a Potter homogenizer. The crude papilla homogenate was first centrifuged at 250 × *g* for 10 min at 4° and the supernatant and the bovine adrenal cortex homogenate were then centrifuged at 30,000 × *g* for 15 min at 4°. This step was repeated three times. Finally, all the pellets were washed twice using homogenizing buffer and were resuspended in 50 mM Tris·HCl buffer, pH 7.4, containing 250 mM sucrose, 0.1 mM EDTA, and 1 mM $MgCl_2$. The membrane preparations were frozen in liquid nitrogen and stored at −70° until used. Protein concentration in membrane preparations was determined using the BCA protein assay kit from Pierce.

Receptor binding assays. The competition binding experiments were performed as described previously (46). Membranes from rat papillae, bovine adrenal cortex, and NIH-3T3 cells, corresponding to 70, 25, and 70 μg of protein, respectively, were incubated for 90 min at 22° in the presence of various concentrations of the unlabeled peptides, in 1 ml of 50 mM Tris·HCl buffer, pH 7.4, containing 0.1 mM EDTA, 0.5% bovine serum albumin, 5 mM $MnCl_2$ (or $MgCl_2$ for NIH-3T3 membranes), and 8–10 pM ^{125}I-rANF(99–126). After incubation, bound ^{125}I-rANF(99–126) was separated from free ligand by rapid filtration through GF/C glass fiber filters that had been pretreated with a 1% polyethyleneimine solution. The tubes were rinsed with 10 ml of ice-cold 50 mM potassium phosphate buffer, pH 7.4, and the radioactivity retained on the filters was measured with a γ counter.

cGMP production assays. Guanylate cyclase activity was determined as described previously (46). Membranes from rat papillae and bovine adrenal cortex, corresponding to 40 and 10 μg of protein, respectively, were incubated for 10 min at 37° in the presence of various concentrations of peptides, in 100 μl of reaction medium containing 50 mM Tris·HCl buffer, pH 7.6, 10 mM theophylline, 2 mM 3-isobutyl-1-methylxanthine, 10 mM creatine phosphate, 10 units of creatine phosphokinase, 4 mM $MnCl_2$, and 1 mM GTP. Incubation was stopped by the addition of 100 μl of 120 mM EDTA and immersion of tubes in boiling water for 3 min. After addition of 1 ml of 50 mM ammonium acetate buffer, pH 7.6, the tubes were centrifuged and cGMP present in the supernatant was isolated by chromatography on alumina columns. cGMP content was determined by radioimmunoassays.

Data analysis. The competition binding curves and concentration-response curves were analyzed with the ALLFIT program, based on a four-parameter logistic equation, to obtain estimates of the IC_{50} or EC_{50} and the slope factor (50). Moreover, the nonlinear least-squares curve-fitting program SCAFIT was used to analyze competition curves according to a model for the binding of competing ligands to one or several independent classes of binding sites (51). A model involving two classes of sites was retained only when the fit to the data was statistically better than that for a single class of sites, as evaluated by a partial *F* test. ED_{50} and K_d values are reported as pD_2 and pK_i, respectively, and expressed as mean ± standard error of several determinations. Comparisons were made using the one-way analysis of variance followed by Student's *t* test for paired or unpaired values. Differences were accepted to be significant at $p < 0.05$.

Results

Potencies of the peptides in competitive binding assays using ^{125}I-rANF(99–126) for labeling of the ANF-R_1 and ANF-R_2 receptors present in different membrane preparations. To evaluate the affinity of the different chimeric analogs for ANF receptor subtypes, their binding properties were determined and compared with those of rANF(99–126) and pBNP32. As shown in Fig. 2A, all peptides were able to compete for ^{125}I-rANF(99–126) binding in bovine adrenal cortex. In this preparation, the competition binding curves for the various peptides tested displayed slope factors close to unity (Table 1) and were all best fitted using a model involving a single class of receptor sites. This is consistent with previous studies that indicated the presence of only one subtype, ANF-R_{1A}, in bovine adrenal cortex (27). As shown by the pK_i values in Table 1, rANF(99–126) and pBNP32 were equipotent in inhibiting the binding of ^{125}I-rANF(99–126) to ANF-R_{1A} receptor in bovine adrenal cortex. The chimeric analog pBNP1, obtained by combining the core of pBNP32 with both the amino- and carboxyl-terminal segments of rANF(99–126), ex-

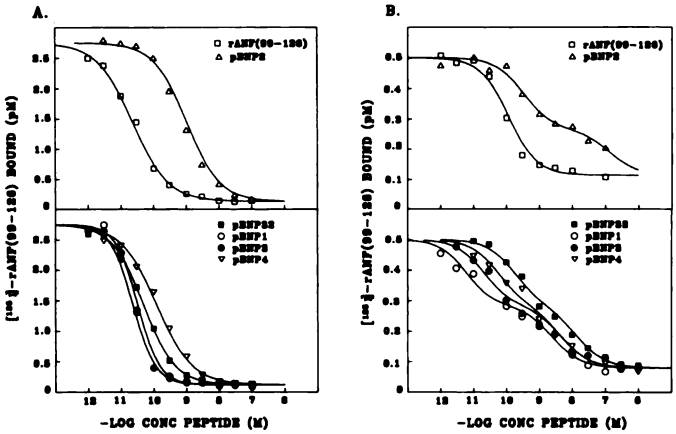

Fig. 2. Competition binding curves of rANF(99–126), pBNP32, and chimeric analogs for the binding of ^{125}I-rANF(99–126) to bovine adrenal cortex (A) and rat papillary (B) membrane preparations.

TABLE 1

Receptor affinities of rANF(99-126), pBNP32, and their related analogs for ANF receptor types in different membrane preparations

Correlation between the pK_i values of various peptides at ANF-R_{1A} receptor found in bovine and rat membranes was 0.83.

Peptide	Bovine adrenal cortex (ANF-R_{1A})		Rat papillae (ANF-R_{1A} and ANF-R_{1B})			NIH-3T3 (ANF-R_2)	
	pK_i[a]	Slope factor[b]	pK_i	Slope factor	Selectivity ratio[c]	pK_i	Slope factor
rANF(99-126)	10.6 ± 0.09	0.9	10.2 ± 0.1	0.9		10.9 ± 0.2	0.9
pBNP32	10.4 ± 0.04	0.9	9.7 ± 0.1[d]			10.6 ± 0.05[d]	0.9
				0.5	100		
			7.7 ± 0.1[d]				
pBNP1	11.1 ± 0.01[d]	1.0	11.3 ± 0.05[d]			10.9 ± 0.1	1.0
				0.4	632		
			8.5 ± 0.1[d]				
pBNP2	9.4 ± 0.05[d]	0.9	9.3 ± 0.1[d]			10.1 ± 0.1[d]	0.9
				0.6	63		
			7.5 ± 0.1[d]				
pBNP3	11.3 ± 0.07[d]	1.2	11.0 ± 0.1[d]			10.8 ± 0.2	1.0
				0.4	560		
			8.3 ± 0.1[d]				
pBNP4	9.9 ± 0.03[d]	0.9	10.4 ± 0.07			10.1 ± 0.09[d]	1.0
				0.4	158		
			8.2 ± 0.2[d]				

[a] pK_i, −log K_d; mean ± standard error of two to six receptor binding assays with ^{125}I-rANF(99-126).
[b] Slope factor of competition binding curves with ^{125}I-rANF(99-126).
[c] Selectivity ratio of corresponding peptides for the ANF-R_{1A} and ANF-R_{1B} receptor subtypes.
[d] $p < 0.05$, data significantly different from rANF(99-126).

hibited higher affinity for ANF-R_{1A} receptor than did the parent compounds. Moreover, replacement of only the amino-terminal portion of pBNP32 with that of rANF(99–126) also resulted in an analog, pBNP3, that showed a higher potency than that of rANF(99–126) for ANF-R_{1A} receptor. However, as shown with analog pBNP4, substitution of only the carboxyl-terminal portion of pBNP32 with that of rANF(99–126) decreased the affinity for ANF-R_{1A} receptor. In addition, the chimeric analog pBNP2, which consists of the core of rANF(99–126) and both the amino- and carboxyl-terminal segments of pBNP32, showed even lower affinity for ANF-R_{1A} receptor. In rat papillary membrane preparations, competition binding curves for pBNP32 and various chimeric analogs all showed biphasic patterns and slope factors smaller than unity, suggesting a heterogeneity of ANF-R_1 receptor (Fig. 2B; Table 1). Furthermore, the binding data for these peptides were significantly better fitted to a model for two classes of receptor sites. These observations support the previous report from our laboratories that indicated the presence in rat papillary membranes of two receptor subtypes, referred to as ANF-R_{1A} and ANF-R_{1B} (46). rANF(99–126) displayed similar affinities for these two receptors, with a pK_i value of 10.2 ± 0.1 (Table 1). Nevertheless, pBNP32 possessed high affinity (pK_i of 9.7 ± 0.1) for the ANF-R_{1A} receptor subtype, which represents approximately 35% of the binding sites, whereas it showed only weak affinity for the ANF-R_{1B} subtype (pK_i of 7.7 ± 0.1). The chimeric analogs pBNP1, pBNP3, and pBNP4 showed higher affinities than did rANF(99–126) and pBNP32 for the ANF-R_{1A} receptor subtype in rat papillae, whereas pBNP2 possessed much lower affinity. In contrast, the pK_i values of various chimeric analogs for the ANF-R_{1B} subtype were similar to that of pBNP32 (Table 1). The rank order of selectivity ratios between the ANF-R_{1A} and ANF-R_{1B} receptor subtypes for the peptides was as follows: pBNP1 > pBNP3 ≫ pBNP4 > pBNP32 > pBNP2.

In addition, characterization of the ring-contracted analog C-ANF in bovine adrenal cortex and rat papillae revealed that this peptide does not exhibit significant inhibition of the binding of ^{125}I-rANF(99–126) at a concentration as high as 1 μM, which is indicative of the absence of the ANF-R_2 receptor type in these preparations (data not shown). This is in agreement with previous reports indicating that these two tissues contain only ANF-R_1 receptor (27, 46, 52). On the other hand, the binding affinities of pBNP1 and pBNP3 were comparable to those of rANF(99–126) and pBNP32 in NIH-3T3 membrane preparations, which contain the ANF-R_2 receptor, whereas pBNP2 and pBNP4 exhibited weaker affinities (Table 1). Therefore, pBNP1 and pBNP3 are more selective analogs for ANF-R_1 receptor than are rANF(99–126) and pBNP32.

Effects of the peptides on ANF-R_1-mediated cGMP production in bovine adrenal cortex and rat papillary membrane preparations. To estimate the agonistic properties of the aforementioned chimeric analogs, we determined their ability to stimulate particulate guanylate cyclase activity related to the ANF-R_1 receptor subtypes present in different tissues and compared their potencies with those of rANF(99–126) and pBNP32. The concentration-response curves for the stimulation of cGMP production by the various peptides are shown in Fig. 3. In the bovine adrenal cortex, the basal level of cGMP production was 20 pmol/mg/min, and all the peptides induced a concentration-dependent stimulation of guanylate cyclase activity with a maximal cGMP accumulation of 120 pmol/mg/min (Fig. 3A). In this preparation, rANF(99–126) and pBNP32 were almost equipotent in stimulating cGMP production, with pD_2 values of 9.8 ± 0.02 and 9.4 ± 0.01, respectively (Table 2). The analogs pBNP1 and pBNP3 exhibited cGMP production potencies higher than those of rANF(99–126) and pBNP32, whereas pBNP2 and pBNP4 had less stimulatory effect. In the rat papillary membrane preparation, the basal level of cGMP production was 2 pmol/mg/min and the maximal cGMP accumulation induced by rANF(99–126) was 16 pmol/mg/min (Fig. 3B). pBNP32 and pBNP2 dose-dependently increased cGMP production, with pD_2 values of 8.0 ± 0.1

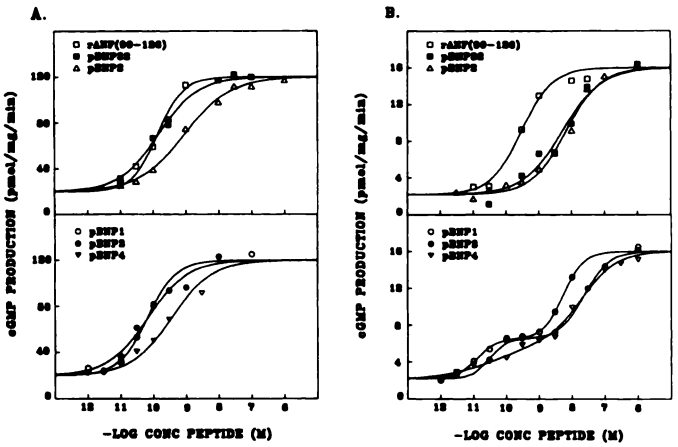

Fig. 3. Concentration-response curves for the stimulation of cGMP production induced by rANF(99–126), pBNP32, and chimeric analogs in bovine adrenal cortex (A) and rat papillary (B) membrane preparations.

TABLE 2

cGMP production potencies of rANF(99-126), pBNP32, and their related analogs for ANF-R_1 receptor subtypes in different membrane preparations

Correlation between the pD_2 values of various peptides at ANF-R_{1A} receptors found in bovine and rat membranes was 0.80.

Peptide	Bovine adrenal cortex (ANF-R_{1A})		Rat papillae (ANF-R_{1A} and ANF-R_{1B})		
	pD_2[a]	Slope factor[b]	pD_2	Slope factor	Selectivity ratio[c]
rANF(99-126)	9.8 ± 0.02	0.9	9.6 ± 0.06	0.9	
pBNP32	9.4 ± 0.01[d]	0.9	8.0 ± 0.1[d]	0.7	
pBNP1	10.1 ± 0.1[d]	1.0	10.9 ± 0.06[d]		
				0.4	504
			8.2 ± 0.02[d]		
pBNP2	8.6 ± 0.1[d]	0.9	7.8 ± 0.2[d]	0.8	
pBNP3	10.3 ± 0.1[d]	1.0	10.6 ± 0.08[d]		
				0.4	400
			8.0 ± 0.06[d]		
pBNP4	9.2 ± 0.01[d]	0.9	10.0 ± 0.1[d]		
				0.5	125
			7.9 ± 0.2[d]		

[a] pD_2, −log ED_{50}; mean ± standard error of two to four experiments on stimulation of particulate guanylate cyclase activity.
[b] Slope factor of concentration-response curves for the stimulation of cGMP production.
[c] Selectivity ratio of corresponding peptides for the ANF-R_{1A} and ANF-R_{1B} receptor subtypes.
[d] $p < 0.05$, data significantly different from rANF(99-126).

and 7.8 ± 0.2, respectively; however, in this assay they were approximately 2 orders of magnitude less potent than rANF(99–126) (pD_2 = 9.6 ± 0.06) (Table 2; Fig. 3B). The analogs pBNP1, pBNP3, and pBNP4 were full agonists in stimulating cGMP production, with a maximal cGMP accumulation of 16 pmol/mg/min. However, the concentration-response curves for these analogs showed biphasic profiles and slope factors smaller than unity, thus revealing that they can discriminate by their differential cGMP production potencies between the ANF-R_{1A} and ANF-R_{1B} receptor subtypes. Interestingly, pBNP1, pBNP3, and pBNP4 were more potent than rANF(99–126) in stimulating cGMP production via ANF-R_{1A} receptor, which mediated about 30% of the maximal response, whereas ANF-R_{1B} receptors were much less sensitive to these analogs. pBNP1 and pBNP3 were more selective analogs, with ratios of selectivity between the two subtypes of 504- and 400-fold, respectively. pBNP4 showed only a 125-fold ratio (Table 2).

Comparative analysis of binding affinities and cGMP production potencies of the various analogs for ANF-R_{1A} receptor. To determine the relationship between the structural requirements of the ANF-R_{1A} receptor for receptor binding and cGMP production, a linear correlation analysis was done in rat and cow. An excellent correlation was obtained between the binding affinities (pK_i) and cGMP production potencies (pD_2) for the various peptides at ANF-R_{1A} receptor in bovine adrenal cortex ($r = 0.99$, $p < 0.01$) and rat papillary ($r = 0.97$, $p < 0.01$) membrane preparations (Fig. 4). On the other hand, the relationship between the binding affinities of the peptides for ANF-R_{1A} receptor in bovine adrenal cortex and rat papillae was rather good, with a regression coefficient of 0.83. Furthermore, the agonistic potencies of the peptides in stimulating cGMP production were comparable between these two tissues, with a regression coefficient value of 0.80 (data not shown). However, all the peptides showed slightly lower affinities and cGMP production potencies for the ANF-R_{1A} receptor in bovine adrenal cortex, compared with those in rat papillae. Moreover, in bovine adrenal cortex the potencies of various peptides were, in decreasing order, as follows: pBNP3 > pBNP1 > rANF(99–126) > pBNP32 > pBNP4 > pBNP2; in rat papillae, pBNP1 and pBNP4 were more potent than pBNP3 and pBNP32, respectively, at the ANF-R_{1A} receptor subtype (Tables 1 and 2).

Discussion

On the basis of the differential affinities and agonistic potencies of pBNP32 and various chimeric analogs for the two binding sites found in rat papillae, the results of the present study indicated a heterogeneity of the ANF-R_1 receptor type. These observations confirm and extend the previous report of Féthière and De Léan (46), which suggested the existence in rat papillae and human glomeruli of two receptor subtypes, termed ANF-R_{1A} and ANF-R_{1B}. rANF(99–126) possessed high affinity for both receptor subtypes, whereas pBNP32 showed high affinity for ANF-R_{1A} and low affinity for ANF-R_{1B}.

Binding assays carried out in rat papillae showed that the substitution of the amino- and carboxyl-terminal segments of rANF(99–126) with those of pBNP32 (pBNP2) decreased substantially the affinity for the ANF-R_{1B} subtype, compared with native rANF(99–126), whereas the ANF-R_{1A} subtype was less sensitive to this structural modification. In addition, pBNP2 displayed a much lower cGMP production potency than did rANF(99–126) in this preparation, suggesting that the presence of amino- and carboxyl-terminal segments of pBNP32 also decreased the stimulation of guanylate cyclase activity. Furthermore, as shown with the chimeric analog pBNP1, the combination of the cyclic portion of pBNP32 with the amino- and carboxyl-terminal segments of rANF(99–126) resulted in a significant increase of the potency at ANF-R_{1A} receptor. In particular, substitution of the amino-terminal segment of pBNP32 with that of rANF(99–126) seemed to contribute largely to the increase in affinity and agonistic potency at ANF-R_{1A} receptor, whereas the replacement of the carboxyl-terminal segment induced only a slight increase in potency, as shown by the analogs pBNP3 and pBNP4, respectively. In contrast, the presence of amino- and carboxyl-terminal portions of rANF(99–126) with the core of pBNP32 did not increase the

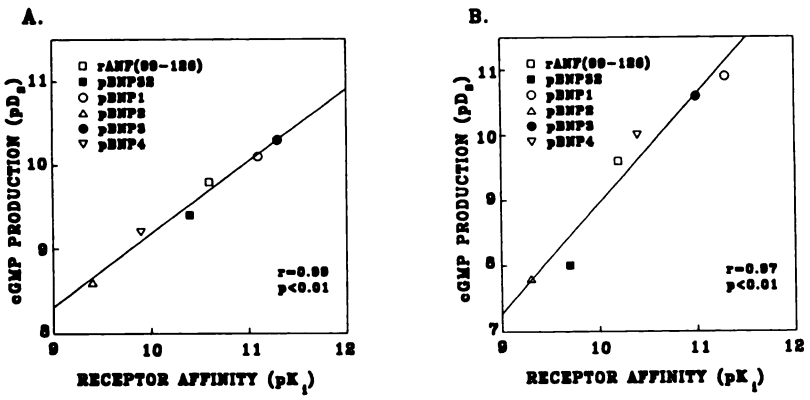

Fig. 4. Correlation analysis of the receptor affinities and cGMP production potencies of various peptides for the ANF-R_{1A} receptor subtype in bovine adrenal cortex (A) and rat papillary (B) membrane preparations.

potency at ANF-R_{1B} receptor. Together, these results suggest that the presence of the ring structure or the amino- and carboxyl-terminal segments of pBNP32 within the peptides contributes to the relative decrease of affinity and agonistic activity at ANF-R_{1B} receptor and therefore seems responsible for the discrimination between the ANF-R_{1A} and ANF-R_{1B} subtypes. Interestingly, a recent study with a reversed-ring analog of rANF(99–126) indicated that the binding and activation of ANF-R_1 receptor required the maintenance of an adequate spatial orientation of the residues found in the disulfide-linked ring with respect to those in the amino- and carboxyl-terminal segments (53). Moreover, NMR spectroscopy studies of pBNP26 and rANF(99–126) indicated several conformational differences between those two peptides, including the differential folding and positioning of their amino- and carboxyl-terminal tails towards the ring structure (54–56). Therefore, the weak potency of pBNP32 and various chimeric analogs, compared with rANF(99–126), at ANF-R_{1B} receptor might be due to the different conformational structures adopted by these peptides.

In addition, the results indicated that the structural requirements of the ANF-R_{1A} receptor subtype present in cow significantly diverged from those in rat. Notably, rANF(99–126) displayed affinity and cGMP production potency higher than those of pBNP32 at the ANF-R_{1A} receptor in rat papillary tissues, whereas the ANF-R_{1A} receptor in bovine adrenal cortex responded similarly to both peptides. Moreover, the replacement of the carboxyl-terminal segment of pBNP32 with that of rANF(99–126) resulted in a slight increase of the potency at ANF-R_{1A} receptor in rat papillae, whereas the opposite effect was observed in bovine adrenal cortex. These differences could be due to species differences in the ANF-R_{1A} receptor, as suggested previously (46). A possible explanation is that the various peptides could be differently degraded in these two tissues. On the other hand, the membrane environment of the ANF-R_1 receptor might possibly contribute to the differences observed between the potencies of the peptides in rat papillae and bovine adrenal cortex. Indeed, the phospholipids of cellular membranes have been reported to play an important role in the interaction of ANF with its receptors, by favoring the formation of the biologically active conformation of peptide (57–58). Furthermore, a recent NMR spectroscopy study with pBNP26 suggested that Arg-25, located in the carboxyl-terminal segment, might be involved in a hydrogen bond with the Ser-14 residue found in the ring structure. This bond might play an important role in determining the three-dimensional structure of pBNP32 (55). Therefore, the substitution of the carboxyl-terminal segment of pBNP32 with that of rANF(99–126) could disrupt the Ser-14/Arg-25 bond and thus affect the formation of the active conformation of pBNP32 in bovine adrenal cortex but not in rat papillae.

An excellent correlation was obtained between the receptor binding affinities and cGMP production potencies of the various peptides at ANF-R_{1A} receptor in rat papillae and bovine adrenal cortex. This suggests that the structural requirements of peptides for receptor occupancy can be comparable to those for guanylate cyclase activation. However, in these tissues the potencies of various peptides for inhibiting the binding of ^{125}I-rANF(99–126) to ANF-R_{1A} receptors were higher than those for stimulating cGMP accumulation. This can be attributed to the different experimental conditions used in these two assays. However, the recognition and guanylate cyclase activation of ANF-R_1 receptor by the peptides could also be differently regulated (47). Indeed, several modulators including ATP and the diuretic amiloride have been reported to alter the binding and activation of particulate guanylate cyclase associated with the ANF-R_{1A} receptor (27, 29, 59), by interacting at allosteric binding sites that could be present on the ANF-R_{1A} receptor. Thus, additional studies are necessary to determine the factors that could be involved in the regulation of ANF-R_{1A} receptor occupancy and the intrinsic activity of various natriuretic peptides.

In addition, our results showed that the chimeric analogs pBNP1 and pBNP3 displayed higher affinities than did rANF(99–126) and pBNP32 for the ANF-R_{1A} receptor subtype but possessed affinities comparable to that of rANF(99–126) for the ANF-R_2 receptor. The ANF-R_2 receptor has been reported to play an important role in the clearance of the natriuretic peptides and seems to contribute substantially to decreasing the plasma ANF concentration (38, 60). Therefore, the high affinities of the analogs pBNP1 and pBNP3 for the ANF-R_{1A} subtype, compared with the ANF-R_2 subtype, suggest that these peptides could have a smaller volume of distribution and could be more effective *in vivo* than rANF(99–126).

Several studies have indicated that most physiological functions of rANF(99–126) and pBNP32 are mediated by the interaction of the peptides with ANF-R_1 receptor and subsequent activation of particulate guanylate cyclase associated with these receptors (17, 37). Nevertheless, the specific role of each receptor subtype and the transduction mechanisms involved in the triggering of the different biological actions of natriuretic peptides are not yet known (2, 4, 60). Our results revealed that

both the ANF-R_{1A} and ANF-R_{1B} receptor subtypes found in rat papillae could be functionally coupled to particulate guanylate cyclase. However, their physiological roles remain unclear. In kidney, ANF has been reported to mediate natriuresis and diuresis after a variety of actions on the vasculature and epithelial cells along the nephron (1, 2, 45, 61–65). In particular, ANF has been suggested to inhibit sodium and water reabsorbtion by interacting with the ANF-R_1 receptor subtype present on the interstitial and medullary collecting duct cells. Interestingly, it has been observed that [Met-O^{110}]-hANF(99–126) has no natriuretic effect at low doses in rat and induces only a diuretic response (66). However, this oxidized form of hANF(99–126) mediated, at high doses, a natriuresis comparable to that of rANF(99–126). Moreover, a similar concentration-dependent natriuretic effect has also been reported for iso-rANF(1–45) (67). Because rat papillae have been proposed to contain only the ANF-R_1 type, this suggests that [Met-O^{110}]-hANF(99–126) and iso-rANF(1–45) could mediate their diuretic effects through ANF-R_{1A} receptor at low doses and could mediate natriuresis through ANF-R_{1B} receptor at high doses. Thus, these results suggest that the ANF-R_{1A} and ANF-R_{1B} receptor subtypes could be activated differently in their physiological functions by ANF, compared with pBNP32 and the chimeric analogs. Nevertheless, the possibility that these two receptor subtypes are different at the level of their amino acid sequences needs to assessed. A second possibility is that the ANF-R_1 receptors present in rat papillae could adopt two distinct conformational states. Both states would display a high sensitivity to rANF(99–126), whereas pBNP32 and various analogs would preferentially recognize one conformation. Thus, further investigations are necessary to discriminate between these two alternative hypotheses.

In conclusion, this study allowed the identification of chimeric analogs that are more selective than pBNP32 for ANF-R_{1A} receptor in rat papillae. Furthermore, these analogs possess affinities for ANF-R_{1A} receptors that are superior to those for the ANF-R_2 receptor type. pBNP1 and pBNP3 might be good pharmacological tools to assess the physiological functions mediated by the ANF-R_{1A} and ANF-R_{1B} receptor subtypes.

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