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Topographical Characterization of the Domain Structure of the Bovine Adrenal Atrial Natriuretic Factor R_1 Receptor†

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Received January 19, 1989; Revised Manuscript Received March 10, 1989

ABSTRACT: We have studied the structure and function of the membrane atrial natriuretic factor R_1 (ANF-R_1) receptor using limited proteolysis and exoglycosidase treatment. Limited digestion with trypsin of the receptor from bovine adrenal zona glomerulosa membranes resulted in the conversion of the native 130-kDa receptor into a single membrane-associated ANF-binding proteolytic fragment of 70 kDa. The 70-kDa fragment bound ANF with enhanced binding affinity but retained intact ANF-R_1 pharmacological specificity and was still sensitive to modulation by amiloride. Trypsin treatment of the membranes produced a dual effect on ANF binding. Low concentrations of trypsin ($\leq$25 μg/mg of protein) increased ANF binding while higher concentrations dose dependently reduced the binding of the hormone. The increase of ANF-binding activity was associated with the formation of the 70-kDa fragment while the loss of ANF binding paralleled the degradation of the 70-kDa fragment. Low concentrations of trypsin drastically decreased the ANF-sensitive guanylate cyclase activity of the membrane fraction. This loss of catalytic activity strongly correlated with the formation of the 70-kDa tryptic fragment. We also evaluated the effect of ANF binding on the susceptibility of the receptor to proteolytic cleavage. The occupied receptor exhibited a greater sensitivity to trypsin digestion than the unoccupied protein, consistent with the hypothesis that hormone binding induces an important conformational change in the receptor structure. On the other hand, the 70-kDa fragment was much more resistant to proteolysis when occupied by ANF, suggesting that the ANF-binding domain forms a very compact structure. Treatment of the membrane receptor with α-chymotrypsin and endoproteinase Glu-C also converted the native protein to a fragment of approximately 70 kDa. The exoglycosidase neuraminidase reduced by $\approx$4 kDa the molecular mass of both the intact receptor and the 70-kDa fragment, indicating that the fragment contains at least one oligosaccharide chain of complex type. These carbohydrate residues appear to reside in the close vicinity of the ANF-binding site as suggested by the inhibiting effect of wheat germ agglutinin on ANF binding. Taken together, these results demonstrate that the ANF-R_1 receptor is a transmembrane protein which consists of at least two functional domains: an extracellular ANF-binding domain and a cytoplasmic guanylate cyclase domain. Those two domains are separated by a protease-sensitive region, distal to the membrane-spanning sequence and which might be involved in receptor metabolism.

Atrial natriuretic factor (ANF)[1] elicits its potent vasodilatory, natriuretic, and diuretic properties by activating highly specific membrane receptors which have been identified in target tissues such as kidney (Napier et al., 1984; De Lêan et al., 1985), adrenal cortex (De Lêan et al., 1984), arteries (Napier et al., 1984; Hirata et al., 1984; Schenk et al., 1985a), and brain (Quirion et al., 1986; Saavedra et al., 1986). Results obtained from detailed pharmacological studies in cultured aortic cells (Leitman & Murad, 1986a; Leitman et al., 1986b;

† This work was supported by a grant from the Medical Research Council of Canada to the Multidisciplinary Research Group in Hypertension.
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[1] Abbreviations: ANF, atrial natriuretic factor; HPLC, high-performance liquid chromatography; PMSF, phenylmethanesulfonyl fluoride; ScGMP-TME, 2′-*O*-monosuccinylguanosine 3′,5′-cyclic monophosphate tyrosyl methyl ester; SDS, sodium dodecyl sulfate; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-ph enylalanine chloromethyl ketone; BS^3, bis(sulfosuccinimidyl) suberate; ED_{50}, 50% efficient dose.

0006-2960/89/0428-5599$01.50/0

Scarborough et al., 1986) and in A10 cells (Napier et al., 1986) and from affinity-labeling experiments (Yip et al., 1985; Misono et al., 1985; Vandlen et al., 1985; Schenk et al., 1985b; Meloche et al., 1986a) have suggested the existence of two subtypes of ANF receptors that have been designated ANF-R_1 and ANF-R_2 (Leitman et al., 1986b). This hypothesis was further confirmed by the purification of the two ANF receptor subtypes from various tissues (Schenk et al., 1987; Shimonaka et al., 1987; Takayanagi et al., 1987; Meloche et al., 1988).

The ANF-R_1 receptor is a monomeric protein of 130 kDa which is coupled to cyclic GMP formation and has low affinity for biologically inactive truncated ANF analogues. The binding affinity of the receptor for ANF is enhanced in the presence of the diuretic amiloride (De Léan, 1986; Meloche et al., 1987), which also potentiates ANF stimulation of guanylate cyclase activity (Heim et al., 1988) and the cellular response to the hormone (De Léan, 1986; Nushiro et al., 1988). The copurification of particulate guanylate cyclase activity with the ANF-R_1 receptor strongly suggests that the receptor protein contains an intrinsic guanylate cyclase domain (Kuno et al., 1986; Paul et al., 1987; Takayanagi et al., 1987; Meloche et al., 1988). On the other hand, a second subtype of ANF-binding protein is also present in various amounts in target and nontarget tissues. The ANF-R_2 receptor is a dimeric protein composed of two disulfide-linked subunits of 65 000 daltons which is not associated with guanylate cyclase activity and which poorly discriminates the biologically active ANF-(99–126) from its metabolites. The primary structure of this ANF receptor subtype has been recently elucidated from its cDNA sequence and appears to consist of a large extracellular domain, a single membrane-spanning sequence, and a very short cytoplasmic domain (Fuller et al., 1988). The ANF-R_2 receptor is currently thought to be associated with tissue uptake and metabolism of the hormone (Maack et al., 1987).

Much less is known about the structure of the ANF-R_1 receptor protein, which is associated with the physiological effects of the hormone and for which the development of long-lasting ANF analogues, antagonists, and mimetics is highly desirable. In the present study, we have used limited proteolysis and exoglycosidase treatment to delineate the functional domains of the membrane ANF-R_1 receptor protein and to probe the conformational changes induced by ANF binding. Our results demonstrate that the ANF-R_1 receptor contains an extracellular, glycosylated, ANF-binding domain, a membrane-spanning region, a unique proteolysis-sensitive region, and a cytoplasmic guanylate cyclase domain.

Materials and Methods

Materials. TPCK-treated trypsin (type XIII), TLCK-treated α-chymotrypsin, endoproteinase Glu-C from *Staphylococcus aureus* V8, neuraminidase from *Clostridium perfringens*, lectins, bovine serum albumin, PMSF, amiloride, dithiothreitol, ScGMP-TME, and alumina (type WN-3) were obtained from Sigma. Rat ANF-(99–126) was from Institut Armand-Frappier, Laval, Canada. Antiserum to cyclic GMP was kindly provided by Dr. Alain Bélanger, Laval University Hospital Centre, Quebec, Canada. Carrier-free $Na^{125}I$ was from Amersham Corp. Bis(sulfosuccinimidyl) suberate and Iodo-Beads were purchased from Pierce Chemical Co. Electrophoresis reagents and molecular weight standards were from Bio-Rad. Other reagents were from Sigma.

Iodination of ANF-(99–126) and ScGMP-TME. ANF-(99–126) was radioiodinated as described previously, and the monoiodinated peptide was purified by reverse-phase HPLC (Ong et al., 1987). The specific activity of monoiodinated ANF-(99–126) was generally around 2000 Ci/mmol of peptide as estimated by self-displacement analysis. ScGMP-TME was iodinated by the method of Patel and Linden (1988).

Preparation of Membranes. Fresh bovine adrenal glands were obtained from the slaughterhouse and placed in cold phosphate-buffered saline. A layer of cortex, corresponding to the zona glomerulosa, was dissected, and membranes were prepared as described previously (Meloche et al., 1986b).

Receptor Binding Experiments. Adrenal zona glomerulosa membranes (60 μg/mL) were incubated with 8 pM ^{125}I-rANF-(99–126) for 90 min at 25 °C, in 50 mM Tris-HCl, pH 7.4, 5 mM $MnCl_2$, 0.1 mM EDTA, and 0.5% heat-inactivated bovine serum albumin. Bound ^{125}I-ANF was separated from free ligand by centrifugation at 30000*g* for 20 min or by rapid filtration on Whatman GF/C filters precoated with 1% poly(ethylenimine). The membrane-bound hormone was counted in a γ counter (LKB1272 CliniGamma) or affinity cross-linked for further experiments.

Affinity Cross-Linking Procedure. Affinity cross-linking of ^{125}I-rANF-(99–126) to zona glomerulosa membranes was performed as previously described (Meloche et al., 1986b, 1987). The concentration of bis(sulfosuccinimidyl) suberate used was 0.1–0.5 mM.

Limited Proteolytic Digestion of Membranes. Zona glomerulosa membranes were washed once in 50 mM Tris-HCl, pH 7.4, and resuspended in the same buffer at a protein concentration of 2 mg/mL. Proteolysis was initiated by addition of an equal volume of buffer containing varying amounts of TPCK-treated trypsin, and the reaction was allowed to proceed for 30 min at 25 °C. The digestion was stopped by addition of PMSF (1 mM final concentration) and centrifugation at 4 °C. Membrane proteins were solubilized in Laemmli sample buffer and analyzed by SDS–polyacrylamide gel electrophoresis. For α-chymotrypsin treatment, the membranes were incubated for 30 min at 25 °C in 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM $CaCl_2$. For endoproteinase Glu-C, the digestion reaction was carried out in 50 mM potassium phosphate and 2 mM EDTA (pH 7.8) for 2 h at 25 °C (Drapeau, 1977).

Neuraminidase Treatment of Membranes. Zona glomerulosa membranes were washed once and resuspended in 50 mM citrate–phosphate buffer (pH 6.2) at a protein concentration of 1 mg/mL. Neuraminidase was added at a final concentration of 2 units/mg, and the reaction was terminated after 30 min at 25 °C by centrifugation at 30000*g* for 20 min at 4 °C. Membrane proteins were then solubilized and analyzed by SDS–polyacrylamide gel electrophoresis as before.

Guanylate Cyclase Assay. Guanylate cyclase activity was assayed by the method of Garbers and Murad (1979). Intact and trypsin-treated membranes were incubated at 37 °C for 10 min in 100 μL of 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 $MnCl_2$ in the absence or the presence of 100 nM ANF. 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 typically 90%. ANF typically stimulated 1.5–3-fold guanylate cyclase activity under these experimental conditions.

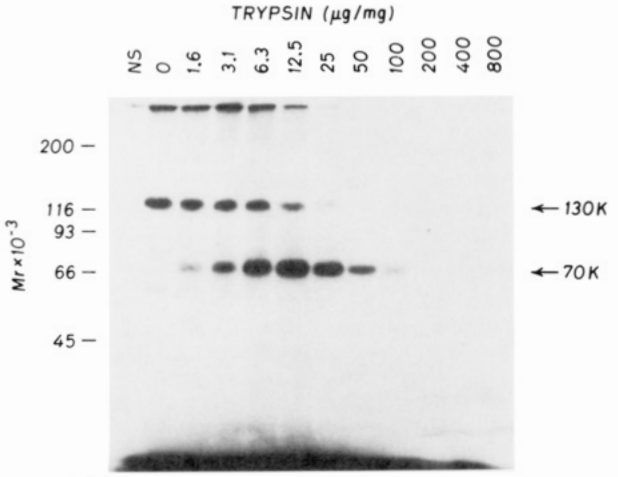

FIGURE 1: Effect of trypsin treatment on membrane ANF receptor. Plasma membranes from bovine adrenal zona glomerulosa were incubated with trypsin for 30 min at 25 °C. Reaction was terminated by addition of 1 mM PMSF. The membranes were then separated by centrifugation and incubated with ^{125}I-ANF for 90 min at 25 °C. The ligand–receptor complex was affinity cross-linked with 0.1 mM BS^3 in phosphate buffer (pH 7.4) for 30 min at 4 °C. The final membranes were dissolved in Laemmli sample buffer and subjected to electrophoresis. Gels were fixed, stained, dryed, and autoradiographed. NS: membranes untreated were incubated with ^{125}I-ANF in presence of 10^{-7} M unlabeled ANF. Molecular mass standards (in daltons) are myosin (200 000), β-galactosidase (116 250), phosphorylase *b* (92 500), bovine serum albumin (66 200), and ovalbumin (45 000).

SDS–Polyacrylamide Gel Electrophoresis. Membrane protein samples were solubilized in Laemmli sample buffer (62 mM Tris-HCl, 2% SDS, 10% glycerol, 0.001% bromphenol blue, pH 6.8) containing 0.1 M dithiothreitol and heated at 100 °C for 3 min. Electrophoresis was performed according to Laemmli (1970) in a 7.5% acrylamide gel or in a 5–10% acrylamide gradient gel prepared by the procedure of Walker (1984). After electrophoresis, the gels were fixed, stained, dried, and autoradiographed on Kodak X-Omat RP film with intensifying screens (Du Pont).

Data Analysis. Monophasic dose–response curves were analyzed by weighted nonlinear least-squares curve fitting using the computer program ALLFIT (De Léan et al., 1978). The program is based on a four-parameter logistic equation and provides the optimal estimates for the initial and the final response values, the ED_{50}, and the slope factor. In the case of the effect of trypsin on the production of the 70-kDa fragment, the biphasic dose–response curves were analyzed with the computer program ASYSTANT (ASYST Software Technologies, Rochester, NY). The seven-parameter equation

$$Y = g + (a - d)/[1 + (X/c)^b] + (d - g)/[1 + (X/f)^e]$$

was applied in order to determine the inital (a) and the final (d and g) response values, the ED_{50} values (c and f) for the ascending and the descending limbs of the biphasic curve, and their corresponding slope factors (b and e).

Statistical testing for differences between groups of replicates of parameter estimates from individual experiments was performed with Student's t test.

Results and Discussion

Limited Proteolysis of the Membrane ANF-R_1 Receptor by Trypsin. The structural domains of the membrane ANF-R_1 receptor were first studied by subjecting adrenal zona glomerulosa membranes to limited trypsin digestion prior to affinity labeling with ^{125}I-ANF. Treatment of the membrane receptor with increasing amounts of trypsin resulted in the progressive conversion of the native 130-kDa protein into a single ANF-binding proteolytic fragment of 70 kDa which was still associated with the plasma membrane (Figure 1). The degradation of the 130-kDa receptor was observed at very low concentration of trypsin (1.6 μg/mg of protein) and was almost complete at 50 μg of trypsin/mg of protein. Higher concentrations of trypsin resulted in the proteolytic degradation of the 70-kDa fragment into smaller fragments undetectable by affinity cross-linking. Incubation of the receptor in the absence of trypsin did not cause any degradation of the protein (Figure 1). When electrophoresis of trypsin-treated membrane receptor was performed under nonreducing conditions in the absence of DTT, the same 70-kDa fragment was observed (data not shown). These results clearly indicate that the 70-kDa proteolytic fragment contains the functional ANF-binding domain and at least one membrane-spanning sequence.

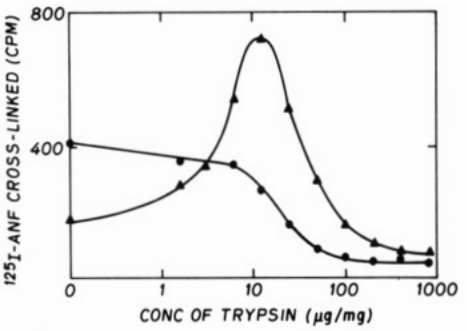

FIGURE 2: ^{125}I-ANF binding of intact and trypsin-treated membrane ANF receptor. The affinity cross-linked ^{125}I-ANF radioactivity corresponding to 130 000-dalton (●) and 70 000-dalton (▲) bands obtained in Figure 1 was counted in a γ counter.

Table I: Effect of Limited Trypsin Digestion on ANF Binding, on ANF-Stimulated Guanylate Cyclase Activity, and on the Structure of the ANF-R_1 Receptor

parameter	ED_{50} of trypsin (μg/mg of protein)[a]
loss of ANF binding	63.0 ± 7.1 (9)[b]
loss of guanylate cyclase activity	6.3 ± 0.7 (7)[c]
formation of 70-kDa fragment	6.0 ± 0.8 (4)[c]
loss of 70-kDa fragment	38.8 ± 2.1 (4)[b]
loss of 130-kDa receptor	21.4 ± 5.9 (4)

[a] Mean ± SEM for the number of experiments shown in parentheses. [b] Different from effect on guanylate cyclase activity ($P < 0.01$). [c] Different from effect on ANF binding ($P < 0.01$).

In order to determine the relative labeling of the intact 130-kDa receptor and the 70-kDa trypsin fragment, zona glomerulosa membranes were incubated with varying concentrations of trypsin, affinity labeled with ^{125}I-ANF, and subjected to SDS–polyacrylamide gel electrophoresis. The 130- and 70-kDa bands were then excised from the gel and counted for ^{125}I. Figure 2 shows that trypsin produced a dose-dependent decrease in the labeling of the native receptor with a half-maximal effect observed at 21 μg/mg of protein (Table I). This loss of the 130-kDa receptor was accompanied by a parallel increase in the formation of the 70-kDa ANF-binding fragment. The maximal amount of the 70-kDa fragment was observed at approximately 12 μg of trypsin/mg of protein and the half-maximal formation at 6 μg/mg of protein (Table I). The amount of labeled 70-kDa fragment decreased at higher trypsin concentrations with 50% degradation observed at 39 μg/mg of protein (Table I). Interestingly, the 70-kDa tryptic fragment incorporated more ^{125}I-labeled ANF than the 130-kDa receptor, suggesting that this fragment might have slightly higher affinity for ANF than

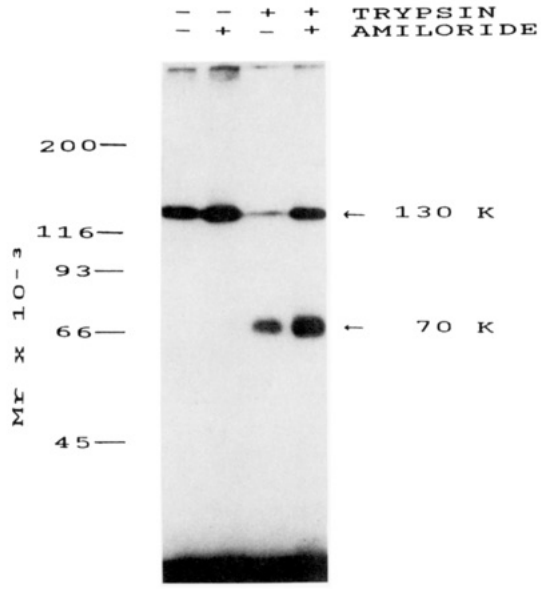

FIGURE 3: Effect of amiloride on intact and trypsin-treated membrane ANF receptor. Amiloride (10^{-4} M) was included during ^{125}I-ANF binding as described in Figure 1.

the intact receptor (Figure 2).

To further analyze the binding characteristics of the 70-kDa fragment, we tested the ability of different ANF analogues to inhibit the labeling of the 70-kDa band by ^{125}I-ANF-(99–126) cross-linking. The ANF-binding 70-kDa fragment displayed the same pharmacological specificity as the intact 130-kDa receptor (data not shown). Specifically, the analogue ANF-(103–123) competed for the labeling of the 70-kDa band with an ED_{50} in the micromolar range, which is typical of the ANF-R_1 receptor subtype (Meloche et al., 1988). We also evaluated the effect of the diuretic amiloride on the labeling of the 70-kDa tryptic fragment. Amiloride has been shown to increase the binding of ANF to its receptor in bovine adrenal zona glomerulosa (De Lêan et al., 1986; Meloche et al., 1987). Figure 3 shows that amiloride still potentiated the labeling of the 70-kDa fragment. Thus, these data indicate that the 70-kDa tryptic fragment, which contains the functional ANF-binding domain, also possesses the same binding properties and specificity as the intact 130-kDa receptor.

Interestingly, the 70-kDa fragment obtained by limited trypsin digestion of the ANF-R_1 receptor has a molecular mass close to the molecular mass (65 kDa) of the ANF-R_2 receptor polypeptide chain (Schenk et al., 1987; Shimonaka et al., 1987). The possibility that the ANF-R_1 and ANF-R_2 receptor subtypes might share the same hormone-binding domain appears however unlikely. As previously mentioned, the 70-kDa tryptic fragment shows a typical ANF-R_1 pharmacological specificity similar to that of the intact receptor and retains its sensitivity to modulation by amiloride, a property which is specific for the ANF-R_1 receptor subtype (Fêthière et al., 1989). A similar conclusion was also reached by Takayanagi et al. (1987) when they compared the tryptic peptide mapping of the two receptor subtypes.

Effect of Trypsin on ANF Binding and Guanylate Cyclase Activity. Treatment of bovine adrenal zona glomerulosa membranes with increasing concentrations of trypsin produced a dual effect on ANF binding. Low concentrations of trypsin (≤25 μg/mg of protein) increased ANF binding while higher concentrations dose dependently reduced the binding of the hormone (Figure 4). The increase in ANF binding was associated with the formation of the 70-kDa fragment, reflecting the apparently higher affinity of this fragment for

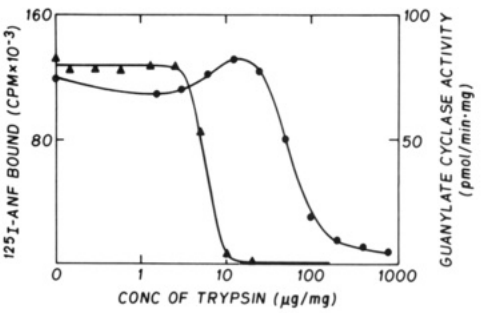

FIGURE 4: Effect of trypsin treatment on membrane ANF binding and guanylate cyclase activities. Plasma membranes were treated with trypsin as described in Figure 1. The membranes were separated by centrifugation and incubated with ^{125}I-ANF for 90 min at 25 °C. Bound ^{125}I-ANF (●) was separated by filtration on Whatman GF/C glass fiber filters precoated with 1% poly(ethylenimine) and counted in a γ counter. Guanylate cyclase activity in the presence of 100 nM ANF (▲) was measured by RIA of cyclic GMP with Mn-GTP as a substrate.

ANF (Figure 2). Maximal binding of ^{125}I-ANF was observed at 12.5 μg of trypsin/mg of protein, where the amount of the 70-kDa fragment is maximal (Figure 2). Higher concentrations of trypsin resulted in the loss of ^{125}I-ANF binding, with 50% reduction observed at 63 μg/mg of protein (Table I). The decrease in ANF binding coincided with the degradation of the 70-kDa fragment (Figure 2). This dual effect of trypsin has also been observed with the insulin receptor, whose treatment with low enzyme concentrations resulted in an initial increase in ^{125}I-insulin binding followed by a loss of binding at longer periods of time (Shoelson et al., 1988). Previous studies have shown that limited proteolysis of the insulin receptor with trypsin leads to an initial increase in binding affinity for insulin accompanied by a subsequent decrease in receptor number (Pilch et al., 1981). Although we have not determined the mechanism of increased ANF binding in our study, we might hypothesize that the removal of a protein fragment results in a conformational relaxation which increases the affinity of the ANF-binding domain.

It has been recently demonstrated that the purified ANF-R_1 receptor contains an intrinsic guanylate cyclase activity (Takayanagi et al., 1987; Meloche et al., 1988). In order to determine whether the membrane ANF-R_1 receptor retains catalytic activity after limited trypsin digestion, zona glomerulosa membranes were treated with various concentrations of trypsin, and guanylate cyclase activity in the presence of ANF was measured in both the supernatant and membrane fractions. The ANF-stimulated guanylate cyclase activity of the membrane fraction was drastically decreased by trypsin treatment with 50% reduction observed at 6 μg/mg of protein (Table I). Similar results were observed for guanylate cyclase activity in the absence of ANF. The ED_{50} value of trypsin for the reduction of ANF-stimulated guanylate cyclase activity was very similar to its ED_{50} on the formation of the 70-kDa fragment (Table I). These results strongly suggest that the loss of the guanylate cyclase activity is due to the removal of a 60-kDa protein fragment from the 130-kDa ANF-R_1 receptor. Under the conditions of this study, we were not able to recover guanylate cyclase activity in the supernatant fraction. It is not known whether the loss of guanylate cyclase activity is due to the degradation of the catalytic domain into smaller inactive fragments or if the guanylate cyclase domain requires native conformation to be active.

The effects of trypsin on ANF binding, on ANF-stimulated guanylate cyclase activity, and on the structure of the ANF-R_1 receptor are summarized in Table I. The data indicate that there is a strong correlation between the formation of the

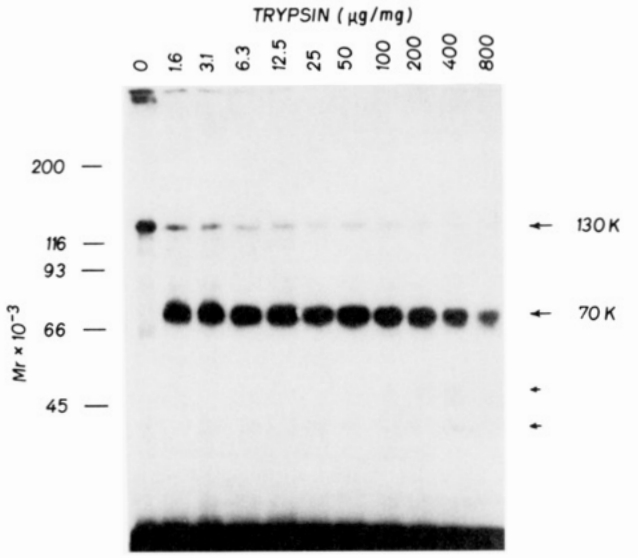

FIGURE 5: Effect of trypsin treatment on ligand-bound membrane ANF receptor. Membranes were first incubated with ^{125}I-ANF and affinity cross-linked. Then, ^{125}I-ANF-bound, affinity cross-linked membranes were treated with trypsin. Electrophoresis and autoradiography were done as in Figure 1.

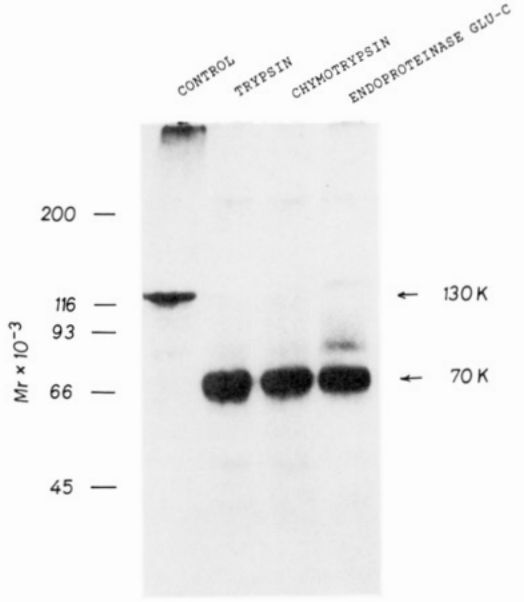

FIGURE 6: Comparison of trypsin, chymotrypsin, and endoproteinase Glu-C treatments on membrane ANF receptor. Plasma membranes from bovine adrenal zona glomerulosa were incubated with ^{125}I-ANF, cross-linked by BS^3, and treated with trypsin (30 μg/mg), chymotrypsin (100 μg/mg), or endoproteinase Glu-C (1000 μg/mg). Electrophoresis and autoradiography were as described in Figure 1.

70-kDa fragment and the loss of guanylate cyclase activity and also a good correlation between the degradation of the 70-kDa fragment and the loss of ANF binding. Taken together, these results demonstrate that the 70-kDa fragment contains the functional ANF-binding domain and at least one membrane-spanning sequence, while the 60-kDa fragment carries the guanylate cyclase domain. The data presented also indicate that the guanylate cyclase domain is much more sensitive to proteolytic inactivation than the hormone-binding domain.

Effect of ANF Binding on the Sensitivity of the Receptor to Proteolysis. We evaluated the effect of ANF binding on the susceptibility of the membrane receptor to proteolytic cleavage. In these experiments, the receptor was first affinity labeled with ^{125}I-ANF and then subjected to limited trypsin digestion as in Figure 1. The results, which are shown in Figure 5, significantly differed from those in Figure 1 in two aspects. First, the occupied receptor exhibited a greater sensitivity to proteolysis than the unoccupied receptor. Even at the lowest concentration of trypsin used (1.6 μg/mg of protein), the intact receptor was almost completely converted into the 70-kDa fragment. Second, the ANF-bound 70-kDa fragment was considerably more resistant to protease digestion when occupied by the hormone. Detectable amounts of the 70-kDa fragment were observed even at a trypsin concentration of 800 μg/mg of protein. At these high concentrations smaller fragments of 41 and 49 kDa were also generated (Figure 5). These smaller fragments, which were not observed when affinity cross-linking was performed after proteolytic cleavage (Figure 1), were presumably inactive and were detectable by autoradiography after long exposure times.

These results are consistent with the hypothesis that ANF binding induces a conformational change in the ANF-R_1 receptor which would render a cleavage site peripheral to the binding domain more accessible to proteolytic attack. This conformational change might be associated with the activation of the guanylate cyclase domain. On the other hand, the hormone-binding domain that is covalently linked to ^{125}I-ANF by chemical cross-linking is itself protected by the occupied hormone, suggesting that this domain forms a very compact structure when bound to ANF. Similar observations have been reported for the insulin receptor (Pilch & Czech, 1980). We cannot however exclude with the currently available methods the contribution of chemical cross-linking to this observed sensitization of the receptor protein to trypsin.

Effects of Other Proteolytic Enzymes on the Structure of the ANF-R_1 Receptor. In addition to trypsin, we also investigated the effects of two other proteolytic enzymes, α-chymotrypsin and endoproteinase Glu-C, on the digestion pattern of the membrane ANF-R_1 receptor. Despite the fact that these enzymes exhibit widely different cleavage-site specificities, they all produced a similar ANF-binding fragment of approximately 70 kDa (Figure 6). The size of the truncated receptor slightly varied between the three enzymes, suggesting that they might be acting at distinct but closely located sites within a similar region of the protein. The fact that the band corresponding to the 70-kDa fragment was broader than the band of the intact 130-kDa protein suggests higher binding activity of these fragments or a heterogeneity in the cleavage sites of the proteolytic enzymes. A minor fragment of 80 kDa was also observed with endoproteinase Glu-C.

We tested the accessibility of the protease-sensitive regions to trypsin in the membrane receptor preparation by comparison with the effects of the enzyme on detergent-solubilized receptor. When the membrane receptor was first solubilized with Triton X-100 and then treated with trypsin, only the 70-kDa fragment was observed (data not shown). This strongly suggests that the proteolysis-sensitive region documented is unique and is readily accessible in the membrane receptor preparation and that further exposure of the receptor protein by the detergent does not reveal additional cleavage sites.

These results document the existence of a region of the ANF-R_1 receptor which is highly susceptible to proteolytic cleavage. We (Meloche et al., 1986a,b) and other (Misono, 1988) have previously reported the acidic pH-dependent cleavage of the ANF-R_1 receptor into a fragment of 68–70 kDa which probably corresponds to the ANF-binding domain described in this study. Thus, the proteolysis-sensitive region of the receptor probably represents an interdomain segment which serves as a hinge within the protein. The wide sus-

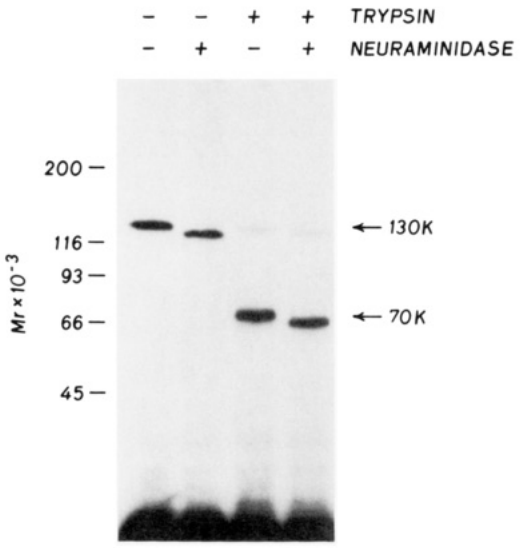

FIGURE 7: Neuraminidase treatment of intact and trypsin-truncated membrane ANF receptor. ^{125}I-ANF-bound, affinity cross-linked membranes were treated with neuraminidase (2 units/mg of membrane protein) for 30 min. SDS–PAGE was done in a 5–10% gradient gel, and autoradiography was as in Figure 1.

ceptibility of this region to proteolytic cleavage suggests that it could be involved in the metabolism of the receptor following ANF binding.

Effects of Neuraminidase and Lectins on the ANF-R_1 Receptor. The ANF-R_1 receptor is a glycoprotein as demonstrated by its sensitivity to neuraminidase (Yip et al., 1985) and its ability to bind to wheat germ agglutinin–agarose (Kuno et al., 1986; Takayanagi et al., 1987). In order to establish the localization of the oligosaccharide chains of the receptor, zona glomerulosa membranes were affinity labeled with ^{125}I-ANF-(99–126) and treated with trypsin and the exoglycosidase neuraminidase. Figure 7 shows that neuraminidase, which cleaves terminal sialic acid residues, decreased by approximately 4 kDa the molecular mass of both the intact receptor and the 70-kDa tryptic fragment, indicating that this fragment contains both the ligand-binding site and the sites of glycosylation. These data suggest that the hormone-binding domain contains complex-type oligosaccharide chains linked to asparagine residues which are probably located proximal to the N-terminal region of the protein (Pollack & Atkinson, 1983). Treatment with neuraminidase did not alter the ANF-binding properties of the receptor, indicating that sialic acid residues are not important for hormone binding (data not shown).

The nature and the localization of the glycosylation sites relative to the hormone-binding domain were further explored by studying the effect of a series of lectins on ^{125}I-ANF binding. Of all the lectins tested, only wheat germ agglutinin altered the specific binding of ANF to the membrane receptor (Figure 8). This lectin, which interacts specifically with *N*-acetylglucosamine and sialic acid residues (Bhavanadan & Katlic, 1979; Goldstein & Poretz, 1986), significantly inhibited ^{125}I-ANF binding at concentrations over 10 μg/mL. These results suggest the presence of complex oligosaccharide chains in the close vicinity of the ANF-binding domain.

Model of the ANF-R_1 Receptor. The results obtained from limited proteolytic digestion and exoglycosidase treatment have allowed us to map the functional domains of the ANF-R_1 receptor protein. The intact receptor of 130 kDa contains at least two functional domains, which are depicted schematically in Figure 9. The ANF-binding domain is located extracellularly and contains at least one oligosaccharide chain of the

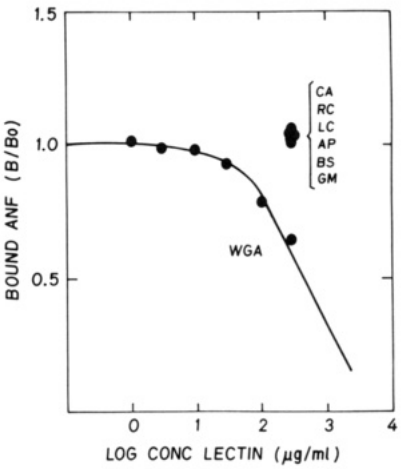

FIGURE 8: Effect of lectins on ^{125}I-ANF binding to membrane ANF receptor. Lectins were incubated together with ^{125}I-ANF and the membrane receptor. Free ^{125}I-ANF was separated on Whatman GF/C glass fiber filters precoated with 1% poly(ethylenimine), and bound ^{125}I-ANF was counted. The lectins used were concanavalin A (CA), *Ricinus communis* agglutinin 60 (RC), lentil lentin (LC), asparagus pea lectin (AP), BS-II (BS), soybean agglutinin (GM), and wheat germ agglutinin (WGA).

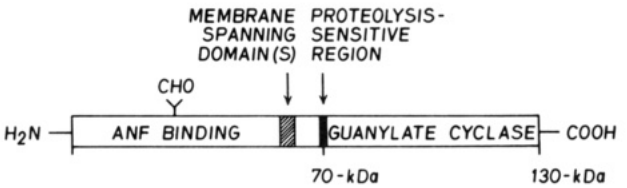

FIGURE 9: Schematic of functional domains of ANF-R_1 receptor. The hypothetical model shows the sequence of an ANF-binding domain, a membrane-spanning domain(s), a proteolysis-sensitive region, a guanylate cyclase catalytic domain, glycosylation sites (CHO), and amino (H_2N–) and carboxyl terminals (–COOH) of the receptor.

complex type. This domain is defined by limited digestion with trypsin, giving rise to a fragment of 70 kDa containing the ANF-binding domain and the membrane anchor sequence. The 70-kDa fragment is not disulfide linked to the remaining segment of the molecule since electrophoresis of the trypsinized receptor under reducing and nonreducing conditions yields the same results (data not shown). This finding contrasts with the results of Misono (1988), who showed that acidic cleavage of the receptor produces a 70-kDa polypeptide disulfide linked to the remaining fragment of the protein. In the schematic model of Figure 9, the receptor protein is oriented so that the extracellular hormone-binding domain represents the N-terminal region of the molecule. No evidence, however, has been obtained in this regard so far.

The exact localization of the proteolysis-sensitive region of the receptor is hypothetical. If we assume that the structure of the ANF-R_1 receptor contains only a single membrane-spanning region, similar to the growth factor receptors (Carpenter, 1987), then this region must lie within the cytoplasmic domain of the molecule. This likely hypothesis is strengthened by the recent determination of the amino acid sequence of sea urchin membrane guanylate cyclase, which contains a single transmembrane sequence (Singh et al., 1988; Chinkers et al., 1989). The guanylate cyclase domain, which is located on the cytoplasmic side of the membrane, has a molecular mass of approximately 60 kDa and appears to be released in solution upon treatment with low concentrations of trypsin.

In summary, we have demonstrated that the ANF-R_1 receptor is a membrane protein that consists of two functionally distinct domains: an extracellular glycosylated ANF-binding

domain and a cytoplasmic guanylate cyclase domain. The binding of ANF induces a conformational change in the receptor structure which is probably related to guanylate cyclase activation. The elucidation of the primary structure of the receptor protein will be necessary for a more complete analysis of these receptor domains.

ACKNOWLEDGMENTS

We thank Lise Laroche for preparing the manuscript.

Registry No. ANF, 85637-73-6; ANF-(99–126), 92046-97-4; ANF-(103–123), 98084-68-5; guanylate cyclase, 9054-75-5.

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