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PURIFICATION AND CHARACTERIZATION OF TWO TYPES OF ATRIAL NATRIURETIC FACTOR RECEPTORS FROM BOVINE ADRENAL CORTEX :GUANYLATE CYCLASE-LINKED AND CYCLASE-FREE RECEPTORS

Ryoichi Takayanagi, Rudolf M. Snajdar, Teruaki Imada, Masaaki Tamura, Kailash N. Pandey, Kunio S. Misono, and Tadashi Inagami

Department of Biochemistry, Vanderbilt University, Nashville, TN 37232

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Summary Atrial natriuretic factor(ANF) receptors with and without guanylate cyclase activity were simultaneously purified to apparent homogeneity from bovine adrenal zona glomerulosa cell membrane fractions. The particulate guanylate cyclase which co-purified with the ANF receptor showed one of the highest specific activity reported. The receptors with or without the guanylate cyclase activity showed high affinities to $ANF_{(99-126)}$. The receptor without the cyclase showed a high affinity to truncated ANF analogs, $ANF_{(103-123)}$ and $ANF_{(105-121)}$, whereas the cyclase-linked receptor had a much lower affinity to these analogs. Both of the receptors migrated as a single band with a molecular weight of 135,000 daltons on SDS-gel electrophoresis under non-reducing conditions. The 135,000 daltons band of the receptor without the cyclase was shifted to a 62,000 daltons band under reducing conditions, but the band for the cyclase-linked receptor was not shifted. These results demonstrated the presence of two subtypes of ANF receptor in bovine adrenal cortex and indicate two different modes of intracellular action of ANF.

Since atrial natriuretic factor (ANF) has been shown to selectively activate particulate guanylate cyclase (1,review 2) and to increase the concentration of intracellular cyclic GMP (cGMP) (review 2,3), it has been suggested that cGMP mediates the biological effects of ANF (review 4). However,whether cGMP is an exclusive or even partial mediator of ANF action has been questioned. The angiotensin II-stimulated aldosterone secretion has never been affected by the addition of various membrane-permeable cGMP analogs (5,6), such as 8-p-chlorophenylthio-cGMP and β-phenyl-1-N^2-etheno-cGMP (7 and our unpublished data). Moreover, it has been shown that there is clear dissociation between cGMP response and ANF-induced diuresis (8) or even vasorelaxation (9). Dissociation between the binding of ANF analogs to the ANF receptor and cGMP accumulation has also been shown and different subtypes of ANF receptor have been proposed (10,11). Recently, 120,000 daltons protein

Abbreviations: ANF,atrial natriuretic factor; TX-100, Triton X-100; HEPES, N-2-hydroxyethyl-N-ethenesulfonic acid; WGA, wheat germ agglutinin; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

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of particulate guanylate cyclase has been co-purified with ANF-binding activity from rat lung (12), whereas affinity labeling experiments have suggested 60,000-70,000 daltons protein as ANF receptor (13,14,15,16).

With the objectives of clarifying the mechanism of ANF receptor functions and the signal transmission, we have purified ANF receptors from bovine adrenal cortex; two distinct types of ANF receptor with different functional properties.

MATERIALS AND METHODS

Materials: $ANF_{(99-126)}$ was obtained from Peninsula Laboratories. $ANF_{(96-126)}$, $ANF_{(102-126)}$, $ANF_{(103-123)}$ and $ANF_{(105-121)}$ were synthesized as described previously (17). The iodination of ANF was done according to Misono et al(18).

Analytical Procedures: ANF-binding activity of solubilized preparations was measured as described by Carrier et al. (19). Binding reached equilibrium in 30 min and was stable for 150 min. Guanylate cyclase activities were assayed essentially as reported by Waldman et al. (1). Photoaffinity labeling of ANF receptors by ^{125}I-4-azidobenzoyl-$ANF_{(102-126)}$ was carried out as described previously (18), except that after photolysis the samples were directly analyzed on SDS-PAGE (7.5% gel) by the method of Laemmli (20) without washing. After being silver-stained (21), the gels were subjected to autoradiography with Kodak XAR-5 film. Iodination of purified receptor was performed using IODO-GEN as reported (22). The protein concentration was determined by fluorescamine with nitrocellulose filter (23).

Purification of ANF receptor: The preparation of membranes from bovine adrenals and the solubilization of the membranes with TX-100 (Pierce Chemical) were done as described previously (18,19). TX-100 extracts were applied to Affi-Gel 10 (BIO-RAD) coupled with $ANF_{(99-126)}$ (1 mg of ANF/ml of gel) at a flow rate of 0.8 ml/min. The column was washed extensively with the solubilizing buffer containing 0 to 1 M NaCl and eluted with 50 mM acetate buffer, pH 5.5, containing 1 M NaCl, 0.5mM EDTA, 0.1% TX-100, 20% glycerol and 0.025% phosphatidylcholine (Sigma). After the eluate was dialyzed against 20 mM HEPES buffer pH 7.5 containing 20% glycerol, 0.025% phosphatidycholine and 0.1% TX-100, the dialyzate was adjusted to 3 mM $MnCl_2$ and 2mM NaN_3, and applied onto a GTP-agarose column (Sigma). After collecting the pass-through fractions, the column was washed and eluted with 5mM GTP. The pass-through fractions and the eluted fractions were separately applied to WGA-sepharose 6 MB (Pharmacia). After extensive wash, the columns were eluted with 0.4 M N-acetylglucosamine.

RESULTS

Purification of ANF receptor - Bovine adrenocortical ANF receptor solubilized with TX-100 was enriched approximately 30,000-fold, and separated into two subtypes, the receptor with and without guanylate cyclase activity(Table 1). The ANF column bound 90% of the ANF-binding activity and 23.5% of the guanylate cyclase activity of the TX-100 solubilized extract. Elution of the loaded gel with 50mM acetate buffer (pH5.5)/1 M NaCl/0.5 mM EDTA resulted in recovery of 42% of the bound receptor and 39% of the bound guanylate cyclase activity. This step attained 6,800-fold purification for ANF receptor and 1,600-fold purification for guanylate cyclase over the membrane extract. In the subsequent chromatography on a GTP column almost all the guanylate cyclase

Table 1. Purification of ANF receptors with or without the guanylate cyclase activity from bovine adrenal cortex

Step	ANF receptor			Guanylate cyclase		
	s. a.[a]	fold purification	% recovery	s. a.[b]	fold purification	% recovery
Membrane fractions	0.20	1	100	0.40	1	100
TX-100 extract	0.55	2.8	87	3.85	9.6	300
ANF-agarose	3,700	19,000	33	6,380	15,700	28

	ANF Receptor with guanylate cyclase						ANF Receptor without guanylate cyclase					
	ANF receptor			G. cyclase			ANF receptor			G. cyclase		
	s. a.[a]	fold purifi-cation	% re-covery	s.a.[b]	fold purifi-cation	% re-covery	s.a.[a]	fold purifi-cation	% re-covery	s. a.[b]	fold purifi-cation	% re-covery
GTP-agarose 5,000	25,000	9		18,000	45,000	17	4,600	23,000	15	1	2.5	0.0
WGA-sepharose 6,800	34,500	5.6		23,100	57,700	9.3	5,710	28,000	10.6	1	2.5	0.0

a. specific activity, pmol of ^{125}I-ANF$_{(99-126)}$/mg protein determined by Scatchard plots.

b. specific activity, nmol of cyclic GMP formed/min/mg protein with Mn-GTP as substrate.

activity and 49% of the ANF receptor activity in the dialysate of the eluates from the ANF column were bound to the GTP gel. The pass-through fractions, which had no guanylate cyclase activity, and the fractions eluted with 5 mM GTP were separately purified further on a WGA column. Final yields were 15.6 μg for the receptor without the cyclase activity and 6.9 μg protein for the receptor with the cyclase from 1066 g of the outer layers of the adrenal cortex. The purified preparations retained the ability to bind ^{125}I-ANF and the guanylate cyclase activity for at least 4 months when stored at -70°C.

Characteristics of the purified ANF receptor and particulate guanylate cyclase - The Scatchard analysis of ^{125}I-ANF$_{(99-126)}$ binding to purified preparations indicated that there is a single class of saturable binding sites with high affinity. The dissociation constant (Kd) values were 68 and 56 pM and Bmax values were 6.8 and 5.7 nmol/mg protein for the cyclase-contanining and -deficient receptors, respectively. The purified particulate guanylate cyclase exhibited typical Michaelis-Menten kinetics with linear Lineweaver-Burk plots as a function of Mn-GTP, and the apparent Michaelis constant for Mn-GTP was 137μM. Competition binding analyses of the purified preparations were performed using varying concentrations of unlabeled ANF peptides against a fixed concentration of ^{125}I-ANF$_{(99-126)}$. Both of the receptors with and without the cyclase showed similar high affinities to ANF$_{(99-126)}$, ANF$_{(96-126)}$, and ANF$_{(102-126)}$. The cyclase-deficient receptor showed a high affinity to truncated ANF analogs, ANF$_{(103-123)}$ and

TABLE 2

Comparison of inhibition constant, Ki's of atrial natriuretic factor analogs for receptors with and without guanylate cyclase purified from bovine adrenal cortex

Peptide	Ki (pM)	
	ANF Receptor without guanylate cyclase	ANF Receptor with guanylate cyclase
ANF(99-126)	53	68
ANF(96-126)	40	45
ANF(102-126)	118	110
ANF(103-123)	150	1.55×10^6
ANF(105-121)	220	10^7

The Ki values were calculated according to Cheng and Prusoff (25).

$ANF_{(105-121)}$, whereas the cyclase-containing receptor had much lower affinity to those ANF fragments (Table 2).

Photoaffinity labeling and SDS-PAGE of receptors - Analyses of the purified receptor labeled with ^{125}I on SDS-PAGE under non-reducing conditions revealed a single band of 135,000 daltons for both receptors with and without the cyclase activity (Fig. 1, lane 1 and 2). Under reducing conditions the 135,000 dalton band of the cyclase-deficient receptor was shifted to a 62,000 daltons band (Fig. 1, lane 4), but the cyclase-containing receptor was not shifted(Fig. 1, lane 3). When the purified preparations were incubated with 4-azidobenzoyl-^{125}I-$ANF_{(102-126)}$, photolyzed and analyzed on SDS-PAGE under reducing conditions, the radioactive bands corresponding to those of the purified receptors were detected(Fig. 2, lane a and d). Inclusion of $10^{-8}M$ $ANF_{(99-126)}$ in the incubation abolished the labeling of the bands of both types of the receptors (Fig. 2, lane b and e), whereas the inclusion of $10^{-8}M$ $ANF_{(105-121)}$ in the incubation prevented the labeling the cyclase-deficient receptor only(Fig. 2, lane f), but the photo-labeling of the band of the cycylase-containing receptor was not displaced by this truncated ANF analog (Fig. 2, lane c). Essentially similar results were obtained with another short fragment, $ANF_{(103-123)}$.

DISCUSSION

Two types of ANF receptor have been purified to apparent homogeneity with high functional activities from bovine adrenal outermost cortex. One had the guanylate cyclase activity, but practically no affinity to truncated ANF analogs, and migrated to the position of 135,000 daltons on SDS-PAGE under reduced or non-reduced conditions. The other type had no cyclase activity,

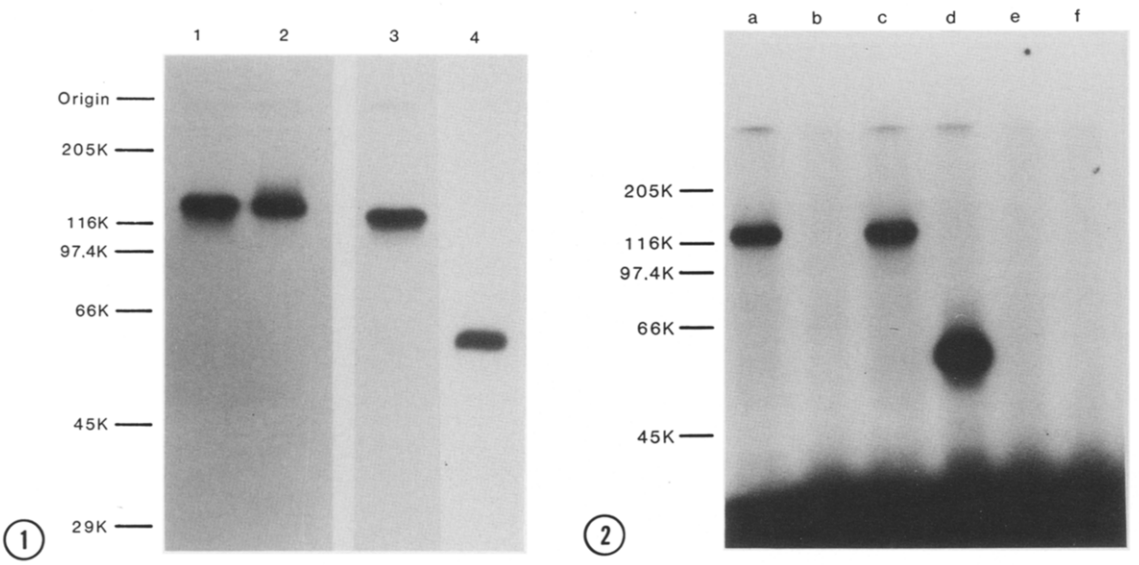

Fig. 1. Analysis of the purified bovine adrenal receptors by SDS-PAGE in 7.5% gels. Purified guanylate cyclase-containing receptor (lane 1 and 3) and -deficient receptor (lane 2 and 4) were ^{125}I-labeled and analyzed under non-reducing (lane 1 and 2) and reducing (lane 3 and 4) conditions (100mM dithiothreitol).

Fig. 2. Photoaffinity labeling of the guanylate cyclase-containing ANF receptor (lane a,b and c) and -deficient ANF receptor (lane d,e and f) purified from bovine adrenals. The ANF receptors were labeled by photolysis with 4-azidobenzoyl ^{125}I-$ANF_{(102-126)}$ in the absence (lane a and d) or presence of 10^{-8}M $ANF_{(99-126)}$ (lane b and e) or 10^{-8}M $ANF_{(105-121)}$ (lane c and f), and analyzed by SDS-PAGE in 7.5% gel under reducing conditions.

but high affinities to the ANF fragments, and migrated to the position of 62,000 daltons upon SDS-PAGE under reducing conditions. Both types of the receptors showed high affinities to longer ANF's, $ANF_{(99-126)}$, $ANF_{(96-126)}$ and $ANF_{(102-126)}$. These characteristics of the two receptors are consistent with recent findings that in intact cultured cells of bovine origin, ANF analogs lacking the C-terminal Phe-Arg-Tyr sequence cannot induce the accumulation of intracellular cGMP but bind to the receptor with a affinity equal to that of the full length $ANF_{(99-126)}$ (10,11). Moreover, one of those ANF fragments, $ANF_{(103-123)}$, has been found to bind much more efficiently to the receptor protein of 66,000 daltons than to that of 130,000 daltons as determined by the radioligand binding followed by affinity cross-linking to the receptor and the analysis on SDS-PAGE(11). We also observed the difference in the affinity of the truncated ANF analogs to the two types of ANF receptor proteins in isolated membranes before solubilizing receptors (to be published). These observations together with the present results suggest that the two types of receptors isolated by the affinity chromatography are present in tissues

rather than being products of artifacts such as proteolysis. The co-purification of the 135,000 daltons ANF receptor activity and the guanylate cyclase activity indicates that the 135,000 dalton receptor molecule is a double functional protein containing guanylate cyclase as an integral part of the receptor molecule analogous to rat lung guanylate cyclase which exhibited an ANF binding activity as shown by Kuno et al (12). The possibility that the guanylate cyclase was a contaminated in the 130,000 dalton receptor can be eliminated by the calculated purity of 92% of the receptor based on the maximal binding capacity to the ligand, estimated molecular weight and protein concentration. Furthermore the fact that the present guanylate cyclase preparation exhibits the highest specific acitity (23.1 μmoles cGMP formed/min/mg protein) ever attained in comparison with 19 μmoles/min/mg protein of the rat lung preparation (12) and 15.2 μmoles/min/mg protein of the sea urchin spermatozoa(24) also strongly indicates that the cyclase is not a minor contaminant. Approximately 25% of the total guanylate cyclase solubilized by TX-100 bound to ANF-agarose. It is likely that various other guanylate cyclases not containing ANF receptor structure exists. The direct evidence that ANF receptor proteins exist in the guanylate cyclase-linked form and the cyclase-deficient form obtained in the present studies raises the possibility that ANF may exert its effects at least by two different pathways involving or not involving cGMP.

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