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ASSOCIATION OF THE ATRIAL NATRIURETIC FACTOR RECEPTOR WITH GUANYLATE CYCLASE IN SOLUBILIZED RAT GLOMERULAR MEMBRANES

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SUMMARY: The elution profile of solubilized rat glomerular membranes from a gel filtration column showed two peaks of ^{125}I-ANF (atrial natriuretic factor) binding (367±21, 156±12 KDa). Over 85% of the total binding for the extract was in the 367 KDa peak. Guanylate cyclase activity was correlated with ^{125}I-ANF specific binding. ANF activation of guanylate cyclase was also observed. As observed previously with particulate membrane, Scatchard-analysis of ANF binding data with the solubilized extract was consistent with a two-site model. Both affinities (Kd's), 4 pM and 1 nM, are within the range of blood concentrations reported for ANF. These observations suggest that most rat glomerular ANF receptors are large molecular complexes coupled with guanylate cyclase in the 300-350 KDa size range.

It has been suggested that the cyclic GMP increases reflect the biologic activity of atrial natriuretic factor (ANF) (1), and that the vasodilator effect of ANF is mediated via increased tissue levels of cyclic GMP (2,3). Furthermore, it has been demonstrated that the activation of guanylate cyclase by ANF is important as an early step of physiological effects of ANF (4-6). Recently, Kuno et al (7) and Paul et al (8) demonstrated that ANF receptor and particulate guanylate cyclase were co-purified. However, the co-purified ANF receptor guanylate cyclase complexes with a molecular mass of 120 KDa (7) and 180 KDa (8) did not retain their ANF activation of their guanylate cyclase activities. Thus, the relationship between ANF binding, guanylate cyclase activity and molecular size requires additional study. Data in the present report suggest that major protein bands (60-70 KDa and 120-140 KDa) demonstrated by affinity labelling procedures from ANF responsive tissues (9-13) represent the molecular weight of ANF receptor subunits.

MATERIALS AND METHODS

Materials: Synthetic rat atrial natriuretic factor (ANF) (rANF III) was obtained from Peninsula Laboratories, Inc.; iodinated ANF(3-[^{125}I]-iodotyrosyl^{28} rat cardionatrin, ^{125}I-rANF) (2000Ci/mmol) was obtained from Amersham. Radioimmunoassay kit for

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cyclic GMP was obtained from Specialty Diagnostics. The gel filtration calibration kits used to determine gel-filtration elution positions were purchased from Pharmacia Fine Chemicals.

Protein concentrations were determined with bicinchoninic acid using a bovine serum albumin (BSA) standard.

Glomerular isolation and membrane separation: The glomerular isolation from male Sprague-Dawley rats and the preparation of glomerular membranes including partially purified ANF receptors were as described previously (14). Rat renal cortices were dissected and the glomeruli were isolated from decapitated male Sprague-Dawley rats (300-350 g) by sieving (250, 150 and 88 μm, respectively) (15) and were stored at -70°C. In order to obtain a partial purification of ANF receptors, glomerular membranes were separated on sucrose gradients (16). The glomeruli were suspended in 30 ml of 50 mM Tris-HCl (pH 7.2), 1 μM aprotinin, 0.1% bacitracin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 5 mM $MgCl_2$ and disrupted in a Parr cell-disrupted bomb. The membrane pellet was suspended in 11 ml of 48% sucrose with a glass tissue homogenizer and 5.5 ml overlayed in two centrifuge tubes with 41%, 37%, 31% and 8.5% aliquots of sucrose in 5 ml, 11 ml, 11 ml and 5 ml volumes, respectively. The gradients were centrifuged at 65,000 x g for 19 hours. After equilibrium floatation on a sucrose gradient, crude membrane protein was distributed in five fractions (F1 to F5, top down). As ANF receptors were concentrated in F1 and F2 of 5 fractions, F1 and F2 fractions were mixed together and diluted with 50 mM Tris-HCl (pH 7.2) and stored at -70°C. This fraction was used for all experiments in this communication.

Solubilization of glomerular membrane ANF receptors: Solubilization conditions were those reported by Carrier et al (17). The pellet of the membrane preparation was resuspended at 6-8 mg/ml in 50 mM Tris-HCl buffer (pH 7.2) containing 10 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS) or 1% Triton x-100, 1 μM aprotinin, 0.5 mM PMSF, 5 mM $MgCl_2$ and incubated for 60 min at 4°C with constant stirring. The material was centrifuged at 50,000 x g for 30 min, and the supernatant was utilized for subsequent experiments.

Gel filtration: The solubilized preparation (1.2-3.2 mg) was subjected to gel filtration at 4°C on a column (1.6 x 100 cm) of Sephacryl S-200 Superfine that was eluted with 50 mM Tris-HCl (pH 7.2) containing 1 μM aprotinin, 5 mM $MgCl_2$, 0.5 mM PMSF, 0.12 M NaCl and 1% Triton x-100. Fractions of 0.345 ml were collected as a flow rate of 0.23 ml/min and assayed for ^{125}I-rANF binding and guanylate cyclase activities. The elution positions of marker proteins were monitored by their absorbance at 280 nm.

ANF binding assay: ^{125}I-rANF receptor-binding conditions in solubilized membrane were those reported by Carrier et al (17). Five to thirty μg portions of solubilized membrane protein were incubated in duplicate with ^{125}I-rANF for 60 min at 22°C in the assay buffer (5 mM Chaps or 0.5% Triton X-100, 50 mM Tris-HCl, pH 7.2, 1 μM aprotinin, 0.1% bacitracin, 0.5 mM PMSF, 5 mM $MgCl_2$, 0.4% BSA) in the presence of 35,000-50,000 cpm of ^{125}I-rANF (14.6-20.8 pM). Non-specific binding was measured in the presence of 10^{-6} M rANF III. The final assay volume was 1 ml. At the end of the incubation period, 200 μl of a solution of bovine gamma-globulin (0.3%) in phosphate-buffered saline (PBS) was added, followed immediately by 2.4 ml of 25% polyethylene glycol 800 (PEG) in PBS and the incubation mixture filtered rapidly on 0.3% polyethylenimine-treated Whatman GF/C filter. The filter was washed three additional times with 3.5 ml of 16% PEG in PBS and the ^{125}I radioactivity on the filter was determined in a PACKARD gamma spectrometer with 75% efficiency. Scatchard-plots of ANF binding were analyzed by non-linear curve-fitting using the LIGAND computer program (18).

Guanylate cyclase activity: Guanylate cyclase activity was measured according to the procedure described by Kimura and Murad (19). Two μg of protein samples were incubated in 50 mM Tris-HCl, pH 7.6, 10 mM theophylline, 0.1 mM dithiothreitol, 15 mM creatine phosphate, 40 μg of creatine phosphokinase, 2 mM $MnCl_2$ and 1 mM GTP in a final volume of 0.2 ml with and without 10^{-7} M rANF III for 15 min at 37°C. The reaction was terminated by the addition of 0.8 ml of 50 mM sodium acetate, pH 4.0, followed by immersion in boiling water for 3 min. Generated cyclic GMP was measured by radioimmunoassay using cyclic GMP assay kit.

RESULTS

The solubilized rat glomerular membrane proteins separated by gel filtration showed two peaks of ^{125}I-ANF binding as shown in figure 1. The molecular sizes of the two peaks were 367±21 KDa (A) and 156±12 KDa (B) (means±SD, n=7). ^{125}I-ANF binding was never observed at a molecular weight (M.W.) less than 100 KDa. There was essentially no difference in this pattern when membranes were solubilized by CHAPS or Triton X-100. The ratio of peak B to peak A was less than 15%.

The eluted fractions also have guanylate cyclase activity, and there was a good correlation between specific binding of ^{125}I-ANF and guanylate cyclase activity as shown in figure 2. Furthermore, the addition of ANF (10^{-7} M) to the eluted samples induced greater generation of cyclic GMP, and the percent increase of cyclic GMP in each fraction in peak A was similar, whereas in peak B, increases in guanylate cyclase activity induced by ANF were small to nonexistent (Fig. 3). Thus, guanylate cyclase activation by added ANF was correlated with the density of ANF receptor binding.

As reported previously (14) LIGAND analysis of Scatchard-plot data of ANF binding to rat glomerular particulate membranes best fit a two-site model. As seen from data in the present report, two-site binding was preserved after solubilization, though the maximum number of binding sites decreased about 40-50% (data not shown). Scatchard plots of ANF binding using the samples from peak A and B are shown in figure 4. Their dissociation constants (Kd) were 3.87 pM and 1.07 nM in peak A, and 1.16 pM and 0.47 nM in peak B, respectively, estimated by the LIGAND computer program.

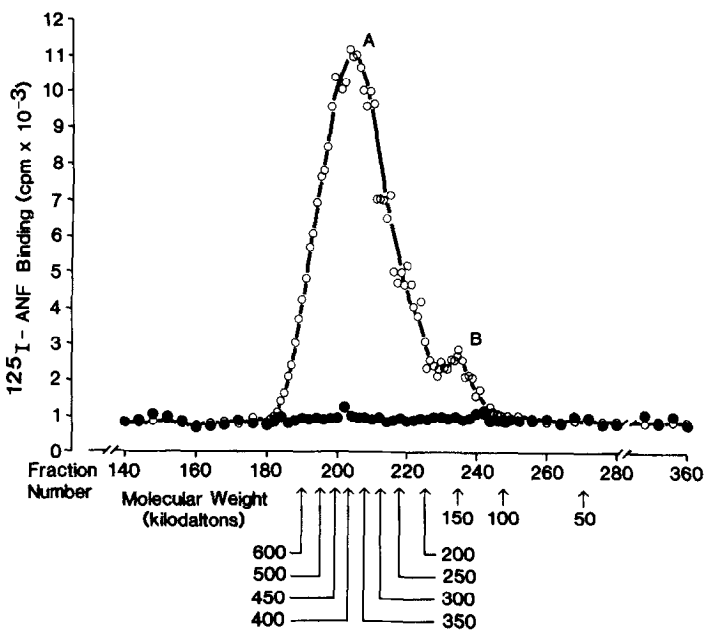

Fig. 1. Gel filtration of solubilized rat glomerular membrane proteins on a column (1.6 x 100 cm) of Sephacryl S-200 Superfine. Fractions were assayed for ^{125}I-ANF binding activity in the absence (o) and presence (•) of 10^{-6} M rANF III. Marker proteins for calibration of molecular weight were albumin (67 KDa), aldolase (158 KDa), catalase (232 KDa), ferritin (440 KDa) and thyroglobulin (669 KDa).

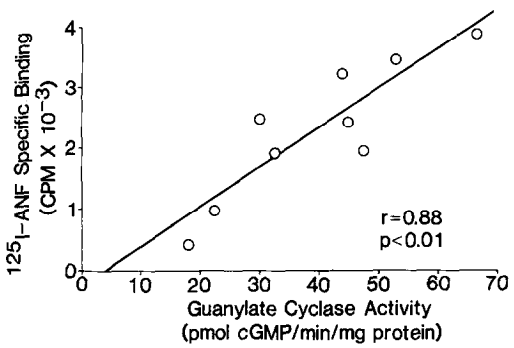

Fig. 2. Relationship between specific binding of ^{125}I-ANF and guanylate cyclase activity in eluted fractions from gel filtration columns.

DISCUSSION

In the present study, we demonstrated that solubilized protein from rat glomerular membranes separated into two peaks of ^{125}I-ANF binding by gel filtration. Moreover, there was a very good correlation between ^{125}I-ANF specific binding and guanylate cyclase activity. This finding suggests that most solubilized rat glomerular ANF receptors exist as a tightly coupled 370 KDa receptor • guanylate cyclase complex. Recently, Ishido et al (20) also demonstrated that bovine lung membrane solubilized with digitonin contained a coupled ANF receptor • guanylate cyclase complex. Kuno et al (7) and Paul et al (8) demonstrated that ANF receptor binding and particulate guanylate

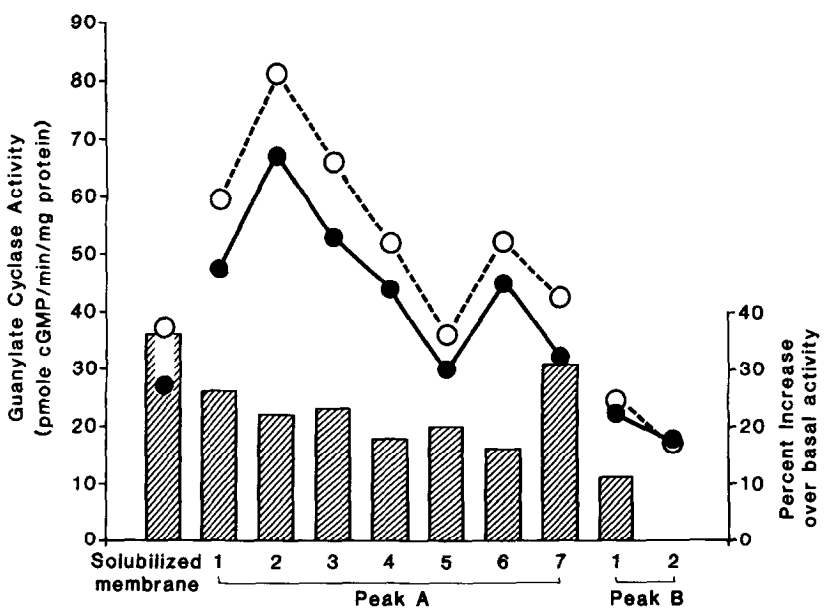

Fig. 3. Effect of rANF III (10^{-7} M) on guanylate cyclase activity. The addition of rANF III (o), enhanced guanylate cyclase activity in peak A and in non-eluted solubilized membrane (left).

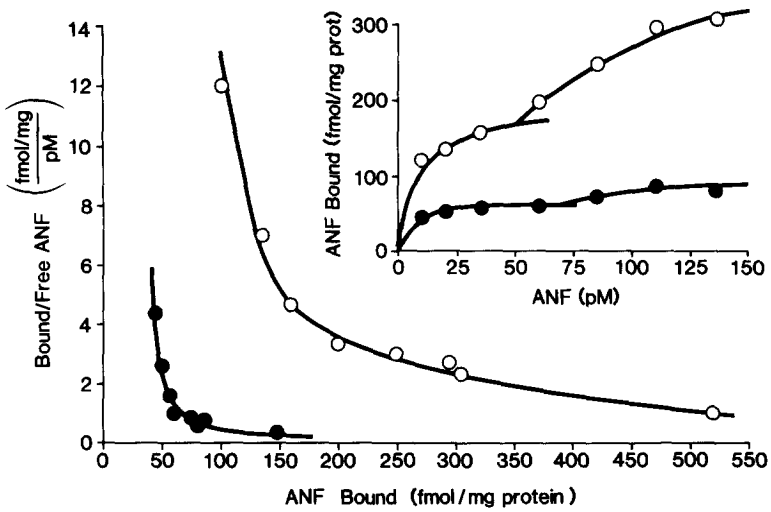

Fig. 4. Scatchard plots of ANF binding to eluted fractions of peak A (o) and peak B (•). The inset shows ^{125}I-ANF binding saturation curves to each fraction.

cyclase were co-purified from rat lung and adrenal cortical carcinoma cells, respectively. These observations indicate that their coupling is very tight. In addition, the activation of guanylate cyclase by ANF was preserved in our eluted samples. It has been suggested that the ANF activation of guanylate cyclase is important as an early step indicating the physiological effects of ANF (4-6). However, the ANF receptor guanylate cyclase complex co-purified by Kuno et al (7) (120 KDa) and by Paul et al (8) (180 KDa) did not show ANF activation of guanylate cyclase.

The present results also indicate that most solubilized ANF receptors are larger molecular complexes than those suggested by affinity labelling and electrophoresis techniques (60-70 and 120-140 KDa) (9-13). In our experiments, no ^{125}I-ANF binding was observed in the M.W. range of less than 100 KDa. This implies that the 60-70 KDa band observed by affinity labelling techniques represents the smallest binding subunit of the ANF receptor but probably does not represent a more native membrane associated form of the ANF receptor.

Separation of solubilized ANF receptors by gel filtration has been achieved in bovine adrenal cortex by Hirose et al (9), in rabbit aorta membrane by Vandlen et al (21) and in human placenta by Sen (22). However, the sizes of ANF receptor complex were not identical. Hirose et al (9) and Sen (22) reported only one peak (140 KDa to 160 KDa) which corresponds to our lower M.W. peak (B) and, to the contrary, Vandlen et al (21) reported one peak (300-350 KDa) corresponding to our higher M.W. peak (A). These differences in M.W. may be due to the differences in species, organs, detergents, or the conditions of gel filtration. Both we (rat glomerular membrane solubilized with CHAPS or Triton X-100) and Ishido et al (20) (bovine lung membrane solubilized with digitonin) observed two peaks, but the ratio of lower M.W. peak (B) to higher M.W. peak (A) was quite different. Our ratio was less than 15% whereas their ratio was over 80%. These latter authors stated that their ratio was technique dependent.

Recently, we reported ^{125}I-ANF binding studies with rat glomerular particulate membrane which suggested two types of physiologically relevant hormonal receptors (14). In the present study we demonstrated that these binding characteristics of particulate membrane were preserved in solubilized membranes. A possibility for explaining these data is that the two site binding pattern seen in LIGAND generated Scatchard plots results from negative cooperativity between sites on receptor protein subunits. These subunits, or complexes thereof (e.g., with guanylate cyclase in peak A), could be present within the A and B peaks that were eluted from gel filtration columns (23). Yet to be resolved is whether the lower M.W. form (B) of ANF receptor, as demonstrated by gel filtration, is a native form or is derived from the larger form (A) during preparation. If this lower type represents a native form, this implies that at least two qualitatively different types of ANF receptor exist, since this minor type (B) was not coupled with ANF activated guanylate cyclase (23).

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