Stoichiometry of the Atrial Natriuretic Factor−R1 Receptor Complex in the Bovine Zona Glomerulosa†

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ABSTRACT: The atrial natriuretic R1 receptor is a membrane protein that is present as an apparently preassociated noncovalent oligomer in the absence of ligand as suggested by steric exclusion studies and cross-linking experiments in physiological and recombinant receptor expression systems. The association state of this receptor oligomer was studied in the presence of amiloride and ATP, two known modulators of the R1 receptor functions with both the intact receptor and a cytoplasmic domain-deleted form obtained by limited proteolysis with trypsin. It was shown by steric exclusion on Superose 6 column that amiloride increased the affinity of ANF for the native and truncated receptor, in contrast with ATP, whose destabilizing effect on ANF binding was abolished by truncation of the cytoplasmic domain. Neither amiloride nor ATP exerts its effects by altering the aggregation state of the receptor. Comparison of the measured number of ANF binding sites with immunoassayable receptor protein revealed that the stoichiometry of ANF binding to the R1 receptor was 1:2. This was confirmed by using an ANF analog that bears a photoactivatable group at both of its ends, showing that ANF, as for the growth hormone/receptor complex, interacts with both the receptor subunits and specifically cross-links a dimeric form of the receptor. The potential pharmacological consequences of this 1:2 stoichiometric ratio of the ANF−receptor complex are discussed.

Pharmacological characterization and molecular cloning have brought new evidence for the existence of types and subtypes of natriuretic peptide receptor. Earlier biochemical studies by Leitman have shown that there are two main types of receptors, i.e., R1 and R2 (Leitman et al., 1986). Molecular cloning studies have subsequently shown that the R1 type (which mediates most of the biological effects of natriuretic peptides) was a monomeric M_r 130 000 protein with a single putative transmembrane spanning domain and a cytoplasmic domain comprising a proximal segment with striking homology with the protein kinase domain of the growth factor receptor family and a distal region homologous with soluble guanylate cyclase (Chinkers et al., 1989; Lowe et al., 1989).

It was also known from biochemical studies that ANF[1] binding and guanylate cyclase activity of the R1 type could be oppositely modulated by the diuretic amiloride and the nucleotide ATP (De Léan, 1986). Amiloride increases ANF binding to ANF-R1, and ATP decreases ANF binding affinity but increases ANF-stimulated guanylate cyclase activity (De Léan, 1986; Kurose et al., 1987; Chang et al., 1990; Larose et al., 1991). These agents appear to interact directly with the receptor and not through accessory proteins since their effects were observed with purified preparation of receptor (Meloche et al., 1988; Larose et al., 1991).

Previous work from our group has shown by steric exclusion chromatography that ANF-receptor appears to exist as an oligomeric form and that amiloride was recruiting this high molecular size form of ANF−receptor complex displaying a Stokes radius of 70 Å. It was then postulated that this complex corresponded to the high-affinity binding component (Meloche et al., 1987). However, it was not known whether increased ANF binding to the 70 Å form could be explained by enhancement of its affinity or by shifting the equilibrium in favor of more receptor oligomers. Primary sequence homologies have evoked the possibility that the mechanism of signal transduction of the R1 type could be analogous to that observed with the receptor tyrosine kinases (Ullrich & Schlessinger, 1990). In those receptor systems, ligand-induced oligomerization appears to be required for receptor activation. Initial results tend to suggest that signal transduction of the ANF-R1 receptor is different. Although ANF-R1 receptor seems dimeric or tetrameric in the bovine zona glomerulosa (Meloche et al., 1987; Iwata et al., 1991), this oligomerization seems ligand-independent (Chinkers et al., 1992; Lowe, 1992). However, as for growth factor receptors, natriuretic peptide receptor dimerization appears to be required for the ligand-induced activation process to occur (Chinkers & Wilson, 1992). Furthermore, previous work on the aggregation state of the ANF-R1 receptor has not studied the stoichiometry of ANF binding to the complex (Iwata et al., 1991; Lowe 1992; Chinkers & Wilson, 1992).

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[1] Abbreviations: ANF, atrial natriuretic factor; BNP, brain natriuretic peptide; C-ANF, [Cys[116]]rat ANF(102-116)-NH_2; BPA-ANF, [Tyr[116],*p*-benzoyl-Phe[125]]rat ANF(99-125); AB-BPA-ANF, [*p*-azidobenzoyl-Ser[99]]-BPA-ANF.

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Since the mechanisms of ATP and amiloride induction on high- or low-affinity forms of the receptor in terms of oligomerization are not known, we have used here steric exclusion chromatography and immunodetection of the R1 receptor protein to demonstrate that these agents appear to act by inducing conformational changes of the receptor without affecting the aggregation state of the receptor and that the receptor exists as a dimer in the membrane before ligand binding. In addition, since some growth factors interact with their dimeric receptor as a 1:2 complex, we studied the stoichiometry of ANF binding, and similar to the mechanism of binding of growth hormone and insulin, we observed that an ANF analog that can covalently cross-link to the receptor binding site through both its amino- and carboxy-terminal tails documents a complex of one molecule of ANF per receptor dimer.

EXPERIMENTAL PROCEDURES

Materials. TPCK-treated trypsin (type XIII), chymotrypsin, thyroglobulin, bovine serum albumin, bovine γ-globulins, protease inhibitors, amiloride, ATP, and glutaraldehyde were obtained from Sigma. *N*-Hydroxysuccinimidyl 4-azidobenzoate (HSAB) was from Pierce. Rat ANF(99-126) was obtained from IAF Biochem (Montréal, Canada), and C-ANF was from Peninsula. BPA-ANF was synthesized by IAF Biochem as described by McNicoll et al. (1992). Carrier-free $Na^{125}I$ was from Amersham, and Iodo-beads were purchased from Pierce Chemical Co. Electrophoresis reagents and molecular weight markers were from Bio-Rad. Superose 6 column and gel filtration standards were obtained from Pharmacia. Octyl β-D-glucopyranoside (octyl glucoside) was from Boehringer Mannheim.

Iodination of Peptides. The peptides were radioiodinated with Iodo-beads according to Ong et al. (1987). Typically, 10 μg of ANF or synthetic peptide was dissolved in 100 μL of 0.5 M KH_2PO_4, pH 7.0. The reaction was started by the addition of 1 mCi of $Na^{125}I$ and two Iodo-beads. The mixture was incubated at 4 °C for 20 min. The iodinated peptide was separated from free iodine on a RP1 cartridge and purified on a Vydac C18 column using a 0.5%/min acetonitrile gradient in 0.1% TFA.

Preparation of Membranes. Bovine adrenal zona glomerulosa membranes were prepared as described previously (Meloche et al., 1988) and stored at −70 °C until used.

Limited Proteolysis with Trypsin. Zona glomerulosa membranes were digested according to Liu et al. (1989). Briefly, the membranes were washed in 50 mM Tris-HCl, pH 7.4, and resuspended in the same buffer at a protein concentration of 2 mg/mL. Proteolysis was performed for 30 min at 25 °C by addition of an equal volume of buffer containing trypsin (5 μg/mg of protein). The digestion was stopped by addition of PMSF (1 mM final concentration), and membranes were washed twice by centrifugation at 30000*g* for 20 min at 4 °C. Membranes were then resuspended in the binding buffer and incubated with ^{125}I-ANF as described in the "Steric Exclusion Chromatography" section. Dose−response curves for the effects of trypsin on the ANF binding in the presence of amiloride or ATP were analyzed by nonlinear least-squares curve fitting using the software ALLFIT for Windows[2] based on a two-step seven-parameter equation derived from the four-parameter logistic equation (Liu et al., 1989). The two-step model was retained only when a statistically better fit was obtained when compared with a one-step model using an *F* test.

Steric Exclusion Chromatography. The binding and chromatography were performed according to Meloche et al. (1987). Adrenal zona glomerulosa membranes (20 μg) were incubated with 8 pM ^{125}I-ANF for 90 min at 25 °C in binding buffer (50 mM Tris-HCl, pH 7.4, 5 mM $MnCl_2$, 0.1 mM EDTA, 0.5% BSA, and 0.1 μM C-ANF). C-ANF was added to prevent the putative interference of ANF-R2 receptors which have been reported by others in bovine adrenal cortex preparations (Takayanagi et al., 1987). Although we have not routinely detected ANF-R2 contamination in our bovine adrenal membrane preparations, we have observed that the inclusion of C-ANF in the binding assay prior to solubilization and size-exclusion chromatography minimized the appearance of the 50 Å peak eluting at a position compatible with a monomeric form of ANF-R1. The membranes were then centrifuged at 30000*g* for 15 min, and the pellet was solubilized at 2 mg/mL protein in 50 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 20% glycerol, 1 μM aprotinin, 1 μM leupeptin, 0.1 μM pepstatin A, and 40 mM octyl glucoside at 4 °C for 60 min. After centrifugation at 30000*g* for 30 min and filtration on 0.22 μm membranes, the supernatant (300 μL) was injected on a Superose 6 column eluted at 0.4 mL/min in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1 mM EDTA, and 40 mM octyl glucoside at 4 °C. Fractions of 0.4 mL were collected. The molecular standards used were as follows: thyroglobulin (85 Å), catalase (52.2 Å), aldolase (48.1 Å), chymotrypsinogen A (20.9 Å), and ribonuclease A (16.4 Å). The void (V_o) and total (V_t) volumes were determined with blue dextran and acetone, respectively. The distribution coefficient K_{av} was calculated from the relation $K_{av} = (V_e - V_o)/(V_t - V_o)$ (where V_e is the elution volume), and the Stokes radius of the receptor was estimated from the linear relationship between $(-\log K_{av})^{1/2}$ and Stokes radius.

Radioimmunoassay of ANF-R1 Receptor. The antibody used for the RIA was developed against the synthetic peptide CS328 (Y-I-T-V-D-H-L-E-F) whose sequence was based on the carboxy-terminal end of a chymotryptic fragment (Met^{173}–Phe^{188}) obtained from the bovine ANF-R1 receptor. A tyrosine was added to the amino terminal in order to allow for iodination. The peptide was coupled to BSA using glutaraldehyde, and 100 μg of conjugate was injected subcutaneously to 1.5 kg rabbits. Antisera were collected after 3 boosts.

The RIA was performed on each chromatographic fraction as follows. Before incubation with CS328, each fraction was pretreated with chymotrypsin in order to increase sensitivity of the RIA by exposing the epitope. Chymotrypsin (2 μg) and 2 mM $CaCl_2$ (final concentration) were added and incubated at 25 °C for 20 h. The fractions were then heated to 100 °C for 4 min. RIA buffer (50 mM sodium phosphate buffer, pH 7.4, 50 mM NaCl, 0.1% BSA, 0.02% NaN_3, 1 μM aprotinin) containing ^{125}I-CS328 (6 pM) and the antibody was added to a final volume of 0.6 mL, and the fractions were incubated overnight at 4 °C. Bound fraction was precipitated by adding 100 μL of 1.5% bovine γ-globulins and 1 mL of 20% PEG 8000. After centrifugation, the

[2] Requests for the software ALLFIT for Windows can be addressed by e-mail: delean@ere.umontreal.ca.

supernatant was discarded and pellets were counted in a γ-counter.

Derivatization of BPA-ANF with HSAB. BPA-ANF (75 μM) was incubated at pH 8.0 in a 0.125 M $NaHCO_3$ buffer with 12.5 mM HSAB overnight at 25 °C in darkness. The reaction was quenched with 0.1 M glycine, pH 7.5, for 1 h.

Receptor Binding Assays. The zona glomerulosa membranes or the trypsin-treated receptor was incubated with ^{125}I-ANF as described in the "Steric Exclusion Chromatography" section. Nonspecific binding was evaluated in the presence of 0.1 μM ANF. The bound fraction was separated by filtration on Whatman GF/C filters precoated with 1% PEI. Filters were washed with 50 mM potassium phosphate buffer, pH 7.4, and counted in a γ-counter.

Saturation curves of ^{125}I-ANF on zona glomerulosa membranes were done in order to evaluate the binding site density. Ligand (specific activity lowered to 50 cpm/fmol with ANF) and membranes were incubated in binding buffer containing 0.1 μM C-ANF for 90 min at 25 °C. The curves were analyzed by nonlinear least-squares regression based on the mass action law (De Léan et al., 1982). Bound fraction was determined by filtration of samples on Whatman GF/C filters precoated with 1% PEI.

For photolabeling studies, zona glomerulosa membranes (80 μg) were incubated with BPA-ANF or AB-BPA-ANF in 2 mL of binding buffer for 90 min at 25 °C in the darkness. Nonspecific binding was evaluated in the presence of 1 μM ANF. The binding buffer was degassed under vacuum and purged with helium before and after the binding in order to remove O_2 from the buffer. The mixture was then exposed to UV for 20 min at 4 °C and washed twice with 50 mM sodium phosphate buffer, pH 7.4. Membranes were then resuspended in sample buffer and run on a 5% reducing SDS–PAGE gel according to Laemmli (1970).

Western Blotting. The antibody used for immunoblotting was raised in rabbits using peptide CS368 (Y-G-E-R-G-S-S-T-R-G) based on the carboxy-terminal sequence Gly^{1021}–Gly^{1029} of the human ANF–R1 receptor flagged with a tyrosine at its amino-terminal end. The immunoglobulin was purified by affinity on a CS368–agarose column before immunoblotting was performed.

SDS–PAGE was followed by blotting proteins to nitrocellulose membrane using a semidry blotting system for 90 min at 1.2 mA/cm^2. The transfer buffer was 25 mM Tris/192 mM glycine/20% CH_3OH. Membranes were blocked overnight at 4 °C in PBS/0.1% Tween-20 (PBST) supplemented with 5% nonfat dry milk. Blots were probed with the CS368 antibody in PBST/0.1% BSA, washed in PBST, and detected by chemiluminescence (ECL kit, Amersham).

RESULTS

Effect of Amiloride and ATP on the Aggregation State of ANF-R1 Receptor. The nonionic detergent octyl glucoside was used to solubilize zona glomerulosa membranes after labeling with ^{125}I-ANF in the presence of the ANF-R2 receptor-specific ligand C-ANF used to ensure selective labeling of ANF-R1 receptor sites. In order to evaluate the mechanism of action of the known modulators of the ANF-R1, amiloride and ATP, the solubilized ANF–receptor complex was injected on a Superose 6 column and the apparent size of the complexes was evaluated before and after treatment with amiloride and ATP.

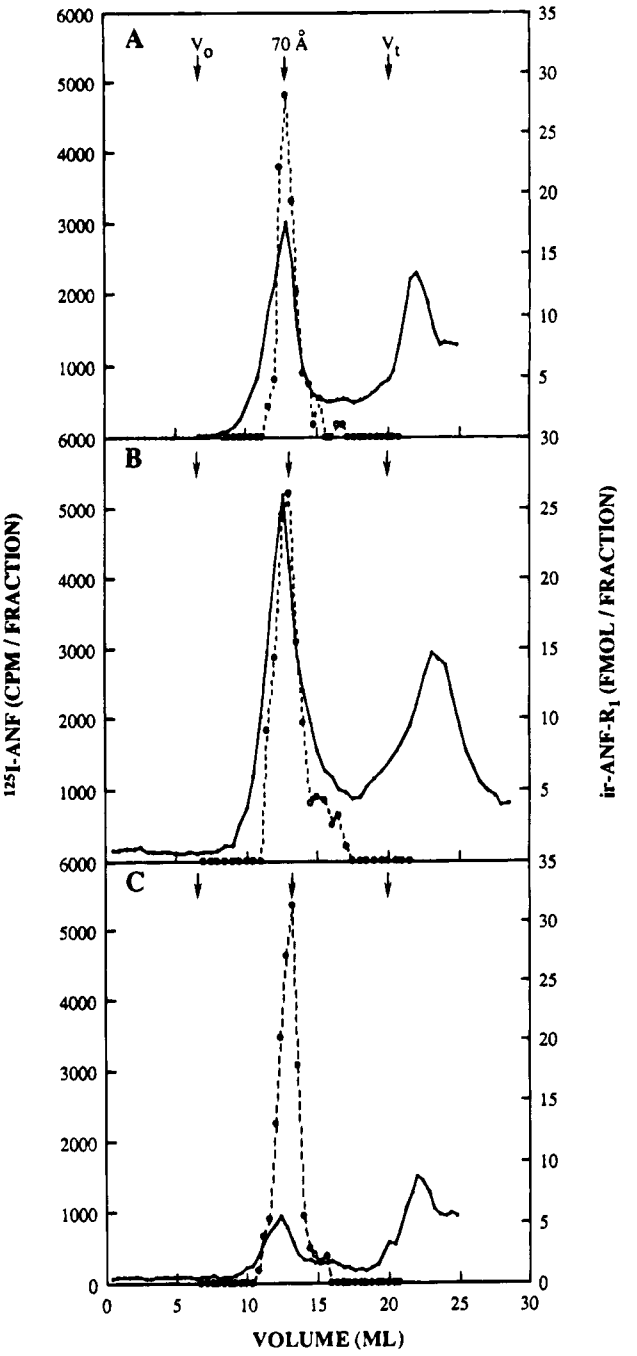

FIGURE 1: Effect of amiloride and ATP on the solubilized ^{125}I-ANF–receptor complex. Adrenal zona glomerulosa membranes were incubated at 25 °C for 90 min with 8 pM ^{125}I-ANF in the presence of 0.1 μM C-ANF in the absence (panel A) or presence (panel B) of 0.1 mM amiloride or 1 mM ATP (panel C). The membranes were then solubilized at 4 °C for 60 min and injected on a Superose 6 gel filtration column according to the Experimental Procedures. The elution was performed at a flow rate of 0.4 mL/min, and 0.4 mL fraction were collected and counted for radioactivity. Then, each fraction was digested with chymotrypsin at 25 °C for 20 h and heated at 100 °C for 4 min, and ANF-R1 receptor was quantified by RIA using CS328 antibody. The void (V_o) and total (V_t) volumes of the column are shown by arrows. Typical profiles of radioactivity (●) and RIA profiles (○) are shown.

The elution profile of the ANF–receptor complex showed one peak of bound ^{125}I-ANF (3000 cpm at maximum peak value) coeluting with immunoassayable receptor protein (Figure 1, panel A). Both peaks eluted at 12.8 mL, corresponding to a Stokes radius of 70 Å as previously observed (Meloche et al., 1987). When ^{125}I-ANF binding

was performed in the presence of 0.1 mM amiloride, an important increase of the ^{125}I-ANF−receptor complex peak (5200 cpm) was observed (Figure 1, panel B). Neither the amount of receptor protein estimated by RIA nor the position of the immunoassayable peak were changed by amiloride. These results suggest that the state of oligomerization of the ANF-R1 is not affected by amiloride and that the observed increase in ANF binding might be due to increased affinity resulting from an allosteric mechanism rather than to a shift in the balance of the receptor dimerization reaction. When 1 mM ATP was included during ^{125}I-ANF receptor prelabeling and solubilization, there was also one peak at 70 Å, but receptor-bound ^{125}I-ANF was decreased from 3000 to 950 cpm at maximum peak values (Figure 1, panel C). In contrast, the elution volume of the peak and the amount of receptor detected by RIA were unchanged by ATP.

Effect of Deleting the ANF-R1 Receptor Cytoplasmic Domain on Modulation of ANF Binding by Amiloride and ATP. The role of the cytoplasmic domain of the receptor in the effect of amiloride and ATP was also studied using a truncated receptor prepared by limited proteolysis with trypsin. The resulting M_r 70 000 membrane-bound fragment contains the extracellular domain, the transmembrane domain, and about 80 amino acids of the cytoplasmic part (Rondeau et al., 1992). The elution profile of the ^{125}I-ANF-prelabeled truncated receptor is shown in Figure 2. As expected, the elution position of the truncated receptor was retarded when compared with that of native receptor (Figure 1). In addition, an earlier peak corresponding to the position of the intact 70 Å receptor was observed, especially in the presence of amiloride (Figure 2, panel B). The position of the ANF−receptor complex peak was distinct from that of the intact receptor but was not affected by amiloride or ATP. However, the amount of ^{125}I-ANF bound to the truncated receptor was significantly increased in the presence of amiloride (from 500 to 3500 cpm at maximum peak values, Figure 2, panel B). In contrast, the inhibitory effect of ATP on ANF binding (376 cpm at maximum peak value) was almost completely lost (Figure 2, panel C, where a 25% loss of ANF binding relative to control is observed) when compared with the intact receptor (Figure 1, panel C, where a 68% loss of ANF binding is observed).

These results suggest that the effect of ATP on the binding of ANF is dependent on the presence of the intracellular domain while amiloride could act at least in part through the extracellular and/or transmembrane domain of the ANF-R1 receptor.

The role of the intracellular domain in the modulation of ANF binding by amiloride and ATP was further characterized by dosing limited proteolysis of the ANF-R1 cytoplasmic domain with increasing concentrations of trypsin prior to incubation with ^{125}I-ANF, in the presence of either 0.1 mM amiloride or 1 mM ATP. The loss of the amiloride effect on ANF binding is better explained by a two-step model with ED_{50} of 12 and 500 μg/mg (Figure 3; $F = 12.5$ in comparison with a one-step model). The first phase corresponds to the ED_{50} of the loss of the inhibitory effect of ATP and to the formation of the M_r 70 000 receptor fragment as reported before (Larose et al., 1991). Above 100 μg/mg trypsin, the proteolysis is less selective for the proteolysis-sensitive cytoplasmic region and the effect of amiloride is gradually lost but still apparent, even at 1 mg of trypsin/mg of protein. These data indicate that part of the effect of

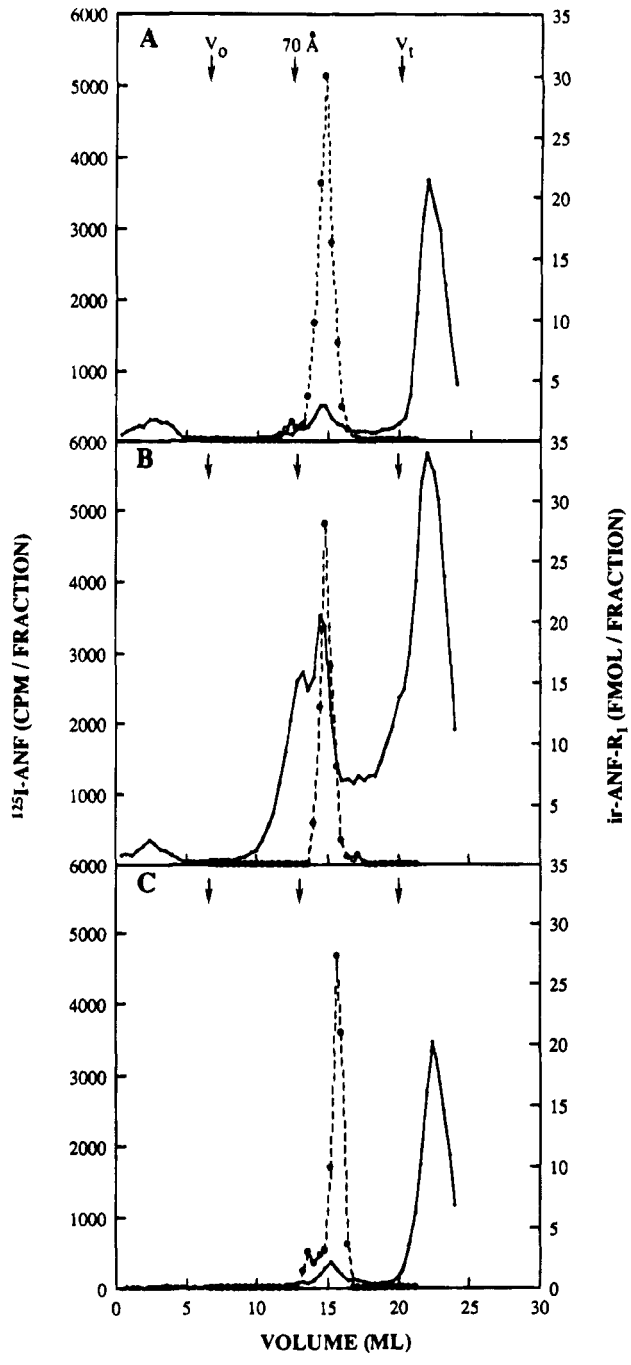

FIGURE 2: Effect of amiloride and ATP on the truncated solubilized ^{125}I-ANF−Receptor Complex. Adrenal zona glomerulosa membranes were digested at 25 °C for 30 min with trypsin in order to obtain a M_r 70 000 membrane-attached fragment. Membranes were washed twice by centrifugation, labeled with ^{125}I-ANF, solubilized, and injected on Superose 6 column as described in the Figure 1 legend. Fractions were counted and RIA was performed as described in Figure 1.

amiloride could require the intracellular domain but that most of its effect depends on the extracellular and/or transmembrane domains. ATP, as reported before, has no effect on the M_r 70 000 fragment (Larose et al., 1991) in agreement with the exclusive role of the cytoplasmic kinase homology domain of the receptor in the effect of ATP.

Assessment of the Stoichiometry of ANF−Receptor Complex. Since the results obtained from the steric exclusion chromatography of the solubilized receptor suggested that ANF was bound to an oligomeric form of the receptor, as confirmed by previous results (Chinkers & Wilson, 1992;

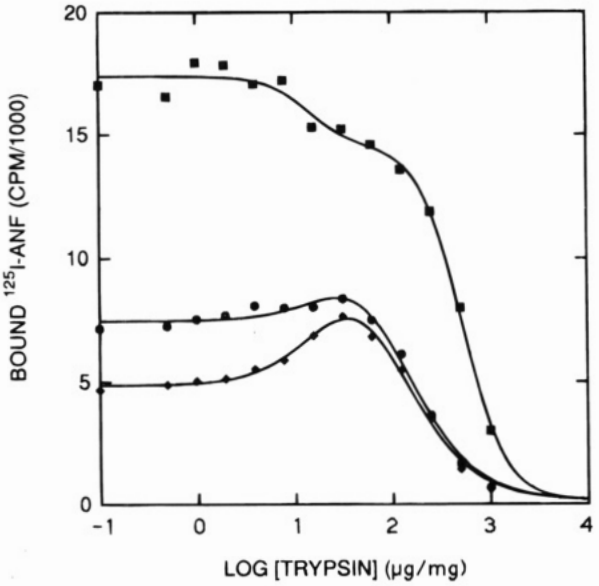

FIGURE 3: Proteolysis of ANF-R1 receptor. Adrenal zona glomerulosa membranes were digested with increasing amounts of trypsin at 25 °C for 30 min and then incubated with ^{125}I-ANF in the absence (●) or in the presence of either 0.1 mM amiloride (■) or 1 mM ATP (♦) at 25 °C for 90 min as described in the Experimental Procedures. Bound fraction was separated by filtration on GF/C filters. Curves were analyzed by least-squares nonlinear regression using the program ALLFIT for Windows as described in the Experimental Procedures section.

Table 1: Estimation of the Stoichiometry of ANF Binding by Saturation and RIA on Membranate and Solubilized Receptor[a]

	membranes (pmol/mg)	soluble fraction (pmol/mg)
R_{sat}[b]	0.40 ± 0.01	0.40 ± 0.06
R_{RIA}[c]	0.87 ± 0.13	0.84 ± 0.12
R_{RIA}/R_{sat}	2.17	2.10

[a] The saturation was performed in the presence of C-ANF. Adrenal zona glomerulosa membranes were incubated with ^{125}I-ANF at 25 °C for 90 min, and bound fraction was separated by filtration on GF/C filters. The solubilized receptor was prepared in 40 mM octyl glucoside, and binding was performed after reconstitution in lipid vesicles as described in the Experimental Procedures section. RIA was done after solubilization in 40 mM octyl glucoside and digestion with chymotrypsin. [b] ANF-R1 density estimated by saturation. Values are mean ± SEM. [c] ANF-R1 density estimated by RIA.

Lowe, 1992), the stoichiometry of the ANF−receptor complex was evaluated by comparing the values of receptor density obtained from receptor saturation using ^{125}I-ANF with those based on immunoassay of the receptor protein. The results indicate one molecule of ANF binding to two molecules of receptor subunit since RIA receptor protein levels are twice those obtained by ANF saturation measurement (Table 1).

Since the ANF-R1 receptor is at least dimeric in its membrane form prior to ANF binding and each dimer appears to bind only one ANF molecule, it was tempting to make a parallel with two other structurally related receptors, i.e., the growth hormone and the insulin receptors. These observations suggested that the ANF receptor could behave as for these receptors whose ligand is interacting with both receptor subunits. In order to show that ANF forms a bridge between those, we derivatized both the amino- and carboxy-terminal ends of ANF with photoactivatable groups in order to obtain a specific bifunctional cross-linking agent. We have previously documented that BPA-ANF is a photoreactive analog of ANF that is covalently bound to the receptor through its C-terminus when exposed to ultraviolet radiation in the absence of oxygen (McNicoll et al., 1992). Since the N-terminal end of BPA-ANF cannot be cross-linked under these conditions, an azido group was attached using HSAB to form AB-BPA-ANF. Using photolabeling, the two photoactivatable functions at each of its ends could then be cross-linked to demonstrate that ANF interacts through its N- and C-terminus with two different receptor subunits. This covalent complex would therefore be apparent on SDS−PAGE.

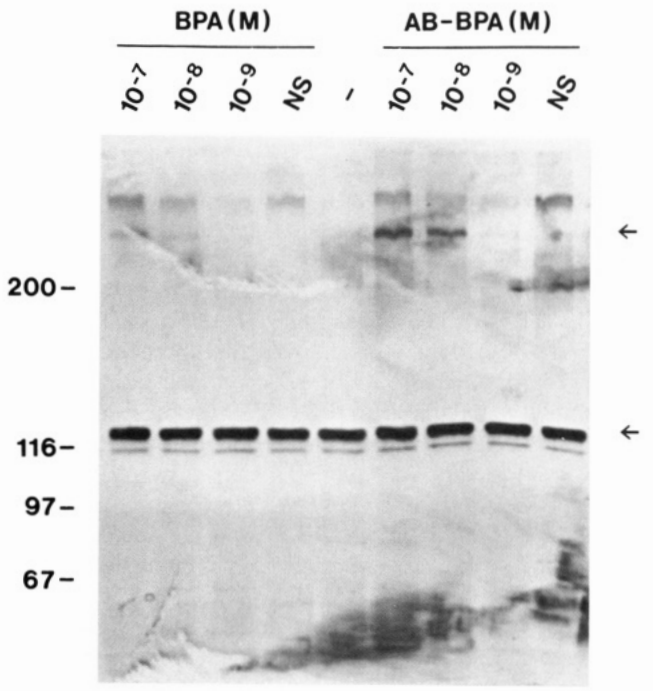

FIGURE 4: Oligomerization of ANF-R1. The ANF-R1 receptor from zona glomerulosa membranes was incubated with BPA-ANF (lanes 1−4) or AB-BPA (lanes 6−9) at 25 °C for 90 min in darkness in the presence (lanes 4 and 9) or absence (lanes 1−3 and 5−8) of 1 μM ANF. The nonspecific binding was made in the presence of 10^{-7} M BPA-ANF or AB-BPA (lanes 4 and 9). The ligands were then cross-linked to the receptor by their C-terminus and/or by its N-terminus (by UV irradiation) as described in the Experimental Procedures. The membranes were then runned on a 5% SDS−PAGE gel and blotted on nitrocellulose, and ANF-R1 receptor was revealed using CS368 antibody raised against the carboxy terminal and a chemiluminescence detection system.

When BPA-ANF was used (Figure 4, lanes 1−4), a major band was present at M_r 122 000 corresponding to the monomeric receptor, together with a minor band at M_r 116 000 which is presumably a deglycosylated form of the receptor (Lowe & Fendly, 1992) present in zona glomerulosa membranes. A faint but specific and concentration-dependent band is present at M_r 250 000, consistent with a dimeric form of the receptor covalently cross-linked by residual oxygen and lipid peroxidation, although these bands are not visible when membranes were irradiated in the absence of ligand (Figure 4, lane 5).

When AB-BPA-ANF was incubated with membranes (Figure 4, lanes 6−9), a significant increase in the intensity of the M_r 250 000 complexes could be observed. This band was also concentration-dependent. The relative intensities of the other bands remained unchanged. Higher molecular mass complexes were apparent in each condition (Figure 4, lanes 1−9) but were not specific (Figure 4, lane 9). This

band might result from a receptor aggregation phenomenon which is independent of the ligand and which probably occurred outside the receptor binding site pocket. The weaker intensity of the M_r 250 000 dimer relative to the monomer can be easily explained by higher efficacy of cross-linking by the BPA residue (around 70%; McNicoll et al., 1992) over the azido substituent (1−2%).

DISCUSSION

The data reported in this paper show that the ANF-R1 receptor is present in its membrane form as an oligomer independent of ANF occupancy and that known modulators of ANF-R1 functions, e.g., amiloride and ATP, do not affect the oligomerization state of the receptor. We also showed that the molecule of ANF binds to a dimer and that both receptor monomers can interact with ANF, suggesting that they might contribute to the formation of the ANF binding site pocket.

The use of nonionic detergent is very common for solubilization of membrane receptors when biological functions should be preserved. We formerly used octyl glucoside to show that there were two ANF-R1 receptor complexes with differing Stokes radii on steric exclusion chromatography profiles (Meloche et al., 1987). When the ANF-R1 receptor from zona glomerulosa membranes was prelabeled with ^{125}I-ANF, solubilized in octyl glucoside, and injected on a Superose 6, there were two apparent ANF−receptor complex forms eluting at 70 and 50 Å, and the 70 Å form was the major one when binding was performed in the presence of amiloride. It was concluded that amiloride promoted a high molecular mass form of the receptor interacting with itself or another receptor molecule. The increased binding observed with this agent could be explained either by increased affinity for ANF or by increased formation of receptor dimer. Because the ANF binding data alone could not discriminate between these two possibilities, we were previously unable to conclude if the high-affinity component observed by binding was associated with the 70 Å form of the receptor. Since then, the development of a ANF-R1-specific polyclonal antibody directed against an extracellular fragment of the bovine receptor enabled us to quantify ANF-R1 protein independently of its binding to ANF. The sensitivity and specificity of this antibody were high enough to enable the dosage of the receptor protein following chromatography.

Although the experiments currently reported were made under the same conditions, the 50 Å peak was highly variable and not always present. It was not observed in the presence of C-ANF, an ANF-R2-specific ligand.

The 70 Å form is probably the homodimeric receptor complex since it was subsequently shown that the effect of amiloride was maintained on the purified receptor (Meloche et al., 1988). The existence of the high molecular mass form of the receptor observed here even in absence of amiloride and ANF is compatible with recent evidence that the ANF-R1 receptor is present in the membrane as a multimeric form prior to ligand binding (Chinkers & Wilson, 1992; Lowe, 1992).

Another known functional modulator of the ANF-R1 receptor is ATP. It was shown that ATP could reduce the ANF binding to its receptor (De Léan, 1986) in bovine zona glomerulosa membranes. As shown in Figure 2, the intracellular part of the receptor (which contains the protein kinase homology domain) is essential for the modulation by ATP since the M_r 70 000 truncated form of the receptor lost almost all of its effect. In contrast to ATP, the effect of amiloride is still present. As the ED_{50} for the disappearance of the effect of ATP is shared by that for the first phase of decrease of the effect of amiloride (Figure 3), we can conclude that amiloride is mainly acting through the extracellular and/or transmembrane domains and that while ATP binding site is in the intracellular kinase homology domain, amiloride acts both intra- and extracellularly. This would explain why ATP inhibition was completely reversed by amiloride and why ATP could only partially reverse the effect of the former (De Léan, 1986). As oligomerization does not seem to be affected by proteolytic cleavage, it therefore supports the observations showing that the extracellular domain appears to be sufficient for oligomerization (Chinkers & Wilson, 1992), as for the growth factor receptors (Hurwitz et al., 1991).

The stoichiometric evaluation of the ANF−receptor complex by ANF binding and RIA is consistent with the presence of a dimer as it suggested that one molecule of ANF was binding to two molecules of receptor in the membrane and in the solubilized form. The existence of partially deglycosylated forms of ANF-R1 receptors in a recombinant receptor system that did not show binding activity and that were separated by SDS−PAGE was reported (Lowe & Fendly, 1992). These nonfunctional forms are minor in the zona glomerulosa membranes (M_r 116 000, Figure 4). The proportion of low glycosylation states of the receptor in expression systems can be relatively more important due to saturation of the post-translational modification process caused by receptor overexpression.

Since ANF binding to ANF-R1 receptor seems to behave somewhat like growth hormone (De Vos et al., 1992) and insulin (Sweet et al., 1987), which interact with two receptor molecules, the nature of the interaction between ANF and the receptor complex was further studied using the bifunctional analog AB-BPA-ANF which can be cross-linked to the receptor by its C- and N-terminal ends. Using this analog as a specific bifunctional cross-linking agent for the ANF-R1, we have demonstrated here that ANF effectively interacts with two receptor molecules. Photolabeling was performed in the absence of oxygen in order to minimize the production of malonedialdehyde from lipid peroxidation, a compound that acts as a nonspecific cross-linker between ANF and its receptor (Larose et al., 1990) but also between primary amines of the receptor protein presumably located outside the ANF binding site. We showed that higher molecular size complexes were present when the receptor was incubated with bifunctional AB-BPA-ANF in agreement with our hypothesis (Figure 4). The M_r 250 000 band of the high molecular mass complexes, which corresponds to the dimeric form of the receptor, was much fainter when BPA was used and would represent cross-linking achieved by the presence of traces of oxygen in the media. The larger sized complexes, while labeled by ANF, are nonspecific and are probably stabilized by a different way than interaction of ANF in the binding site.

In conclusion, our results suggest that conformational changes of the receptor would account for the two affinity forms of the receptor induced by amiloride and ATP and reported before (De Léan, 1986; Meloche et al., 1987). If

the receptor is present as a tetramer, in the membrane (Iwata et al., 1991) or in expression system as reported by other groups (Chinkers & Wilson, 1992; Lowe, 1992; Jewett et al., 1993), this would mean that two ANF molecules would be bound per tetramer, each ANF molecule forming a bridge between two receptor molecules. On the basis of steric exclusion chromatography and specific cross-linking with AB-BPA-ANF, our results suggest rather the presence of dimer as the main form of receptor aggregate. It cannot be excluded that overexpression can increase artifactually the proportion of tetrameric receptors. The presence of complexes of higher order in zona glomerulosa membranes would be formed by interactions of dimers, but ANF would not be directly implicated in these. The interactions would not take place in the binding site but rather near the membrane between the conserved cysteine residues as suggested by Hirose and colleagues (Iwata et al., 1991; Itakura et al., 1994). The dimer would therefore represent the simplest activatable form of guanylate cyclase as suggested by the spontaneous dimerization of the soluble GC domain of the rat ANF-R1 receptor (Thorpe et al., 1991).

Upon ANF binding, intracellular domains would be brought together by conformational changes in order to activate the guanylate cyclase domain. This is suggested by the fact that when guanylate cyclase domain is expressed alone, it forms an active dimer (Thorpe et al., 1991), and by the results of Chinkers and Wilson (1992) who showed that truncated receptor lacking most of the cytoplasmic domain blocked guanylate cyclase activation of the native receptor. This mechanism is reminiscent of the signal transduction mechanism of the growth factor family (Ullrich & Schlessinger, 1990). The difference resides in the fact that ANF receptor oligomerization is ligand-independent. An important observation is that both ends of ANF can interact with two different receptor molecules, as observed with the growth hormone (De Vos et al., 1992) and insulin receptor (Sweet et al., 1987). It will be of interest to know if, like the growth hormone, ANF binds sequentially to each receptor molecule and if both ends interact with the same region of the extracellular domain. In addition, the contribution of both receptor monomers interacting with ANF to the overall ligand binding specificity and pharmacological properties of the receptor remains to be assessed. Further works will be needed to study the mechanism and the role of this peculiar interaction.

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