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Distinct Properties of Atrial Natriuretic Factor Receptor Subpopulations in Epithelial and Fibroblast Cell Lines

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SUMMARY

We have characterized two atrial natriuretic factor (ANF) receptor subtypes, designated ANF-R_1 and ANF-R_2, in two established cell lines that express exclusively one receptor subtype. The ANF-R_1 receptor is selectively expressed by the kidney epithelial cell line LLC-PK_1. It is a 130-kDa protein that has a much higher affinity for the biologically active forms of ANF than for its metabolites. The binding of ANF to this subtype is potentiated by amiloride and by divalent cations. The activation of the ANF-R_1 receptor leads to an accumulation of cyclic GMP that is only partially inhibited by methylene blue. The ANF-R_2 receptor, which is expressed selectively by the fibroblast cell line NIH-3T3, is a 130-kDa protein composed of two disulfide-linked subunits of 64-kDa. Activation of this subtype by saturating concentrations of ANF does not appear to elicit cyclic GMP production. However, supraphysiological concentrations of ANF induce a nonsaturable accumulation of cyclic GMP with an apparent ED_{50} in the high micromolar range. In contrast to the ANF-R_1 subtype, the stimulation of cyclic GMP production is completely abolished by methylene blue. This subtype recognizes the active forms of ANF as well as its metabolites, and the binding is insensitive to amiloride and is decreased by divalent cations. These two cell lines can serve as models for studying the differential regulatory properties of ANF-R_1 and ANF-R_2 subtypes. In addition, we have also characterized the two ANF receptor subtypes in rat kidney glomeruli, where they show the same structure and pharmacological characteristics as in the two model cell lines.

ANF is a peptide hormone actively involved in the regulation of fluid and electrolyte balance, the renin-angiotensin system, smooth muscle tone, and blood pressure homeostasis (for review, see Refs. 1–5). ANF exerts its effects by activating specific high affinity receptor sites that were first identified in rabbit aorta (6), bovine adrenal zona glomerulosa (7), vascular smooth muscle and endothelial cells (8, 9), kidney glomeruli and collecting tubules (6, 10), and specific brain areas (11). The association between the effects of ANF and the production of cyclic GMP has been well established (12, 13). The hormone directly activates a membrane-associated form of guanylate cyclase to increase the intracellular levels of cyclic GMP (13). This form of the enzyme is distinct from soluble guanylate cyclase, which is known to respond to the vasodilator sodium nitroprusside (13). The role of cyclic GMP as a second messenger of the vasorelaxant effect of ANF has been well documented (14). However, the cyclic nucleotide does not appear to mediate ANF actions in other tissues, such as adrenal cortex (15, 16).

Structure-activity relationship studies have clearly identified the C-terminal fragment ANF-(99–126) as the biologically active form of the hormone and have demonstrated the crucial role of an intact disulfide bridge, Cys^{105}–Cys^{121}, as well as of the amino acids Phe^{124} and Arg^{125} (17–19). This initially led to the notion of a homogeneous class of pharmacological receptors mediating all the effects of ANF. But evidence for more than one class of ANF receptors has recently accumulated. Comparative studies showed that the major circulating form, ANF-(99–126), was much more potent than the metabolite ANF-(103–123) in bovine adrenal cortex receptor binding studies (19), in relaxing rabbit aortic strips (17, 18), in producing natriuresis in bioassay rats (17), in decreasing aldosterone production (19), and in stimulating cyclic GMP formation in cultured endothelial and smooth muscle cells (20, 21). In contrast, the weakly active metabolite ANF-(103–123) was almost as potent as ANF-(99–126) in receptor binding studies in cultured aortic endothelial cells (20), smooth muscle cells (21), and A10 cell line (22) and in relaxing chick rectum (17). In addition, biochemical characterization of the ANF receptor by affinity cross-linking or photoaffinity labeling revealed the presence of a 130-kDa binding protein in adrenal cortex and kidney cortex (23–25), a 60–70-kDa protein in A10 smooth muscle cell line (22) and cultured smooth muscle cells (26), and both types of binding proteins in aorta membranes (27) and cultured endothelial cells

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ABBREVIATIONS: ANF, atrial natriuretic factor; C-ANF, des[Gln^{18}-Ser^{19}-Gly^{20}-Leu^{21}-Gly^{22}]-ANF-(102–121)-NH_2; SDS, sodium dodecyl sulfate.

(28). This led to the original proposal by Leitman *et al.* (28) that two distinct ANF receptor subtypes exist. The ANF-R_1 receptor, which is coupled to particulate guanylate cyclase, is composed of a single 130-kDa polypeptide and selectively recognizes with high affinity only the biologically active forms of ANF-(99–126). The ANF-R_2 receptor, which is not coupled to guanylate cyclase, is composed of two disulfide-linked 60-kDa subunits and has high affinity for weakly active truncated forms of ANF. It has been proposed by Maack *et al.* (29) that the ANF-R_2 subtype might be associated with the cellular uptake and the metabolic clearance of the hormone, rather than with transmembrane signalling. This hypothesis would be consistent with the lack of selectivity of this receptor subtype for metabolites of ANF and with its primary structure, which displays no significant cytoplasmic domain (30). Thus, ANF-R_2 receptor might be analogous to low density lipoprotein and transferrin receptors, which act as cell docking and translocation proteins (31, 32).

Many target tissues, such as kidney and aorta, seem to contain both ANF receptor subtypes in variable proportions. This receptor dualism complicates the interpretation of experimental results on the cellular function of each receptor subtype. In order to investigate in more detail the molecular characteristics of each ANF receptor subtype, we have identified two cell lines, the epithelial cell line LLC-PK_1 and the fibroblast cell line NIH-3T3, which exclusively express the ANF-R_1 or ANF-R_2 receptor, respectively. The pharmacological profile, receptor properties, effect on cyclic GMP formation, and subunit structure of the receptors were studied in each cell line in order to fully characterize each receptor subtype. The results obtained indicate that LLC-PK_1 and NIH-3T3 cells can be used as model systems for each ANF receptor subtype. Furthermore, our results provide a rational basis for delineating the proportion of each subtype in target tissues (e.g., kidney glomeruli) and contribute to an explanation of the unexpected stimulation of cyclic GMP by ANF in tissue that do not appear to be physiological targets of ANF.

Experimental Procedures

Materials. The porcine kidney epithelial cell line LLC-PK_1 was purchased from American Type Culture Collection (ATCC CL 101) and the embryonic fibroblast cell line NIH-3T3 was a gift from Dr. P. Jolicoeur (IRCM, Canada). Cell culture media were obtained from GIBCO (Grand Island, NY). Carrier-free $Na^{125}I$ and radioiodinated cyclic GMP were purchased from Amersham Corp. (Oakville, Canada). The anti-cyclic GMP antibody was generously provided by Dr. A. Bélanger (CHUL, Canada). Rat ANF-(99–126), rat ANF-(103–126), and rat ANF-(103–123) were obtained from Institut Armand-Frappier (Laval, Canada) and C-ANF peptide was a gift from Dr. Julian Adams (Biomega, Canada). ^{125}I-ANF-(99–126) was prepared by radioiodination of ANF-(99–126) using the solid phase IODO-BEADS method (33). The specific activity of the monoiodinated peptide was typically 2000 Ci/mmol. Bis(sulfosuccinimidyl)suberate and IODO-BEADS were obtained from Pierce Chemical Corp. (Rockford, IL). Reagents for electrophoresis and molecular weight standards were purchased from Bio-Rad (Mississauga, Canada). Bovine serum albumin, leupeptin, aprotinin, pepstatin A, amiloride, and lysozyme chloride were from Sigma Chemical Co. (St. Louis, MO).

Cell culture. LLC-PK_1 and NIH-3T3 cells were grown as monolayers in Dulbecco's modified Eagle medium that was supplemented with 5% fetal bovine serum and 100 units of penicillin-streptomycin in a 5% CO_2 water-saturated atmosphere. Confluent cells were trypsinized and subcultured every 3–4 days. For membrane preparation, the trypsinized cells were seeded onto 75-cm^2 flasks, whereas for cyclic GMP stimulation experiments the cells were seeded onto 24-well cluster plates. Experiments were performed when the cells reached confluence.

Cyclic GMP determination. The confluent cell monolayers were washed twice with serum-free HAM F12 medium and were incubated in a final volume of 1 ml of the same medium that contained 0.02% lysozyme chloride, 0.5 mM 3-isobutyl-1-methylxanthine, and varying concentrations of ANF analogs, in the absence or presence of 10^{-4} M methylene blue. After incubation, the medium was removed and the extracellular levels of cyclic GMP were determined by direct radioimmunoassay, using the second antibody precipitation technique (34) with some modifications. The assay was performed in acetate buffer, pH 6.2. First, 100 μl of cyclic nucleotide standards or 50 μl of samples diluted to 100 μl with buffer were acetylated with 5 μl of triethylamine/acetic anhydride (2:1), as described (35). Then, 100 μl of ^{125}I-labeled 2′-*O*-monosuccinyl cyclic-GMP tyrosyl methyl ester (10,000 cpm), diluted in acetate buffer containing 1% normal rabbit serum, and 100 μl of anti-cyclic GMP antibody, diluted in buffer containing goat anti-rabbit immunoglobulin, were added. The amount of antibody used bound 30% of the total radioligand. The assay mixture was incubated 16–18 hr at 4° in the dark. The free ligand was separated from bound ligand by addition of 1 ml of cold buffer and centrifugation for 20 min at 1800 × *g*. The supernatant was aspirated and the pellet was counted in a γ counter. Each sample was assayed in duplicate and the data were expressed as picomol of cyclic GMP/well.

Preparation of membranes. Confluent LLC-PK_1 and NIH-3T3 cells were washed three times with 10 ml of ice-cold saline and were detached with a rubber policeman. The cells were then centrifuged 10 min at 600 × *g*. The supernatant was aspirated and the cell pellet was resuspended in 10 volumes of buffer A (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 then homogenized two times for 10 sec, setting 6) with a Polytron homogenizer, followed by homogenization with a Potter homogenizer. The homogenate was diluted with 1 volume of buffer B (50 mM Tris·HCl, 0.1 mM EDTA, 1 mM $MgCl_2$, 10^{-6} M aprotinin, 10^{-6} M leupeptin, and 10^{-7} M pepstatin A, pH 7.4) and centrifuged 10 min at 250 × *g*. The pellet was discarded and the supernatant was centrifuged at 30,000 × *g* for 15 min. The pellet obtained was washed twice in buffer B and resuspended by gentle homogenization in buffer C (50 mM Tris·HCl, 250 mM sucrose, 0.1 mM EDTA, and 1 mM $MgCl_2$, pH 7.4). The membranes were frozen in liquid nitrogen and stored at −70° until used. Protein concentration was measured using the Bradford protein assay kit (Bio-Rad).

Isolation of glomeruli. Rat glomeruli were isolated according to the graded sieving method of Misra (36) with some modifications. After decapitation of adult Sprague-Dawley rats, the kidneys were removed and kept in ice-cold buffer D (0.9% NaCl, 10^{-7} M aprotinin, 10^{-6} M leupeptin, and 10^{-7} M pepstatin A) until dissected. Each kidney was cut longitudinally and the medulla was removed. All subsequent steps were performed in siliconized glassware. The cortical tissue was minced with scissors and then pressed through a 500-μm stainless steel sieve with the help of a pestle. The fragments passing through were collected from the undersurface of the sieve and dispersed in buffer D. The first pass-through fraction was gently filtered through a 190-μm sieve and washed extensively. The filtrate was then centrifuged three times at 250 × *g* for 3 min and the resulting pellet, containing the glomeruli, was resuspended in 100 ml of buffer D and passed through a 140-μm sieve. The sieve was washed several times and the resulting filtrate was passed through a 46-μm sieve. Glomeruli remaining on the sieve were recovered and washed extensively on a 94-μm sieve. These two last steps were repeated until the glomeruli preparation was 95% pure. At this point, glomeruli visualized under light microscopy were devoid of tubules and vessels. The crude glomeruli membrane preparation was prepared by homogenization as described in the precedent section.

Receptor binding assay. Binding studies with intact membranes were carried out as previously described (7). Briefly, membranes from LLC-PK_1 cells (40 μg/ml), NIH-3T3 cells (70 μg/ml), LLC-PK_1 plus

NIH 3T3 cells (40 μg/ml), and rat kidney glomeruli (30 μg/ml) were incubated with 8 pM ^{125}I-ANF-(99–126) and varying concentrations of unlabeled competing peptides. The binding assay was carried out at 25° for 90 min in 1 ml of buffer E (50 mM Tris·HCl, 0.1 mM EDTA, 5 mM $MnCl_2$, and 0.5% heat-inactivated bovine serum albumin, pH 7.4), in the presence or absence of 10^{-4} M amiloride. Membrane-bound ^{125}I-ANF-(99–126) 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). ^{125}I-ANF-(99–126) retained on the filters was counted in an LKB 1272 CliniGamma counter, with 80% efficiency.

Affinity labeling protocol. Affinity cross-linking of the ANF receptor was performed as described by Meloche *et al.* (25). Membranes were incubated for 16 hr at 10° with 10 pM ^{125}I-ANF-(99–126) in buffer E, in the presence or absence of indicated agents. The final concentration of plasma membranes was 100 μg/ml for LLC-PK_1, 50 μg/ml for NIH-3T3, and 40 μg/ml for rat kidney glomeruli. After incubation, the membranes were washed and incubated with 0.1 mM bis(sulfosuccinimidyl)suberate for 30 min at 4°. Then, the reaction was quenched and the membranes were centrifuged. The washed pellet was resuspended in sample buffer (62 mM Tris·HCl, pH 6.8, 2% SDS, 10% glycerol, 0.001% bromphenol blue), containing 5% 2-mercaptoethanol, and was heated at 100° for 3 min.

SDS-polyacrylamide gel electrophoresis. Electrophoresis was carried out on slab gels using the discontinuous buffer system of Laemmli (37) with a 7.5% acrylamide separating gel. Amounts of 100–200 μg of solubilized membrane proteins were loaded on each lane. After electrophoresis, the gels were fixed, stained with Coomassie blue R-250, destained, and dried before autoradiography on Kodak X-Omat RP film, exposed at −70° with enhancing screens (DuPont, Wilmington, DE).

Data analysis. Dose-response curves for cyclic GMP production were analyzed with the ALLFIT program, based on a four-parameter logistic equation (38), in order to obtain estimates of the ED_{50} of the ANF analogs. Competition binding curves were first analyzed with the same program to obtain the ED_{50} and the corresponding slope factor of the curves. Then, a nonlinear least-squares curve-fitting program was used to analyze each competition curve according to a model for the binding of competing ligands to one or several independent classes of binding sites (39). A model involving two classes of sites was retained only when the goodness of the fit was statistically better than that for a single class of sites, as evaluated by a partial F test. ED_{50} values are reported as pD_2 or $-\log ED_{50}$, and equilibrium binding constants are reported as pK or $-\log K_d$.

Results

Pharmacological profile of ANR-R_1 and ANF-R_2 subtypes. The established cell lines LLC-PK_1 and NIH-3T3, which selectively express the ANF-R_1 and ANF-R_2 subtypes, were used to exemplify the differential selectivity of the two receptor subtypes for metabolites of the active hormone ANF-(99–126). Table 1 summarizes the potency of these ANF analogs in receptor binding studies and cyclic GMP production studies in the two model systems. The porcine kidney epithelial cell line is representative of the ANF-R_1 subclass. Fig. 1, *upper*, illustrates the binding characteristics of the different ANF analogs in LLC-PK_1 membranes. The ANF-R_1 receptor has a very high affinity for ANF-(99–126), moderately recognizes the metabolite ANF-(103–126), and has a very low affinity for the metabolite ANF-(103–123). This order of potency of ANF analogs is the same as that obtained for inhibition of aldosterone secretion (19), stimulation of cyclic GMP production (20, 21), and receptor binding studies in bovine adrenal zona glomerulosa cells (19). Fig. 1, *lower*, illustrates the binding properties of the same analogs in NIH-3T3 cell membranes. Although the biologically active form ANF-(99–126) has the same potency as in the kidney cell line, the two other analogs are clearly more potent than in the other system. The ANF-(103–126) displays a 3-fold higher potency whereas the potency of the metabolite ANF-(103-123) is increased 400-fold, relative to the ANF-R_1 model. The widely divergent binding properties of the ANF-R_1 and ANF-R_2 subtypes for ANF-(103-123) suggested that this metabolite might be used to discriminate the two receptor subtypes and to assess their relative proportion in target tissues. In order to test this hypothesis, membranes prepared from LLC-PK_1 and NIH-3T3 cells, both specific carriers of pure ANF-R_1 and ANF-R_2 subtypes, respectively, were mixed in equivalent proportions and used for ANF binding assays. The resulting competition curves are illustrated in Fig. 2. The biologically active form ANF-(99–126) and the active metabolite ANF-(103–126) competed for ^{125}I-ANF-(99–126) binding in the mixed receptor subtype preparation with almost the same potency as in pure ANF-R_1 and ANF-R_2 membrane preparations. In contrast, the competition curve for ANF-(103–123) displayed an intermediate plateau,. consistent with the ability of this metabolite to actively compete with ^{125}I-ANF-(99–126) for ANF-R_2 sites. The occupation by ^{125}I-ANF-(99–126) of the remaining ANF-R_1 sites is not competed with by ANF-(103–123) and the competition curve reaches a plateau.

Affinity labeling of ANF receptor subtypes. Analysis of the molecular weight and subunit composition of the ANF receptor subtypes was performed by SDS-polyacrylamide gel electrophoresis following affinity cross-linking of ^{125}I-ANF-(99–126) to LLC-PK_1 and NIH-3T3 membranes. Fig. 3 illustrates the autoradiograms of the gels obtained under reducing conditions. The ANF-R_1 subtype in LLC-PK_1 cells migrated as a 130-kDa protein whereas the ANF-R_2 subtype in NIH-3T3 cells showed a protein band of 64-kDa. In the absence of reducing agent, both receptor subtypes migrated at 130-kDa (data not shown).

Modulation of ANF receptor subtypes by amiloride. We have previously reported that the potassium-sparing di-

TABLE 1

Correlation between cyclic GMP production and receptor binding affinity of different ANF analogs in the ANF-R_1 and ANF-R_2 model systems

Values are mean ± standard error of the number of experiments in parentheses.

ANF Analog	ANF Receptor Binding		Cyclic GMP Production	
	ANF-R_1	ANF-R_2	ANF-R_1	ANF-R_2
	pK		pD_2	
ANF-(99–126)	10.7 ± 0.1 (6)	10.9 ± 0.02 (4)	9.7 ± 0.2 (5)	6.7 ± 0.2 (5)
ANF-(103–126)	9.1 ± 0.06 (3)	9.9 ± 0.06 (3)	7.8 ± 0.6 (2)	7.6 ± 0.2 (2)
ANF-(103–123)	6.0 ± 0.5 (3)	9.3 ± 0.3 (2)	5.7	6.7 ± 0.2 (4)

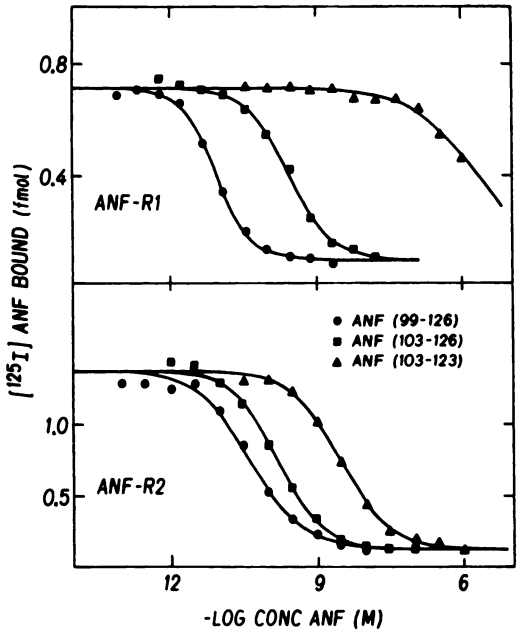

Fig. 1. Competition binding curves of ANF analogs for the binding of ^{125}I-ANF-(99–126) to LLC-PK$_1$ membranes (*upper*) and NIH-3T3 membranes (*lower*). LLC-PK$_1$ membranes (40 μg/ml) and NIH-3T3 membranes (70 μg/ml) were incubated at 25° for 90 min with 8–10 pM ^{125}I-ANF-(99–126) and varying concentrations of the competing peptides. Bound ^{125}I-ANF-(99–126) was separated from free ligand by rapid filtration through GF/C filters. The curves were simultaneously analyzed by computer according to a model for one or two classes of binding sites based on mass action law (39). The *solid lines* represent the computerized least squares fit of the data.

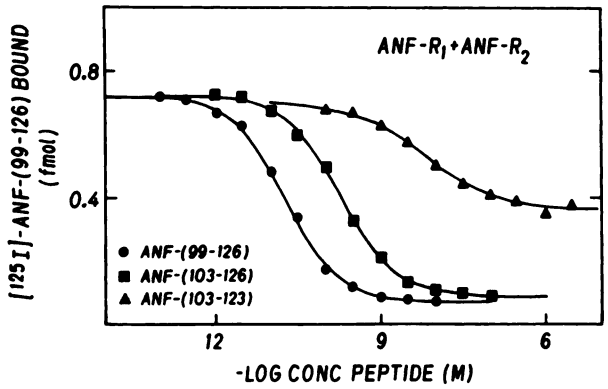

Fig. 2. Competition curves of ANF analogs for the binding of ^{125}I-ANF-(99–126) to a mixed population of receptor sites. Membranes from LLC-PK$_1$ and NIH-3T3 cells were prepared as described in experimental procedures and were mixed in equivalent proportions. This mixed preparation (40 μg/ml) was incubated at 25° for 90 min with 8–10 pM ^{125}I-ANF-(99–126) and varying concentrations of the competing peptides. Binding data were analyzed as in Fig. 1.

uretic amiloride increases the binding of ANF to high affinity receptors in bovine adrenal zona glomerulosa and potentiates the inhibitory action of ANF on aldosterone secretion (40, 41). We also demonstrated that amiloride stabilizes a high molecular weight form of the receptor (41). In this study, we have tested the specificity of amiloride by comparing its effect on the binding of ^{125}I-ANF-(99–126) to both ANF receptor subtypes. Fig. 4 shows that amiloride increases by about 2-fold ^{125}I-ANF-(99–126) binding to the ANF-R$_1$ subtype whereas the ANF binding to the ANF-R$_2$ receptor was insensitive to the diuretic. Amiloride did not change the nonspecific binding and

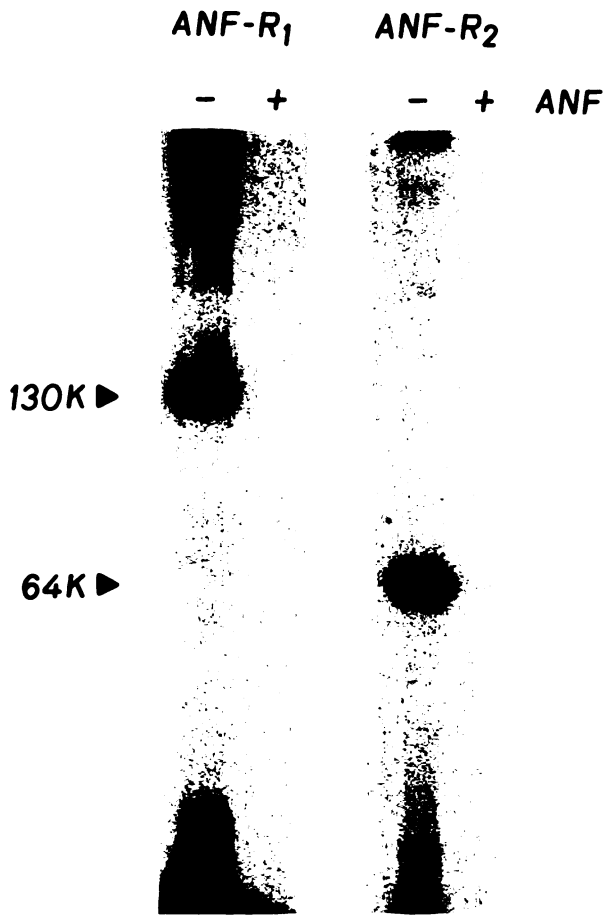

Fig. 3. Affinity labeling of ANF-R$_1$ and ANF-R$_2$ receptor subtypes. Cell membranes were incubated for 16 hr at 10° with 10 pM ^{125}I-ANF-(99–126) in the presence or absence of 10^{-7} M unlabeled ANF-(99–126). The cross-linking reaction was performed by incubating the membranes with 0.1 mM bis(sulfosuccinimidyl)suberate for 30 min at 4°. The reaction was quenched, and the washed membranes were solubilized in sample buffer containing 5% 2-mercaptoethanol. The membrane proteins were then subjected to SDS-gel electrophoresis on a 7.5% acrylamide gel. Autoradiograms of the fixed dried gels are shown after 4-day exposure. Molecular weight protein 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).

its potentiating effect on the ANF-R$_1$ receptor was fully competed with by unlabeled ANF-(99–126). This finding indicates that amiloride selectively modulates the ANF-R$_1$ subtype.

Modulation by divalent cations. We have investigated the effect of divalent cations on the binding of ANF to both receptor subtypes. Among the various cations tested, the most potent effects were obtained with Mn^{2+}. Fig. 5 shows that this divalent cation potentiates the binding of ^{125}I-ANF-(99–126) to the ANF-R$_1$ subtype and inhibits its binding to the ANF-R$_2$ subtype, both in a dose-dependent manner. All divalent cations tested had the same effect on both receptor subtypes, with a very broad spectrum of efficacy, whereas monovalent cations had no effect on either ANF-R$_1$ or ANF-R$_2$ subtype.

Stimulation of cyclic GMP production by ANF analogs. We have also examined the intracellular events associated with the activation of the two receptor subtypes by measuring the potency of ANF analogs as stimulators of cyclic GMP production. The different potencies of the metabolites are summarized in Table 1. Fig. 6 shows that, in the ANF-R$_1$ system of LLC-PK$_1$ cells, which is coupled to particulate gua-

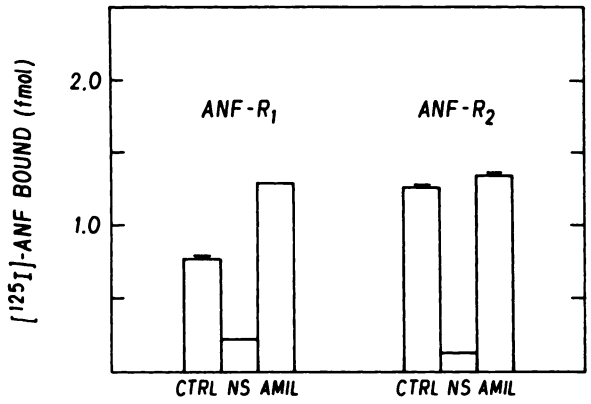

Fig. 4. Effect of amiloride on the binding of ^{125}I-ANF-(99–126) to the ANF-R_1 and ANF-R_2 receptor subtypes. Membranes of LLC-PK$_1$ and NIH-3T3 cells were incubated with 8–10 pM ^{125}I-ANF-(99–126) in the absence (*CTRL*) or presence of 10^{-4} M amiloride (*AMIL*) at 25° for 90 min. Specific ^{125}I-ANF-(99–126) binding was calculated from the difference between total binding and binding in the presence of 10^{-7} M ANF-(99–126) (*NS*). Results shown represent the mean ± standard error of three experiments.

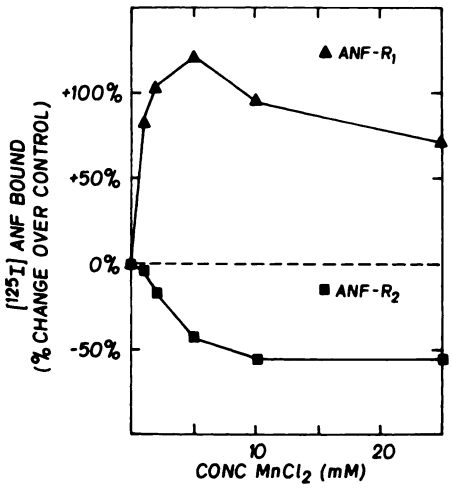

Fig. 5. Effect of $MnCl_2$ on the binding of ^{125}I-ANF-(99–126) to the ANF-R_1 and ANF-R_2 subtypes. Membranes from LLC-PK$_1$ and NIH-3T3 cells were incubated with 8–10 pM ^{125}I-ANF-(99–126) and varying concentrations of $MnCl_2$ for 90 min at 25°. The data are from a single experiment representative of three separate experiments.

nylate cyclase (28), ANF-(99–126) dose-dependently stimulates, by up to 5-fold, cyclic GMP production with a pD_2 of 9.7, which is commensurate with its pK of 10.7 for the inhibition of ^{125}I-ANF-(99–126) binding in the same system (Fig. 1). In addition, the rank potency order of the metabolites ANF-(103–126) and ANF-(103–123) on cyclic GMP production was the same as that for their receptor binding activity (Table 1), indicating that the ANF-stimulated cyclic GMP production is clearly receptor coupled. In the case of the ANF-R_2 system of NIH-3T3 cells, the results were strikingly different. ANF-(99–126) had no effect at concentrations below 10^{-8} M and then progressively stimulated cyclic GMP formation, with an apparent pD_2 of 6.7, with no plateau being reached even at 1 μM. Similar low potency was also observed for ANF metabolites. This nonsaturability of the dose-response curve has also been reported in the A10 smooth muscle cell line (42), in which the ED$_{50}$ of several ANF analogs in cyclic GMP production or

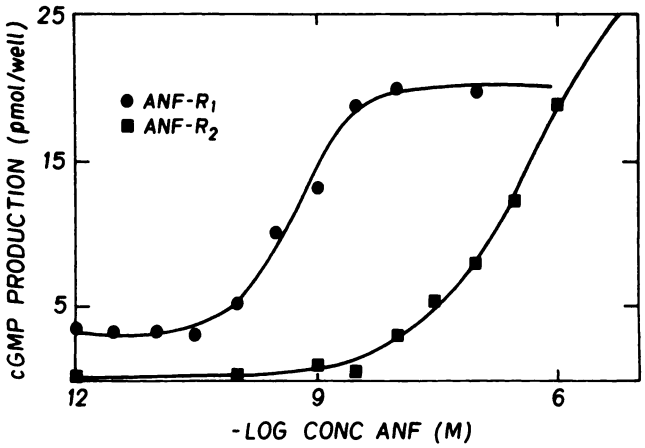

Fig. 6. Dose-response curves of ANF-(99–126) on the stimulation of cyclic GMP production in LLC-PK$_1$ cells (ANF-R_1) and NIH-3T3 cells (ANF-R_2). Confluent cells were incubated with varying concentrations of ANF-(99–126) in the presence of 0.5 mM 3-isobutyl-1-methylxanthine. Cyclic GMP was measured in the extracellular medium by direct radioimmunoassay, as described in Experimental Procedures. Each *data point* represents the average of duplicate cyclic GMP levels in four adjacent wells.

receptor binding assays were compatible with an ANF-R_2 system.

In other experiments, we have used methylene blue, which is a known inhibitor of the soluble guanylate cyclase (43), in order to attempt to understand the mechanisms by which ANF might increase the production of cyclic GMP. We observed that 100 μM methylene blue completely abolished the increase in cyclic GMP induced by ANF-(99–126) in the ANF-R_2 system, whereas it only partially inhibited the response in the ANF-R_1 system (Fig. 7).

ANF receptors in renal glomeruli. Many target organs of ANF, such as endothelial cells (28) and kidney glomeruli (44), have been shown to carry both receptor subtypes. In order to further investigate the distinct molecular properties of these subtypes, it seemed relevant to assess their relative proportions in target tissues. We have used membranes prepared from rat kidney glomeruli to evaluate the binding profile of different ANF analogs in a typical heterogeneous system (Fig. 8). In the absence of amiloride (Fig. 8, *left*), the competition curve of ANF-(103–123) was shallow, with a slope factor smaller than 1, suggesting that this analog interacts with more than one class of binding sites. Analysis of binding data according to a model for several independent classes of binding sites (39) documented the presence of two populations of binding sites with pK values of 7.9 and 9.4, respectively, and receptor density of 74 and 195 fmol/mg of protein. These results clearly indicate that in rat tissues the analog ANF-(103–123) has a much higher affinity for the ANF-R_1 receptor, compared with the porcine model cell line or with bovine tissues, but has a comparable affinity for ANF-R_2 receptors from other species. The analog C-ANF, which is a highly specific ligand for the ANF-R_2 subtype (29), competed only for 60% of the specific ^{125}I-ANF-(99–126) binding. Computer analysis of the data indicated that C-ANF binds to a single population of sites, with a pK of 9.0. These results indicate that C-ANF is a more selective analog than ANF-(103–123) for the ANF-R_2 subtype, especially in rat tissues.

The selective effect of amiloride on the ANF-R_1 subtype is

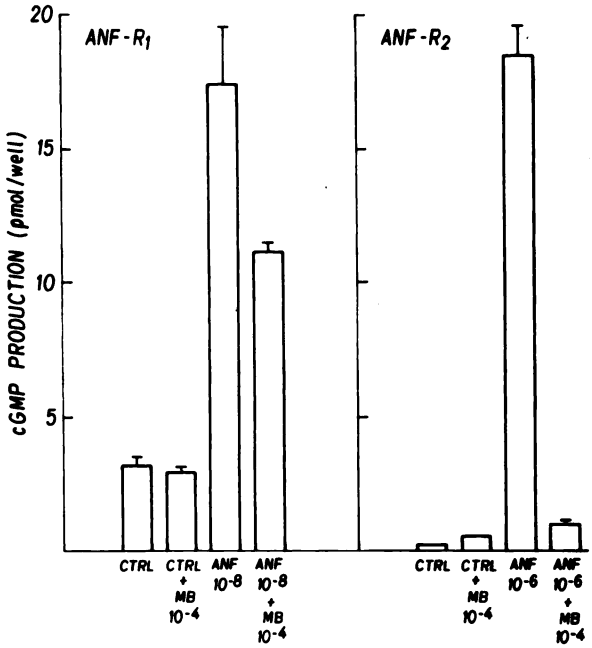

Fig. 7. Effect of methylene blue (*MB*) on the ANF-induced cyclic GMP accumulation in LLC-PK$_1$ cells (ANF-R$_1$) and NIH-3T3 cells (ANF-R$_2$). Confluent cells were incubated with maximally stimulating concentrations of ANF-(99–126) in the presence or absence of 10^{-4} M methylene blue. Cyclic GMP was measured in the extracellular medium by direct radioimmunoassay. Results are presented as the mean ± standard error of four experiments.

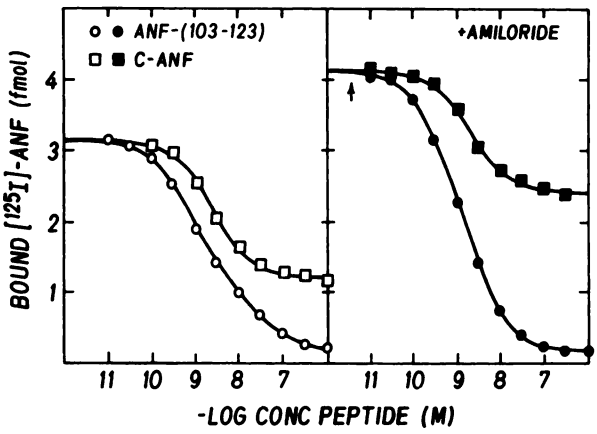

Fig. 8. Competition curves of ANF analogs for the binding of ^{125}I-ANF-(99–126) to rat kidney glomeruli membranes. Kidney glomeruli membranes (30 μg/ml) were incubated at 25° for 90 min with 8–10 pM ^{125}I-ANF-(99–126) and varying concentrations of the competing analogs in the absence (*open symbols*) or presence (*closed symbols*) of 10^{-4} M amiloride. Binding data were analyzed by computer as in Fig. 1.

shown in Fig. 8, *right*. In the presence of amiloride, the competition curve of C-ANF is characterized by an increase in total binding, which correlates with an increase in nondisplaceable binding. Inasmuch as C-ANF does not compete for binding to the ANF-R$_1$ subtype, this increase in total binding reflects the specific potentiation of ^{125}I-ANF-(99–126) binding to the ANF-R$_1$ subtype.

The heterogeneity of ANF receptor sites in rat kidney was finally confirmed by affinity cross-linking of ^{125}I-ANF-(99–126) to glomerular membrane preparations, followed by SDS-polyacrylamide gel electrophoresis. The autoradiograms of the gels obtained under reducing conditions showed the labeling of two protein bands that migrated at 130 and 64 kDa (Fig. 9). Amiloride potentiated the labeling of the 130-kDa band, whereas the 64-kDa band was not affected. Fig. 9 shows the complete inhibition of the labeling of both receptor proteins by unlabeled ANF-(99–126), indicative of their specificity. In contrast, C-ANF only abolished the labeling of the 64-kDa band, consistent with its selectivity for the ANF-R$_2$ receptor subtype.

When similar experiments were repeated in bovine kidney glomeruli, C-ANF and ANF-(103-123) equally discriminated between the two ANF receptor subtypes, in accordance with the predictions based on the model cell lines (data not shown). These contrasting results can be explained by species differences in the affinity of ANF-(103–123) for the ANF-R$_1$ receptor.

Discussion

The data presented in this paper support and further extend the hypothesis of two subtypes of ANF receptors that was recently proposed by Leitman *et al.* (28). We have identified and characterized two cell lines that selectively express the ANF-R$_1$ and ANF-R$_2$ receptors and have investigated their distinctive properties (Table 2).

The kidney epithelial cell line LLC-PK$_1$ is the prototypical

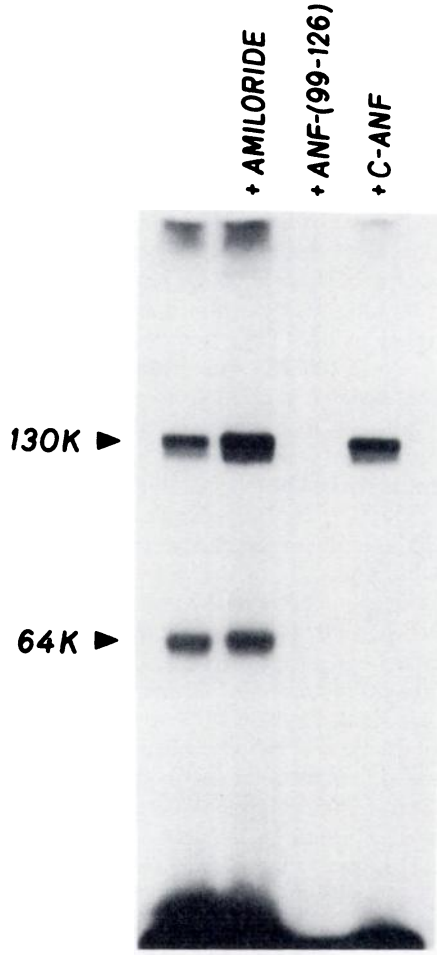

Fig. 9. Affinity cross-linking of ^{125}I-ANF-(99–126) to rat kidney glomeruli membranes in the absence or presence of 10^{-7} M ANF-(99–126), 10^{-7} M C-ANF, and 10^{-4} M amiloride. Affinity cross-linking and SDS-polyacrylamide gel electrophoresis were performed as described in Fig. 3. An autoradiogram of the fixed dried gel is shown after 6-day exposure.

TABLE 2
Differential properties of ANF receptor subtypes

	ANF-R_1	ANF-R_2
Affinity for ANF metabolites	Low	High
Modulation by amiloride	Yes	No
Effect of Mn^{2+}	Potentiation	Inhibition
Subunit structure	Monomer of 130 kDa	Homodimer of 64 kDa
Potency on cyclic GMP production	High	Low
Sensitivity to methylene blue	Low	High
Distribution	LLC-PK_1 Zona glomerulosa Endothelial cells Vascular smooth muscle cells Kidney glomeruli	NIH-3T3 A10 Endothelial cells Vascular smooth muscle cells Kidney glomeruli Platelets

tissue for the ANF-R_1 subtype. The receptor discriminates very well the native hormone ANF-(99–126) from its metabolites. The order of potency of the ANF analogs in competing for ^{125}I-ANF-(99–126) binding was ANF-(99–126) > ANF-(103–126) > ANF-(103–123). The affinity of the ANF-R_1 subtype for these ANF analogs was the same as that obtained in binding studies with adrenal zona glomerulosa membranes (19) and correlates well with the biological activity of the analogs in different systems. In fact, ANF-(103–123) was shown to be a very poor stimulator of cyclic GMP production in endothelial and vascular smooth muscle cells (20, 21) and a very weak inhibitor of aldosterone production (19). The ANF-R_2 subtype expressed by the mouse embryonic fibroblast cell line NIH-3T3 recognizes all the fragments of the hormone with similar affinity. This order of potency of ANF analogs has also been reported in competition binding experiments in A10 smooth muscle cells (22), rat kidney cortex (29), and vascular endothelial and smooth muscle cells (20, 21). From these results, it is clear that the C-terminal residues Phe^{124} and Arg^{125}, crucial for the binding of ANF to the ANF-R_1 receptor (19), are not required for its binding to the ANF-R_2 subtype. Synthesis of N- and C-terminal-truncated fragments of ANF-(99–126) will be very useful in elucidating the physiological role of the ANF-R_2 subtypes and the contribution of ANF metabolites to the overall effect of the hormone.

The ability of the metabolite ANF-(103–123) to discriminate between ANF-R_1 and ANF-R_2 subtypes is, however, not so clear in the rat species. As seen in Fig. 8, this metabolite displayed only a 32-fold selectivity for the ANF-R_2 subtype in rat kidney glomeruli. The C-ANF analog, which not only lacks the C-terminal amino acids but which also displays a shorter loop, is, however, completely selective for the ANF-R_2 subtype in the rat. These findings suggest that the loop structure is a very crucial discriminating moiety of the ANF molecule, whereas the C-terminal Phe^{124} and Arg^{125} also contributes to the subtype selectivity to a degree varying among species.

Results from affinity labeling experiments showed that the binding subunit of the ANF-R_1 receptor is present on a 130-kDa protein, whereas the ANF-R_2 binding protein has a molecular weight of 64-kDa. These experiments were performed using cell membranes prepared in the presence of protease inhibitors of various specificities (EDTA, aprotinin, leupeptin, and pepstatin A) in order to exclude the possibility that the ANF-R_2 subtype might result from the proteolysis of the ANF-R_1 receptor. The 130-kDa ANF-R_1 receptor has also been identified in adrenal cortex (24, 25), kidney cortex (23) and glomeruli (44), aorta (27, 41), endothelial cells (28), lung (45), placenta (46), and lung fibroblasts (47). The 60–70-kDa ANF-R_2 receptor is found in aorta membranes (27), vascular smooth muscle cells (26), endothelial cells (28), A10 cell line (22), kidney glomeruli (44), and lung fibroblasts (47). The presence of ANF-R_2 receptor sites in nonphysiological target tissues such as fibroblasts is compatible with their putative role as clearance sites.

We have previously reported that various cations can modulate the binding of ANF to adrenal zona glomerulosa membranes (7). High concentrations of monovalent cations slightly increased specific binding by reducing nonspecific binding, whereas low concentrations of divalent cations were more potent in increasing ANF binding. The results of this study indicate that divalent cations have an opposite effect on the binding of ANF to the ANF-R_1 and ANF-R_2 subtypes. The effect of divalent cations on the ANF-R_2 subtype was also reported in A10 smooth muscle cells, where all cations tested inhibited the binding of ANF (22). A modulatory effect of divalent cations has also been observed for other receptors, such as the enkephalin receptors (48).

The finding that amiloride selectively modulates the binding of ANF to the ANF-R_1 subtypes provides a very useful tool in discriminating the two receptor subtypes in computer-modeled competition binding curves of heterogeneous receptor populations. Use of amiloride in competition binding curves with an ANF analog specific for the ANF-R_2 receptor might help to reveal and enhance low amounts of ANF-R_1 receptor subtype, which otherwise might be unnoticed. The low amount of the ANF-R_1 subtype can be detected by an increase in the amount of nondisplaceable ^{125}I-ANF-(99–126) binding due to the selective potentiating effect of amiloride on the ANF-R_1 subtype. When this method was applied to rat kidney glomeruli, we found that approximately 60% of the receptor population consisted of the ANF-R_2 subtype. ANF receptor heterogeneity detected in competition binding curves was confirmed by autoradiography of affinity-labeled membranes. Hamada *et al.* (44) also reported the labeling of two bands of 75 and 140 kDa in rat kidney glomeruli, the former being of greater intensity, which confirms the apparent majority of ANF-R_2 receptor sites in rat kidney glomeruli. The proportion of ANF-R_1 receptors documented in our study is, however, in contradiction with the results of Maack *et al.* (29).

It is well known that many effects of ANF are accompanied by an increase in cyclic GMP production within target cells (12, 13) via activation of particulate guanylate cyclase (13). The duality of ANF receptor subtypes is also reflected in the differential ability of each subtype to elicit cyclic GMP production; only the 130-kDa ANF-R_1 receptor is associated with cyclic GMP production at physiological concentrations of ANF. The interaction of ANF with the dimeric ANF-R_2 receptor is not directly associated with significant increase in cyclic GMP formation until increasingly high concentrations of the hormone are reached. Our results in NIH-3T3 cells are in perfect agreement with those of Nambi *et al.* (42), who reported in A10 smooth muscle cells that the ANF-R_2 receptor showed an ANF-sensitive cyclic GMP production at high ANF concentrations. The mechanism by which ANF increases cyclic GMP produc-

tion in cells expressing the ANF-R_2 receptor is not understood. However, the fact that methylene blue completely abolishes this effect might lead us to conclude that ANF could indirectly activate the soluble guanylate cyclase or that these cells might contain a particulate guanylate cyclase isozyme that is sensitive to methylene blue. The second hypothesis would be more compatible with the observation that sodium nitroprusside, which is a known activator of soluble guanylate cyclase, has no stimulatory effect on cyclic GMP production in NIH-3T3 cells (data not shown). The distinctive dose-response profile of cyclic GMP production in tissues containing the ANF-R_2 receptor and the fact that the receptor is not retained on a GTP affinity column and is not associated with guanylate cyclase activity (49, 50) also rules out the possibility that the ANF-R_2 subtype is associated with the same particulate isozyme as the ANF-R_1 subtype. Furthermore, the large dissociation between the binding affinity of ANF to the ANF-R_2 subtype and its potency on cyclic GMP production strongly indicates that the receptor is not directly involved in the cyclic GMP response of the peptide.

The role of the ANF-R_2 receptor is not clear at the present time. It was recently postulated that ANF-R_2 receptors are biologically silent and that they might serve as clearance sites for the hormone (29). Some reports also showed that the guanylate cyclase-free subtype was down-regulated and probably internalized into lysosomal structures (51, 52). These results are, however, not conclusive because the studies were done in heterogeneous systems. Use of homogeneous ANF receptor models, e.g., those documented in this report, will enable us to study the regulation of each receptor and its contribution to the metabolism of the hormone and hopefully will lead to proper assignment of the specific role of each ANF receptor subtype in the mediation of ANF effects.

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