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Atrial Natriuretic Factor R_1 Receptor from Bovine Adrenal Zona Glomerulosa: Purification, Characterization, and Modulation by Amiloride†

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ABSTRACT: The atrial natriuretic factor (ANF) R_1 receptor from bovine adrenal zona glomerulosa was solubilized with Triton X-100 and purified 13 000-fold, to apparent homogeneity, by sequential affinity chromatography on ANF–agarose and steric exclusion high-performance liquid chromatography. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and silver staining of the purified receptor preparation in the absence or presence of dithiothreitol revealed a single protein band of M_r 130 000. Affinity cross-linking of ^{125}I-ANF to the purified receptor resulted in the labeling of the M_r 130 000 band. The purified receptor bound ANF with a specific activity of 6.8 nmol/mg of protein, corresponding to a stoichiometry of 0.9 mol of ANF bound/mol of M_r 130 000 polypeptide. Starting with 500 g of adrenal zona glomerulosa tissue, we obtained more than 500 pmol of purified receptor with an overall yield of 9%. The purified receptor showed a typical ANF-R_1 pharmacological specificity similar to that of the membrane-bound receptor. The homogeneous M_r 130 000 receptor protein displayed high guanylate cyclase activity [1.4 μmol of cyclic GMP formed min^{-1} (mg of protein)$^{-1}$] which was not stimulated by ANF. This finding supports the notion that the ANF binding and the guanylate cyclase activities are intrinsic components of the same polypeptide. Finally, the purified ANF-R_1 receptor retained its sensitivity to modulation by amiloride, suggesting the presence of an allosteric binding site for amiloride on the receptor protein.

Atrial natriuretic factor is a peptide hormone secreted mainly by the atrial cardiocytes to produce a variety of biological effects, including diuresis and natriuresis, relaxation of vascular smooth muscle, and inhibition of aldosterone secretion (Cantin & Genest, 1985; Needleman et al., 1985; Atlas, 1986; Lang et al., 1987). ANF[1] exerts these effects through interaction with specific membrane receptors present in all target tissues (Napier et al., 1984; De Léan et al., 1984; Hirata et al., 1984; Schenk et al., 1985a). Affinity labeling experiments have revealed the presence of two distinct ANF binding proteins with apparent M_r of 60 000–70 000 and 120 000–140 000 (Yip et al., 1985; Misono et al., 1985; Vandlen et al., 1985; Schenk et al., 1985b; Meloche et al., 1986a). Studies in cultured endothelial cells and vascular smooth muscle cells have demonstrated a clear dissociation between ANF binding and ANF-mediated cyclic GMP accumulation (Leitman & Murad, 1986; Leitman et al., 1986; Scarborough et al., 1986). On the basis of these pharmacological and structural criteria, the existence of two subtypes of ANF receptor, designated ANF-R_1 and ANF-R_2 by

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[1] Abbreviations: ANF, atrial natriuretic factor; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; MES, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography.

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Leitman et al. (1986), has been proposed. The ANF-R_1 receptor is a nonreducible protein of M_r 130 000 that is coupled to cyclic GMP formation and has low affinity for truncated ANF analogues. This receptor is responsible for the known biological effects of the peptide. The recent findings that particulate guanylate cyclase from rat lung (Kuno et al., 1986) and rat adrenocortical carcinoma cells (Paul et al., 1987) copurify with ANF binding activity suggested that these two activities might reside in the same M_r 120 000–180 000 protein. This was further substantiated by the recent purification of an ANF receptor with very high guanylate cyclase activity from bovine adrenocortical cells (Takayanagi et al., 1987). On the other hand, the ANF-R_2 receptor is a M_r 130 000 protein composed of two disulfide-linked subunits of M_r 65 000 that is not coupled to cyclic GMP formation and binds shorter ANF analogues with high affinity. The purification of the receptor protein (Schenk et al., 1987; Shimonaka et al., 1987) and the cloning of the cDNA encoding the bovine receptor (Porter et al., 1987) have been recently reported. Although no biological effects have been attributed to the ANF-R_2 receptor, recent studies suggest that it might serve as specific storage-clearance binding sites for the hormone (Maack et al., 1987).

Previous studies from this laboratory have documented that the ANF receptor from bovine adrenal zona glomerulosa shows a typical ANF-R_1 order of potency (De Lêan et al., 1985) and exists almost exclusively as a M_r 130 000 monomeric protein (Meloche et al., 1986b). Recently, we have identified two distinct molecular forms of the M_r 130 000 receptor with different apparent affinities (Meloche et al., 1987). The proportion of these two forms can be modulated by the diuretic amiloride, which increases the high-affinity binding component and the high molecular weight form of the receptor, suggesting that the latter form might represent a ternary complex of the receptor with an amiloride-sensitive protein. However, a definitive answer to this hypothesis necessarily requires the purification and reconstitution of the receptor with putative effector components. The objective of the present studies was to develop a simple procedure for the high-yield purification of the ANF-R_1 receptor in quantities sufficient for the rigorous characterization of the structure and function of the protein. In this report, we describe the large-scale purification of the bovine adrenal ANF-R_1 receptor with stoichiometric ANF binding properties and high guanylate cyclase activity. The purified receptor is still sensitive to modulation by amiloride.

Experimental Procedures

Materials. Rat ANF-(99–126) was obtained from Bio-Mega, Laval, Canada. Rat ANF-(103–126) and rat ANF-(103–123) were from Institut Armand-Frappier, Laval, Canada. ^{125}I-ANF-(99–126) was prepared by radioiodination of ANF-(99–126) according to the solid-phase Iodo-Beads method described by Ong et al. (1987). The specific activity of the monoiodinated peptide was typically 2000 Ci/mmol. Antiserum to cyclic GMP was kindly provided by Dr. Alain Bêlanger, Centre Hospitalier de l'Université Laval, Laval, Canada. Carrier-free ^{125}INa and ^{125}I cyclic GMP were from Amersham Corp. Iodo-Beads, Triton X-100, Tween 20, and bis(sulfosuccinimidyl) suberate were from Pierce Chemical Co. Aprotinin, leupeptin, pepstatin A, PMSF, bovine serum albumin, phosphatidylcholine (type X-E), amiloride, *N*-acetyl-D-glucosamine, and alumina (type WN-3) were from Sigma. Poly(ethylene glycol) 8000 and glutaraldehyde were from J. T. Baker Chemical Co. Affi-Gel 10, SDS, and molecular weight standards for electrophoresis were from Bio-Rad. Accell QMA was from Waters. Wheat germ agglutinin–agarose was from Pharmacia P-L Biochemicals. Other biochemical reagents were from Sigma.

Preparation of ANF–Agarose. Affi-Gel 10 (20 mL) was mixed with 10 mg of ANF-(99–126) dissolved in 20 mL of 0.1 M MES (pH 6.5) and gently shaken for 5 h at 4 °C. After incubation, 2 mL of 1 M glycine (pH 8.0) was added, and the gel was allowed to react for an additional 2 h at 4 °C. The gel was then washed extensively with 0.1 M sodium acetate, 0.5 M NaCl, and 1 mM EDTA (pH 4.0) and with 0.1 M Tris-HCl, 0.5 M NaCl, and 1 mM EDTA (pH 9.0) in alternance and finally with 50 mM Tris-HCl, 100 mM NaCl, and 0.1 mM EDTA (pH 7.4). The yield of coupling was estimated by including ^{125}I-ANF-(99–126) in the reaction mixture and was found to be over 95% each time (final concentration of 0.16 μmol of ANF/mL of gel.

Preparation of Membranes. Bovine adrenal glands were obtained shortly after slaughter and placed in cold phosphate-buffered saline. A 0.5-mm layer of cortex, corresponding to the zona glomerulosa, was dissected, and membranes were prepared as previously described (Meloche et al., 1986b) with the following modifications. The adrenal zona glomerulosa was homogenized in 20 mM $NaHCO_3$, 2 mM EDTA, 10^{-4} M PMSF, 10^{-6} M aprotinin, 10^{-6} M leupeptin, and 10^{-6} M pepstatin A, and the membrane pellet was washed twice with the same buffer. Membranes were frozen in liquid nitrogen and stored at −70 °C until used.

Solubilization Procedure. All operations were carried out at 4 °C. The membrane suspension was centrifuged, and the pellet was resuspended in 50 mM Tris-HCl, 100 mM NaCl, 20% glycerol, 0.1 mM EDTA, 10^{-5} M PMSF, 10^{-7} M aprotinin, 10^{-6} M leupeptin, 10^{-6} M pepstatin A, and 1% Triton X-100 (pH 7.4) at a protein concentration of 4 mg/mL. The suspension was stirred for 60 min, and insoluble material was removed by centrifugation at 35000*g* for 60 min. The clear supernatant was used immediately for purification of the ANF receptor.

ANF–Agarose Affinity Chromatography. All purification procedures described below were performed at 4 °C. Triton X-100 solubilized membranes were loaded directly at a flow rate of 0.5 mL/min onto an ANF–agarose column (1.5 × 11 cm) protected by a precolumn of native Affi-Gel 10 (1.5 × 3 cm) and equilibrated with buffer A (50 mM Tris-HCl, 100 mM NaCl, 10% glycerol, 0.05% phosphatidylcholine, 0.1 mM EDTA, 0.1% Triton X-100, pH 7.4). After removing the precolumn, the affinity column was washed with 25 volumes of buffer B (50 mM Tris-HCl, 1 M NaCl, 10% glycerol, 0.05% phosphatidylcholine, 0.1 mM EDTA, 10^{-7} M aprotinin, 10^{-6} M leupeptin, 10^{-6} M pepstatin A, 0.1% Triton X-100, pH 7.4), followed by 25 volumes of buffer C (20 mM Hepes, 10% glycerol, 0.05% phosphatidylcholine, 0.1 mM EDTA, 10^{-7} M aprotinin, 10^{-6} M leupeptin, 10^{-6} M pepstatin A, 0.1% Triton X-100, pH 7.4) at a flow rate of 0.5 mL/min. The gel was then mixed with 10 mL of buffer D (50 mM sodium acetate, 1 M NaCl, 10% glycerol, 0.05% phosphatidylcholine, 0.1 mM EDTA, 0.1% Triton X-100, pH 5.0) and gently shaken for 10 min. The proteins were eluted by gravity with the above buffer in 10-mL fractions into tubes containing 0.75 mL of 1 M Hepes buffer (pH 8.0). Fractions were assayed for ^{125}I-ANF binding activity.

Steric Exclusion HPLC. Fractions with ANF binding activity from the affinity column were pooled and dialyzed against 5 volumes of buffer E (20 mM Hepes, 10% glycerol, 0.05% phosphatidylcholine, 0.1 mM EDTA, 0.1% Triton X-100, pH 7.4) for 24 h with one change of dialysis buffer. The dialyzed sample was applied at a flow rate of 0.25 mL/min to a 1-mL column of Accell QMA equilibrated with

buffer E containing 50 mM NaCl. The column was washed with 10 mL of the latter buffer and eluted with 3 mL of buffer F (20 mM Hepes, 0.5 M NaCl, 10% glycerol, 0.05% phosphatidylcholine, 0.1 mM EDTA, 1% Triton X-100, pH 7.4). The concentrated receptor was injected in 750-μL aliquots onto a Superose 6 (Pharmacia P-L Biochemicals) steric exclusion column (1 × 30 cm) equilibrated with buffer G (20 mM Hepes, 150 mM NaCl, 0.05% phosphatidylcholine, 0.1 mM EDTA, 0.1% Triton X-100, pH 7.4). The column was eluted with buffer G at a flow rate of 0.3 mL/min, and fractions of 0.5 mL were collected and assayed for ^{125}I-ANF binding activity.

Receptor Binding Assays. Binding assays with intact membranes were as described previously (Meloche et al., 1987). Binding to soluble receptor was assayed by a poly(ethylene glycol) precipitation technique throughout the purification procedure. Solubilized receptor was incubated with 8 pM ^{125}I-ANF-(99–126) and varying concentrations of competing ligands for 90 min at 25 °C in a total volume of 1 mL of 50 mM Tris-HCl, 0.05% phosphatidylcholine, 0.1 mM EDTA, 0.1% bovine serum albumin, and 0.01% Triton X-100 (pH 7.4). After incubation, 1 mL of 20% poly(ethylene glycol) in 50 mM Tris-HCl and 0.1 mM EDTA (pH 7.4) was added, and the tubes were left at 4 °C for 30 min. Bound ^{125}I-ANF was separated from free ligand by rapid filtration through 1% polyethylenimine-treated GF/C filters (Whatman), followed by washing with ice-cold 10 mM Tris-HCl (pH 7.4). The filters were counted for radioactivity in a LKB 1272 Clini-Gamma counter with 81% efficiency. Averages of duplicate determinations of bound ^{125}I-ANF were used for data analysis. Competition curves were analyzed by nonlinear least-squares curve fitting (De Léan et al., 1978, 1982).

Protein Determination. Protein concentrations in membrane and solubilized fractions were determined by the bicinchoninic acid method (Smith et al., 1985). After ANF affinity chromatography, protein contents were measured with a silver-binding assay (Krystal, 1987). The samples were dialyzed extensively against 0.2% SDS and supplemented with Tween 20 prior to assay. Bovine serum albumin was used as standard for each assay.

Guanylate Cyclase Assay. Guanylate cyclase activity was assayed following the procedure of Garbers and Murad (1979). Enzyme preparation (membrane or solubilized fraction) was incubated at 37 °C for 10 min in 100 μL of a reaction mixture containing 50 mM Tris-HCl, pH 7.6, 10 mM theophylline, 2 mM 3-isobutyl-1-methylxanthine, 10 mM creatine phosphate, 10 units of creatine phosphokinase, 1 mM GTP, and 4 mM $MnCl_2$. The reaction was initiated with the addition of sample and terminated by the addition of 20 μL of 120 mM EDTA, followed by immersion in boiling water for 3 min. The incubation mixture was centrifuged, and the cyclic GMP content of the supernatant was quantified by radioimmunoassay (Steiner et al., 1972; Harper & Brooker, 1975) after separation on an alumina column (White & Zenser, 1971). The recovery of cyclic GMP from alumina chromatography was 90%.

Affinity Cross-Linking Procedures. Affinity cross-linking of plasma membranes was performed as previously described (Meloche et al., 1986b) with 0.1 mM bis(sulfosuccinimidyl) suberate. Purified receptor (100 pM) was affinity labeled by incubation with 100 pM ^{125}I-ANF-(99–126) for 90 min at 25 °C in 1 mL of 50 mM Hepes, 0.05% phosphatidylcholine, 0.1 mM EDTA, and 0.01% Triton X-100, pH 7.4. After incubation, bis(sulfosuccinimidyl) suberate was added to a final concentration of 0.05 mM, and the reaction was allowed to proceed for 30 min at 4 °C. The reaction was quenched, and the samples were lyophilized to dryness and dissolved in 0.2 mL of sample buffer.

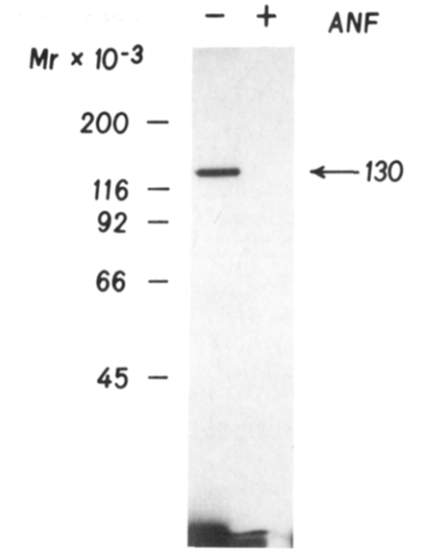

FIGURE 1: Affinity cross-linking of ^{125}I-ANF to bovine adrenal zona glomerulosa membranes. Adrenal membranes (20 μg/mL) were incubated for 90 min at 25 °C with 10 pM ^{125}I-ANF-(99–126) in the absence or in the presence of 10^{-7} M ANF-(99–126). Cross-linking was performed by incubation at 4 °C for 30 min with 0.1 mM bis(sulfosuccinimidyl) suberate. After the reaction was quenched, the washed membranes were solubilized in sample buffer containing 20 mM dithiothreitol and subjected to SDS gel electrophoresis on a 7.5% acrylamide gel. An autoradiogram of the fixed, dried gel is shown after a 3-day exposure. Molecular weight protein standards are as follows: myosin, 200 000; β-galactosidase, 116 250; phosphorylase *b*, 92 500; bovine serum albumin, 66 200; and ovalbumin, 45 000.

SDS–Polyacrylamide Gel Electrophoresis. Protein samples were solubilized in sample buffer (final concentration of 62 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.001% bromphenol blue) in the presence or absence of 20 mM dithiothreitol and heated at 100 °C for 3 min. Electrophoresis was carried out on slab gels by using the discontinuous buffer system of Laemmli (1970) with a 7.5% acrylamide separating gel. Following electrophoresis, the gels were stained with silver nitrate according to the method of Heukeshoven and Dernick (1985). When iodinated samples were processed, the gels were fixed, stained with Coomassie Blue R-250, and dried prior to autoradiography on Kodak X-Omat RP film at −70 °C with intensifying screens (Du Pont).

Results

Membrane Preparation and Solubilization. Bovine adrenal zona glomerulosa was chosen as a source of ANF receptor for several reasons. As shown in Figure 1, affinity cross-linking of ^{125}I-ANF to zona glomerulosa plasma membranes revealed a single-labeled band of apparent M_r 130 000, corresponding to the R_1 subtype of ANF receptors. The order of potency of ANF analogues in competing for ^{125}I-ANF binding to these membranes was typical of ANF-R_1 pharmacology (Figure 5A). Furthermore, this tissue possesses a relatively high concentration of ANF-R_1 receptor. Starting with 500 g of bovine adrenal zona glomerulosa, we obtained membrane preparations that contained 5.5 nmol of receptors with a specific activity in the range of 500 fmol of ^{125}I-ANF binding/mg of protein.

The zona glomerulosa membranes were solubilized with purified Triton X-100. Under the conditions used, 105% of the binding activity and 77% of membrane proteins were released in a soluble form with a 1.4-fold increase in specific

Table I: Purification of the ANF Receptor from Bovine Adrenal Cortex[a]

step	protein (mg)	act.[b] (pmol)	sp act. (pmol/mg)	yield (%)	x-fold purifcn
membranes	10700	5550	0.52	100	1
Triton X-100 extract	8203	5834	0.71	105	1.4
ANF–agarose	0.894[c]	1149	1285	21	2476
Superose 6	0.074[c]	504	6844	9	13187

[a] The ANF receptor was purified from 500 g of bovine adrenal zona glomerulosa. All the purification steps were performed as described under Experimental Procedures. The results are from one preparation, which is representative of three experiments. [b] As measured by ^{125}I-ANF-(99–126) binding. [c] Determined by silver-binding assay after dialysis against 0.2% SDS (Krystal, 1987).

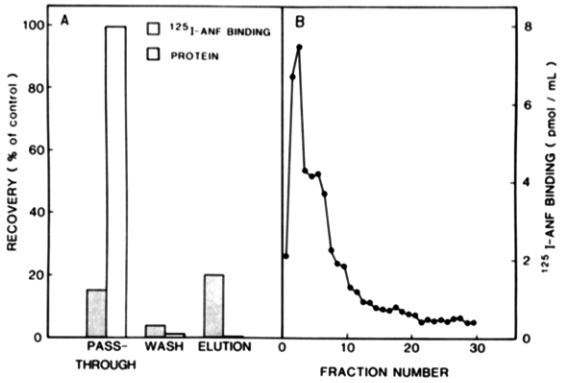

FIGURE 2: ANF–agarose chromatography of Triton X-100 solubilized bovine adrenal cortex ANF-R_1 receptor. Solubilized membranes (2800 mL containing 5.8 nmol of ^{125}I-ANF binding activity) were applied to a 20-mL column of ANF–agarose protected by a precolumn of native gel and equilibrated with buffer A. The affinity column was then washed at high ionic strength with buffer B, washed at low ionic strength with buffer C, and eluted at pH 5.0 with buffer D as described under Experimental Procedures. Fractions of 10 mL were collected and assayed for ^{125}I-ANF binding activity. (A) Recovery of ^{125}I-ANF binding activity and proteins at the various steps of the chromatography expressed as a function of the total amount applied to the column. (B) Elution profile of ^{125}I-ANF binding activity from the ANF–agarose column.

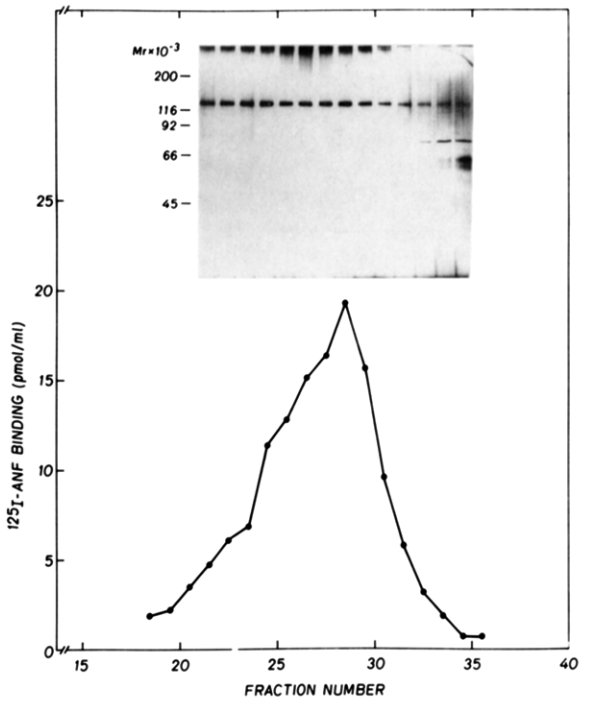

FIGURE 3: Steric exclusion HPLC of affinity-purified bovine adrenal cortex ANF-R_1 receptor. Receptor-containing fractions (340 mL with 1.1 nmol of ^{125}I-ANF binding activity) from ANF–agarose chromatography were pooled, dialyzed, and concentrated to 3 mL by anion-exchange chromatography as described under Experimental Procedures. The concentrated receptor was then chromatographed in 750-μL aliquots on a Superose 6 steric exclusion column equilibrated and eluted with buffer G. Fractions of 0.5 mL were collected and assayed for ^{125}I-ANF binding activity. The elution profile of one chromatographic run is shown. Additionally, 30-μL aliquots of the active fractions were subjected to SDS gel electrophoresis on a 7.5% acrylamide gel and silver stained. The resulting gel (top inset) is shown for the corresponding fractions.

activity (see Table I). The soluble ANF-R_1 receptor showed the same binding properties as the native membrane receptor.

Purification of the ANF-R_1 Receptor. The ANF-R_1 receptor was purified from the Triton X-100 extract by ANF affinity chromatography and steric exclusion HPLC. When the solubilized membranes were applied to the ANF–agarose column, more than 99% of the proteins passed through while 85% of ANF binding activity bound to the gel (Figure 2A). Extensive washing of the column at high ionic strength followed by washing at low ionic strength resulted in very little loss of activity (typically <5%). The receptor was eluted nonspecifically by lowering the pH to 5.0. More than 75% of the eluted binding activity was recovered in the first five column volumes (Figure 2B). This affinity chromatography step resulted in a 1800-fold purification over the Triton X-100 extract, with a typical yield of 20%. We also evaluated several other elution conditions, including alkaline pH (pH 10.0), reducing agents (0.1 M dithiothreitol), high-salt buffers (4 M NaCl, 4 M $MgCl_2$), chaotropic agents (4 M urea, 2 M NaSCN), organic solvents (20% ethylene glycol), 100 mM EDTA, and high concentrations of ionic detergents. Only two conditions, acidic pH (4.5–5.5) and 3% sodium cholate, were successful in eluting ANF binding activity (data not shown). Elution at pH 5.0 gave optimal recovery.

Final purification to homogeneity of the ANF-R_1 receptor was achieved by steric exclusion HPLC. Prior to this step, the pooled fractions from the ANF–agarose column were dialyzed to lower the NaCl concentration and applied to a small column of Accell QMA. The receptor was eluted at high ionic strength in the presence of 1% Triton X-100 with an overall yield of 80%. This step did not increase the specific activity of the preparation but was very effective in concentrating the receptor. Figure 3 shows a typical elution profile of ANF binding activity from a Superose 6 steric exclusion column. The receptor emerged as a symmetrical peak containing about 55% of the applied binding activity. This step resulted in a 5-fold purification over the pooled ANF–agarose fractions. The silver staining pattern of the peak receptor fractions revealed the presence of a single M_r 130000 protein (Figure 3, top inset).

The results obtained from a typical ANF-R_1 receptor purification are summarized in Table I. The receptor was purified 13000-fold with an overall yield of 9% of the initial ANF binding activity in intact membranes. Starting with 500 g of adrenal zona glomerulosa, we obtained 500 pmol of pure ANF-R_1 receptor.

Purity, Structure, and Identity of the Receptor Protein. The purified receptor was subjected to SDS–polyacrylamide gel electrophoresis in the absence or presence of dithiothreitol. Silver staining of the gel obtained under reducing conditions revealed the presence of a single band of M_r 130000 (Figure

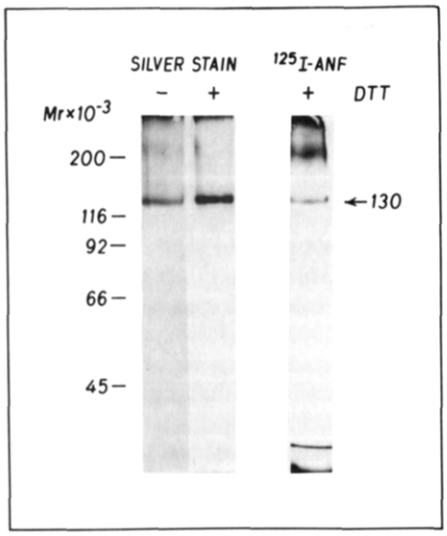

FIGURE 4: Silver staining and affinity labeling of the purified ANR-R_1 receptor following SDS–polyacrylamide gel electrophoresis. Aliquots (250 ng of protein) of the pooled fractions from steric exclusion HPLC were solubilized in sample buffer in the absence or presence of 20 mM dithiothreitol and subjected to SDS gel electrophoresis on a 7.5% acrylamide gel. Following electrophoresis, the gel was silver-stained by the method of Heukeshoven and Dernick (1985). Purified receptor (100 fmol) was incubated with ^{125}I-ANF, cross-linked with bis(sulfosuccinimidyl) suberate, and electrophoresed on a 7.5% acrylamide gel as described under Experimental Procedures. The resulting autoradiogram of the fixed, dried gel is shown after a 2-day exposure.

Table II: Affinities of Membrane-Bound and Purified Bovine Adrenal Cortex ANF-R_1 Receptor for ANF Analogues[a]

analogue	K_d (M) membrane bound	purified
ANF-(99–126)	2.2×10^{-11}	1.2×10^{-11}
ANF-(103–126)	2.0×10^{-9}	1.1×10^{-9}
ANF-(103–123)	9.0×10^{-7}	3.4×10^{-7}

[a] For each ANF analogue, competition curves were performed as described in the legend to Figure 5. K_d values were calculated by nonlinear least-squares regression analysis of binding data according to the mass action law (De Lêan et al., 1982). The values indicated are from a single experiment with triplicate determinations of each point and are representative of three different preparations.

4). The mobility of the band was not affected, or even slightly decreased, when the sample was pretreated with 20 mM dithiothreitol. An amount of 250 ng of purified receptor was applied to the gel for silver staining, and the limit of sensitivity of the method was below 10 ng of protein, so that a single contaminant of less than 5% of total protein could be detected.

To demonstrate that the purified M_r 130 000 protein contains the ANF binding site, an aliquot of the purified preparation was incubated with ^{125}I-ANF, cross-linked with bis(sulfosuccinimidyl) suberate, and analyzed by SDS gel electrophoresis. Autoradiography of the gel showed a major labeled band at M_r 130 000 coincident with the silver-stained protein band (Figure 4). These results indicate that the purified ANF-R_1 receptor from adrenal zona glomerulosa consists of a M_r 130 000 polypeptide chain.

Binding Characteristics and Enzymatic Activity of the Purified ANF-R_1 Receptor. The binding of ANF to the purified receptor was assessed by a poly(ethylene glycol) precipitation technique. Computer analysis of binding data indicated that the purified receptor bound ^{125}I-ANF in a saturable manner with a dissociation constant (K_d) of 16 pM and a binding capacity of 6.8 nmol/mg of protein. This K_d value is in good agreement with the K_d of 23 pM for ^{125}I-ANF binding to adrenal zona glomerulosa membranes. The specificity of the receptor was investigated by competition binding studies with various ANF analogues. The purified receptor showed a typical ANF-R_1 pharmacological specificity similar to that of the membrane-bound receptor (Figure 5). Table II summarizes the dissociation constants of ANF analogues for the membrane-bound and purified receptor. These results indicate that the binding characteristics of the receptor are maintained throughout the purification procedure.

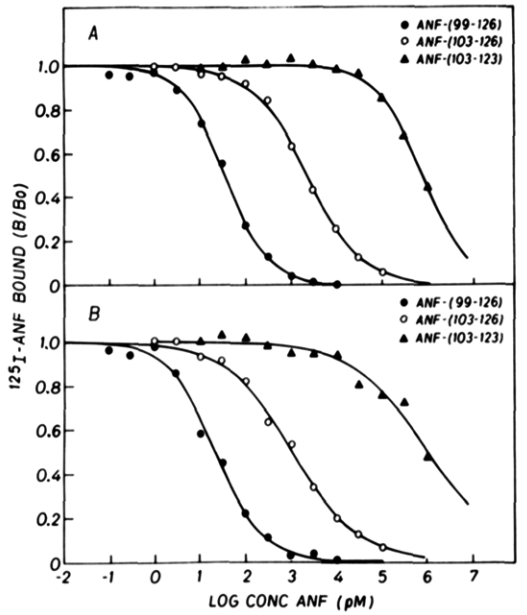

FIGURE 5: Competition curves of ANF analogues for the binding of ^{125}I-ANF-(99–126) to crude adrenal zona glomerulosa membranes (A) and purified receptor (B). Adrenal zona glomerulosa membranes (21 μg/mL) or purified receptor (1.2 ng of protein) was incubated at 25 °C for 90 min with 8 pM ^{125}I-ANF-(99–126) and varying concentrations of the indicated ANF analogues. Bound ^{125}I-ANF was determined by rapid filtration of GF/C filters (A) or by precipitation with 10% poly(ethylene glycol) followed by filtration on GF/C filters (B). The curves were obtained from a single representative experiment with triplicate determinations of each point. The curves were analyzed simultaneously by computer with a four-parameter logistic equation (De Lêan et al., 1978) and with a model for several independent classes of binding sites according to the mass action law (De Lêan et al., 1982). The solid line represents the computerized least-squares fit of the data.

The guanylate cyclase activity of the purified receptor was evaluated by measuring the production of cyclic GMP. The enzymatic protein exhibited typical Michaelis–Menten kinetics as a function of MnGTP (data not shown). The apparent Michaelis constant (K_m) for MnGTP was 170 μM, and the V_{max} was 1.4 μmol of cyclic GMP formed min^{-1} (mg of protein)$^{-1}$. This represents a 9500-fold purification over the enzymatic activity present in bovine adrenal zona glomerulosa membranes. The purified guanylate cyclase activity was not stimulated by ANF.

Modulation of the ANF-R_1 Receptor by Amiloride. We have previously shown that amiloride enhances ^{125}I-ANF binding to bovine adrenal zona glomerulosa membranes (De Lêan, 1986; Meloche et al., 1987). The development of a purification procedure for the ANF receptor has permitted the rigorous characterization of the modulatory effect of amiloride on the receptor. Figure 6 shows that the potentiating effect of amiloride on ANF binding persists at the successive purification steps of the receptor. The binding of ^{125}I-ANF to the steric exclusion HPLC-purified receptor was increased by 46% in the presence of 100 μM amiloride. The effect was more pronounced on the membrane-bound receptor compared to that on the solubilized receptor. Figure 7 shows competition

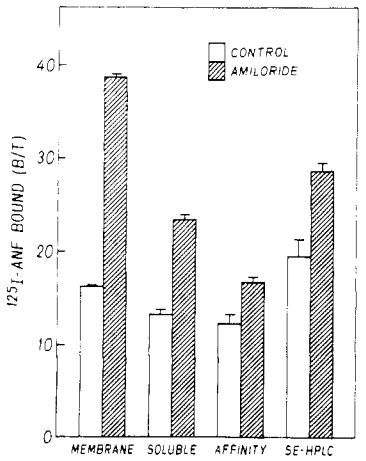

FIGURE 6: Effect of amiloride on the binding of ^{125}I-ANF to bovine adrenal cortex ANF-R_1 receptor at different stages of purification. Binding assays were performed by incubating membrane-bound or solubilized receptor at different purification steps at 25 °C for 90 min with 8 pM ^{125}I-ANF in the absence or presence or 100 μM amiloride. Bound ligand was determined as described in the legend to Figure 5. Specific ^{125}I-ANF binding was calculated from the difference between total binding and binding in the presence of 10^{-7} M ANF-(99–126). Results are expressed as the mean ± SE of triplicate determinations.

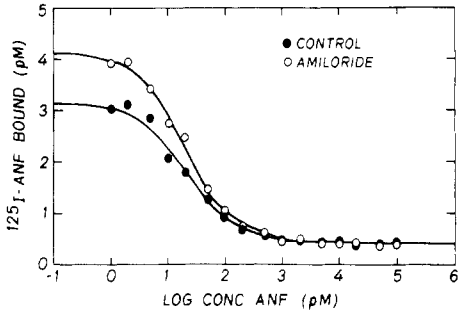

FIGURE 7: Effect of amiloride on competition of ANF-(99–126) for the binding of ^{125}I-ANF-(99–126) to purified ANF-R_1 receptor. Competition curves were performed as described in the legend to Figure 5 in the absence or presence or 100 μM amiloride. Binding data were analyzed as in Figure 5. The data are from a single experiment representative of three separate experiments.

binding curves of ANF-(99–126) in the absence and presence of 100 μM amiloride. Computer analysis of the curves revealed that amiloride decreased the K_d of ANF-(99–126) from 10.3 pM to 3.5 pM, while the density of binding sites was not changed.

Discussion

We have described a simple two-step procedure for the large-scale purification of the ANF-R_1 receptor from bovine adrenal zona glomerulosa. This tissue represents an excellent source of the ANF-R_1 receptor subtype. The receptor was first solubilized from adrenal zona glomerulosa membranes with the detergent Triton X-100. This step resulted in the recovery of 105% of ANF binding activity, probably due to the unmasking of receptor binding domains during the solubilization process. The solubilized receptor was then purified to apparent homogeneity by sequential affinity chromatography on ANF–agarose and steric exclusion HPLC. The key step in the purification scheme was the ANF affinity chromatography step. We compared a number of affinity columns prepared by coupling different concentrations of ANF, by its N-terminal or C-terminal moiety, to various agarose matrices. Optimal results were obtained by immobilizing low concentrations of ANF-(99–126) on an Affi-Gel 10 matrix. The N-terminal coupling of ANF-(99–126) resulted in higher recovery of ANF binding activity although, surprisingly, acceptable results were obtained by coupling the peptide by its C-terminal end with a water-soluble carbodiimide. It is possible that part of the coupling on carboxyl residues involves the Asp 111 residue in the ANF molecule. A low concentration (0.16 mM) of ligand immobilized on the gel facilitated the elution of the receptor from the column. The ANF–Affi-Gel 10 column gave rise to minimal nonspecific hydrophobic adsorption because of the hydrophilic spacer of the matrix and was very stable over time. We found that elution at pH 5.0 was optimal for complete and reversible dissociation of the ligand–receptor complex. A similar nonspecific elution (pH 5.0) was used for the successful purification of the ANF-R_2 receptor (Schenk et al., 1987). The anion-exchange step following affinity chromatography was essential to concentrate the receptor 25–100-fold prior to steric exclusion HPLC. The binding of the receptor to the Accell QMA column confirmed the acidic nature of the protein. The purification to homogeneity of the receptor was achieved by selectively pooling the first fractions of the ANF binding activity peak following chromatography on a Superose 6 steric exclusion column. Protease inhibitors of various specificities (aprotinin, leupeptin, pepstatin A, PMSF, EDTA) were included from the initial membrane preparation step to the final purification step to prevent proteolytic degradation of the receptor protein.

SDS–polyacrylamide gel electrophoresis of the purified receptor preparation showed a single protein band of M_r 130 000, either in the absence or presence of dithiothreitol. Affinity cross-linking with ^{125}I-ANF demonstrated that the purified M_r 130 000 protein contains the ANF binding site. The fact that the purified receptor had a molecular weight similar to that of the affinity-labeled membrane-bound receptor (Figure 1) proved that the structural integrity of the protein was maintained during the purification process. In addition to the M_r 130 000 band, we also observed a labeled band of M_r ~250 000 following affinity labeling of the purified receptor. This band likely corresponds to a receptor dimer covalently linked by the cross-linking agent, since protein staining of the same material shows a unique M_r 130 000 band (Figure 4). Consistent with this idea is the finding that the labeling of this M_r ~250 000 band is not visible in the membrane fraction until we reach millimolar concentrations of bis(sulfosuccinimidyl) suberate (data not shown). In the purified fraction, the cross-linking of receptor monomers would be favored by the large excess of cross-linking agent over the receptor protein. The purified receptor bound to wheat germ agglutinin–agarose and was specifically eluted with *N*-acetyl-D-glucosamine, indicating that it contains carbohydrate residues (data not shown). The specific activity of the purified preparation was 6.8 nmol/mg of protein on the basis of silver-binding assay. If we assume a M_r of 130 000 for the receptor protein, the stoichiometry of ANF binding would be 0.9 mol of ANF bound/mol of receptor. This further confirms that the receptor isolated by the described procedure is homogeneous.

The pharmacological properties of the purified receptor were very similar to those found in membrane preparations from bovine adrenal zona glomerulosa. The purified receptor showed a high affinity for ANF-(99–126) and bound ANF analogues with the appropriate ANF-R_1 order of potency. This indicates that the functional integrity of the receptor protein was maintained throughout the procedure.

While this work was in progress, Takayanagi et al. (1987) reported the purification of two distinct forms of ANF receptors from bovine adrenocortical cells. The two forms

showed all the characteristics of the R_1 and R_2 subtypes of ANF receptors. They used a three-step purification procedure including a GTP–agarose step to separate both forms of receptors. In the present study, we have developed a simpler purification procedure that results in higher yields of pure receptor and is suitable for the large-scale isolation of the ANF-R_1 receptor. In contrast with Takayanagi et al., we did not observe significant amounts of the ANF-R_2 receptor in adrenal zona glomerulosa on the basis of results from affinity labeling experiments (Figure 1) and competition binding studies (Figure 5). One possible explanation for this discrepancy could be related to differences in the procedures employed for membrane preparation. In our study, we carefully dissected the zona glomerulosa layer using a scalpel, while Takayanagi et al. used a microtome to cut the outermost slices of the cortex.

The purified M_r 130 000 receptor protein displayed high guanylate cyclase activity with a specific activity of 1.4 μmol of cyclic GMP formed min^{-1} (mg of protein).$^{-1}$ The homogeneity of the purified protein as determined by the presence of a single silver-stained band and by the stoichiometry of ANF binding clearly supports the notion that the binding and enzymatic activities coexist in a single transmembrane protein. This confirms the results of Takayanagi et al. (1987), although the specific activity of the purified guanylate cyclase is somewhat lower than the value obtained by the latter group. This difference can be explained, at least in part, by the use of a pH 5.0 elution buffer, which was shown to cause a significant inactivation of guanylate cyclase during affinity chromatography (Takayanagi et al., 1987). The particulate guanylate cyclase lost its response to ANF during the purification procedure, which is in agreement with previous observations (Kuno et al., 1986; Paul et al., 1987; Takayanagi et al., 1987). The presence of two activities on a single polypeptide chain for the ANF-R_1 receptor is reminiscent of the structure of the growth factor receptors that contain both a ligand binding site and a growth factor sensitive tyrosine kinase activity (Carpenter, 1987). However, the role of the guanylate cyclase activity in the ANF-mediated inhibition of aldosterone secretion is still obscure.

The present studies demonstrate that the purified ANF-R_1 receptor retains its sensitivity to modulation by amiloride. Amiloride increased the receptor affinity for ANF-(99–126) by a factor of 3, without significant changes in the density of binding sites. These observations indicate that amiloride binds to the ANF-R_1 receptor on a site different from the ligand-binding site, presumably an allosteric site. This is the first direct demonstration of a positive modulatory effect of amiloride on a receptor protein. It has been recently demonstrated by Nunnari et al. (1987) that amiloride analogues accelerate the rate of [^{3}H]yohimbine dissociation from purified α_2-adrenergic receptor, suggesting that these agents bind to an allosteric site on the receptor protein. Further studies will be required to elucidate the role of this allosteric site on the ANF-R_1 receptor and to investigate the possible relationships between receptors that are positively or negatively modulated by amiloride analogues.

In summary, we have described the complete purification of the ANF-R_1 receptor from bovine adrenal zona glomerulosa using ANF affinity chromatography and steric exclusion HPLC. The isolation of substantial amounts of purified receptor will facilitate the structural and functional characterization of the receptor protein.

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Registry No. ANF, 85637-73-6; MnGTP, 56444-94-1; amiloride, 2609-46-3; guanylate cyclase, 9054-75-5.

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Fourier Transform Infrared Study of Fully Hydrated Dimyristoylphosphatidylglycerol. Effects of Na^+ on the *sn*-1′ and *sn*-3′ Headgroup Stereoisomers

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ABSTRACT: Molecular packing and the thermotropic phase behavior of fully hydrated ammonium salts of 1,2-dimyristoyl-*sn*-glycero-3-phosphatidyl-*sn*-1′-glycerol (1′-DMPG) and the corresponding 3′ stereoisomer (3′-DMPG) as well as the effects of 300 mM NaCl on these lipids were studied by Fourier transform infrared (FTIR) spectroscopy. The ammonium salts of both stereoisomers show similar thermotropic phase behavior and have an order–disorder phase transition at approximately 21 °C. While complexing with Na^+, however, an incubation of liposomes at +6 °C for 3 days results in significant structural differences between liposomes of 1′-DMPG and 3′-DMPG. In the presence of 300 mM NaCl the infrared spectra for 3′-DMPG reveal the appearance of a more solidified lipid nominated here as the highly crystalline phase with a transition into the liquid-crystalline state at a significantly higher temperature (approximately at 33 °C) than that for 1′-DMPG (approximately at 23 °C). Crystal field splitting resulting from interchain vibrational coupling is observed in the CH_2 scissoring mode of the 3′-DMPG(Na^+) complex in the highly crystalline phase (T < 33 °C); i.e., the acyl chains are packed in a rigid orthorhombic- or monoclinic-like crystal lattice. At temperatures above the transition at 33 °C the acyl chains of 3′-DMPG(Na^+) give rise to infrared spectra indicative of hexagonal packing. The latter type of hydrocarbon chain packing is also found for the ammonium salts of 1′-DMPG and 3′-DMPG without Na^+ as well as for 1′-DMPG with Na^+. In addition, the binding of Na^+ to 3′-DMPG causes narrowing of the bands associated with the interfacial and polar headgroup regions of 3′-DMPG and thus reveals reduced motional freedom. This demonstrates that Na^+ binds tightly to 3′-DMPG, leading to the immobilization of the entire phospholipid polar headgroup. Such effects by Na^+ are not observed for 1′-DMPG.

Interactions of metal cations with membrane phospholipids have recently been the focus of increasing interest. This is due to observations that in membranes containing negatively charged phospholipids changes in membrane morphology (Verkleij et al., 1984), fluidity (Träuble et al., 1976), and phospholipid phase transitions (Watts et al., 1978; Jähnig, 1976; Träuble, 1977) can be triggered isothermally by changing the ionic strength, pH, or membrane potential (Kinnunen & Virtanen, 1986). Thus, the lipid–ion interactions are likely to play an important role in the regulation of the structure and function of biological membranes.

Characteristically lipid–cation interactions induce tighter packing of the acyl chains and stabilization of the bilayer, resulting in increased phase transition temperature and enthalpy (Amey & Chapman, 1983). Ca^{2+} in particular has long been recognized as a fusion-promoting agent. Its mode of interaction is based on its complexing to the headgroup moieties of membrane lipids. FTIR[1] spectroscopy has been

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