Ligand Binding-Dependent Limited Proteolysis of the Atrial Natriuretic Peptide Receptor: Juxtamembrane Hinge Structure Essential for Transmembrane Signal Transduction†

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ABSTRACT: The atrial natriuretic peptide (ANP) receptor is a 130-kDa transmembrane protein containing an extracellular ANP-binding domain, a single transmembrane sequence, an intracellular kinase-homologous domain, and a guanylate cyclase (GCase) domain. We observed that the receptor, when bound with ANP, was rapidly cleaved by endogenous or exogenously added protease to yield a 65-kDa ANP-binding fragment. No cleavage occurred without bound ANP. This ligand-induced cleavage abolished GCase activation by ANP. Cleavage occurred in an extracellular, juxtamembrane region containing six closely spaced Pro residues and a disulfide bond. Such structural features are shared among the A-type and B-type ANP receptors but not by ANP clearance receptors. The potential role of the hinge structure was examined by mutagenesis experiments. Mutation of Pro_{417}, but not other Pro residues, to Ala abolished GCase activation by ANP. Elimination of the disulfide bond by Cys to Ser mutations yielded a constitutively active receptor. Pro_{417}, and Cys_{423} and Cys_{432} forming the disulfide bond are strictly conserved among GCase-coupled receptors, while other residues are largely variable. The conserved Pro_{417} and the disulfide bond may represent a consensus signaling motif in the juxtamembrane hinge structure that undergoes a marked conformational change upon ligand binding and apparently mediates transmembrane signal transduction.

Atrial natriuretic peptide (ANP)[1] is a 28-residue peptide hormone produced in and secreted by the heart atrium (*1*). ANF exerts potent natriuretic and vasodilatory effects (*2, 3*) leading to lower blood pressure and volume. ANP also suppresses vascular smooth muscle cell hypertrophy and growth (*4*) and induces cardiac myocyte apoptosis (*5*). Thus, ANP plays a critical role in regulating circulation and vascular biology. Anomalies in ANP and ANP receptor functions have been implicated in hypertension, cardiac hypertrophy and dilatation, heart failure, and other cardiovascular diseases. Naturally occurring peptides homologous to ANP have been identified (*6*). Brain natriuretic peptide (BNP), originally found in the brain (*7*), is secreted mainly from the heart and shows activities similar to ANP (*8*). C-type natriuretic peptide (CNP) (*9*), present mainly in the brain, shows much weaker natriuretic and vasorelaxant activities. CNP is thought to be involved in the central, rather than peripheral, control of body fluid homeostasis.

The activities of these natriuretic peptides are mediated by cell-surface receptors coupled to cGMP as the intracellular second messenger. ANP receptors are found in the target organs of ANP, including the kidney, adrenal cortex, and vascular beds, with K_d values generally ranging from 0.1 to 1 nM. The receptors in the cell membranes from these organs show binding selectivity in the order ANP > BNP ≫ CNP. The receptors exhibiting this order of specificity are referred to as A-type natriuretic peptide receptors (NPR-A). NPR-A receptors have been purified and characterized (*10*−*12*) and cloned (*13, 14*). B-type natriuretic peptide receptors (NPR-B) that show different binding specificity in the order CNP ≫ ANP > BNP have also been found (*15*). NPR-B receptor is thought to mediate the actions of CNP in the brain, regulating body fluid equilibrium through the central nerve system (*16*). More recently, NPR-B has also been identified in shark rectal gland where it regulates Cl^- secretion (*17*). Apart from the GCase-coupled NPR-A and NPR-B, receptors not coupled to GCase have been described (*18*). These receptors bind not only ANP, BNP, and CNP, but also biologically inactive truncated peptides, such as atriopeptin I (AP-I) (*2*), with high affinity. These receptors, referred to as ANP clearance receptors (C-receptors), are localized primarily in the lung (*19*) and are thought to function in removing excess ANP from the circulation. Most of the known actions of natriuretic peptides, such as natriuresis,

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[1] Abbreviations: ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; AP-I, atriopeptin I; GCase, guanylate cyclase; KHD, kinase-homologous domain; NPR-A, A-type natriuretic peptide receptor; NPR-B, B-type natriuretic peptide receptor; NPR-ECD, extracellular domain of the ANP receptor; C-receptor, ANP clearance receptor; N_3Bz-^{125}I-ANP, $N_{4\alpha}$-azidobenzoyl ^{125}I-ANP(4−28).

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vasodilation, and anti-mitotic functions, are mediated by NPR-A.

The ANP receptor belongs to a family of transmembrane receptors coupled to GCase. These receptors include NPR-A and NPR-B, sea urchin egg peptide receptors (*20, 21*), receptors for the heat-stable enterotoxin and endogenous peptides guanylin and uroguanylin (*22*−*24*), and possibly membrane-bound GCases recently identified in the retina (*25*) or the eye (*26, 27*), olfactory organ (*28*), sensory neurons of *Caenorhabditis elegans* (*29, 30*), and fruit fly (*31, 32*). All these molecules have the domain structures similar to that of the ANP receptor and, therefore, are likely to share a common signal transduction mechanism.

The ANP receptor is a 130-kDa transmembrane protein consisting of a single-chain polypeptide that contains an extracellular ANF-binding domain, a single transmembrane sequence, an intracellular kinase-homologous regulatory domain (KHD), and a GCase enzyme domain (*13*). Binding of ANP to the extracellular domain activates the GCase domain by an as yet unknown mechanism. Cytosolic GCase, a heme-containing enzyme involved in nitric oxide-induced vasorelaxation, is a heterodimer (*33*). It has been speculated that GCase activation may be mediated by ANP-induced receptor dimerization (*34, 35*). Dimer and oligomer forms of the receptor have been observed in cells overexpressing cloned ANP receptors (*36, 37*). However, such dimers and oligomers were preexisting, independent of the presence of ANP. We have also found that the extracellular domain of the ANP receptor (NPR-ECD) expressed in a soluble form spontaneously self-associates to a dimer, in the absence of ANP, at high protein concentrations (*38*). The spontaneous dimerization without ANP clearly indicates that receptor dimerization alone is not sufficient for nor is the direct mechanism of GCase activation. It is apparent, therefore, that the effect caused by ANP binding, presumably a conformational change, is transferred across the cell membrane to activate the intracellular GCase domain. The mechanism responsible for such a transfer of activation signal remains unknown.

In the present report, we describe proteolytic cleavage of the ANP receptor in the isolated membranes by endogenous or exogenously added proteases when the receptor was bound with ANP. No cleavage occurred without bound ANP. Furthermore, this cleavage abolished GCase stimulation by ANP. Based on these findings, we hypothesize that the ANP receptor contains a specific region near the membrane forming a hinge-like structure that undergoes a large conformational change upon ANP binding, and that this ANP-induced conformational change mediates signal transduction across the cell membrane. To examine this hypothesis, we generated a series of point mutations of highly conserved Pro residues and Cys residues in the region and examined their effects on cGMP stimulation by ANP. From these studies, we obtained evidence suggesting a pivotal role of the juxtamembrane hinge structure in transmembrane signal transduction.

EXPERIMENTAL PROCEDURES

Materials. Rat ANP(1−28), ANP(4−28), and AP-I were synthesized as described previously (*39*). Rat BNP and rat CNP were obtained from Peninsula Laboratories (Belmont, CA). [^{125}I]NaI (specific activity, 2.1 μCi/pmol) was from DuPont (Boston, MA). ^{125}I-ANP(4−28) and N_3Bz-^{125}I-ANP were prepared as described (*40*). COS-1 cells were obtained from the American Type Culture Collection (Rockville, MD). DNA restriction enzymes, T4-DNA ligase, and bacitracin were from United States Biochemical (Cleveland, OH). Phenylmethanesulfonyl fluoride, aprotinin, leupeptin, and pepstatin were obtained from Boehringer Mannheim (Indianapolis, IN). Oligonucleotides were synthesized by Integrated DNA Technologies, Inc. (Coraville, IA). Antibodies against the purified extracellular domain of rat ANP receptor (*38*) were produced using the services of Biodesign International (Kennebunk, ME). Biotin-conjugated goat anti-rabbit IgG was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). The molecular weight standards for SDS−PAGE were obtained from Bio-Rad (Hercules, CA).

All other reagents used were of analytical grade or of highest available grades. The sequence alignment was done using the computer program Clustal-V (*41*).

Preparation of ^{125}I-Streptavidin. Streptavidin (Boehringer Mannheim, Indianapolis, IN) was radioiodinated using chloramine-T. Radioiodinated streptavidin was isolated by gel filtration chromatography on a Sephadex G-25 column.

Expression of Rat ANP Receptor in COS-1 Cells. cDNA encoding the entire sequence of NPR-A was cloned in the eukaryotic expression vector pcDNA3 from Invitrogen (Carlsbad, CA) to obtain a construct designated pcDNA3-NPRA (*38*). COS-1 cells were transfected with pcDNA3-NPRA using the DEAE-dextran method (*42*). Two days after transfection, cells were divided into 6-well plates and cultured for an additional 24 h before being assayed for cGMP production and the number of ANP-binding sites.

Preparation of Plasma Membranes from Bovine Adrenal Cortex. Adrenal cortex tissues were prepared from bovine adrenal glands procured from a local slaughterhouse. Cell membranes were prepared by a series of differential centrifugation and then partially purified by sucrose density centrifugation as previously described (*40*). The membrane suspension was divided into small aliquots and stored at −80 °C. The protein concentration was determined using a dye-binding assay kit from Bio-Rad and rabbit γ-globulin as the standard.

Preparation of COS-1 Cell Membranes. The membranes were prepared using the Parr-bomb method (*43*). Briefly, the transfected cells were collected from the culture plates using a scraper followed by centrifugation. The cells were resuspended in 25 volumes of phosphate-buffered saline containing leupeptin (20 μg/mL), phenylmethanesulfonyl fluoride (0.4 mM), benzamidine (20 μg/mL), bacitracin (1 mg/mL), aprotinin (20 μg/mL), and EDTA (1 mM). The cells were then disrupted in a Parr-bomb apparatus. The cell nucleus was removed by centrifugation at 500*g* for 5 min. The membranes were collected by centrifugation at 30000*g* for 25 min and were resuspended in phosphate-buffered saline at the membrane protein concentration of 2 mg/mL. The membrane suspension was divided into 100-μL aliquots, rapidly frozen in a dry ice−ethanol bath, and stored at −80 °C.

Ligand-Induced Proteolytic Cleavage of the ANP Receptor in Bovine Adrenal Membranes. Adrenal membranes (20 μg) were incubated with 0.5 nM N_3Bz-^{125}I-ANP (approximately 200 000 cpm) in 100 μL of 50 mM Tris-HCl (pH 7.5)

containing 0.15 M NaCl at 0 °C for 1 h to allow binding. The mixture was then incubated at 23 °C for varying periods of time, followed by an additional 1-h incubation at 0 °C. The membrane suspension was then photolyzed to effect photoaffinity labeling as described (*40*). The membranes were collected by centrifugation at 6000*g* for 3 min. The material was solubilized in SDS−PAGE sample buffer with or without dithiothreitol and electrophoresed in a 8% polyacrylamide pre-cast gel from Novex (San Diego, CA) according to the manufacturer's protocol. Radioactive bands were detected by autoradiography. In control experiments, the membranes were incubated without N_3Bz-^{125}I-ANP during the initial 1-h incubation at 0 °C and subsequent incubation at 23 °C. N_3Bz-^{125}I-ANP was then added, and the membrane suspension was incubated at 0 °C for 1 h to allow binding. Photolysis and SDS−PAGE separation were carried out in the same manner as for the experimental samples above.

Ligand-Induced Proteolytic Cleavage of Rat ANP Receptor Expressed in COS-1 Cell Membranes. The experiments to test ligand-induced cleavage of the receptor were carried out in a manner similar to those with the adrenal membranes above. The membranes (10 μg) prepared from COS-1 cells expressing the wild-type rat ANP receptor were incubated with 0.5 nM N_3Bz-^{125}I-ANP (approximately 100 000 cpm) in 50 μL of 20 mM Tris buffer (pH 7.4) containing 0.15 M NaCl and 15% sucrose at 0 °C for 1 h to allow binding. Thermolysin (0.1 μg) in a 1-μL aliquot was then added, and the suspension was incubated at 23 °C for varying periods of time. After addition of EDTA (5 mM) to inhibit thermolysin, the suspension was incubated for an additional 1 h at 0 °C and photolyzed. The membranes were solubilized in the sample buffer with or without dithiothreitol and separated by SDS−PAGE. In control experiments, the membranes were incubated without N_3Bz-^{125}I-ANP during the initial 1-h incubation at 0 °C and subsequent incubation at 23 °C. N_3-Bz-^{125}I-ANP was added after the addition of EDTA, and the suspension was incubated at 0 °C for 1 h followed by photolysis.

Construction of the Mutant Receptors. The *Bam*HI−*Xba*I fragment excised from pcDNA3-NPRA was subcloned into the pUC19 vector. The mutants were generated by using the Quick-Change mutagenesis kit from Stratagene (La Jolla, CA). The mutations were confirmed by endonuclease restriction digestion and DNA sequencing. The mutated fragment excised from the pUC19 construct was then ligated with the remaining fragments of pcDNA3-NPRA to obtain a mutant receptor expression construct.

Measurement of the Expression Level of the ANP Receptor at the Cell Surface of Intact COS-1 Cells by Antibody Binding Assay. The transfected COS-1 cells in a culture dish were incubated with antibodies against the extracellular domain of the rat ANP receptor for 1 h at 4 °C. After being washed 4 times with Dulbecco's modified Eagle's medium containing 1% BSA, the cells were incubated with the biotin-conjugated goat anti-rabbit IgG antibodies for 30 min. The cells were washed with the same medium and then incubated with ^{125}I-streptavidin (320 000 dpm/pmol) for 30 min. After washing, the cells were lysed in 1 mL of 0.2 M NaOH and counted in an ICN 10/600 Plus γ-counter (Huntsville, AL).

Natriuretic Peptide Binding Assay with Intact COS-1 Cells. Natriuretic peptide binding activity of the wild-type and mutant receptors expressed on the intact COS cell surface was assayed according to the method described previously (*44*). Data were analyzed by iterative nonlinear regression analysis to calculate the binding affinity and the number of binding-site values (*45*).

Characterization of Ligand Binding Specificity by Competitive Binding Assay. The ligand specificity of the wild-type and mutant receptors was examined by competitive binding assay using membrane preparations from transfected COS-1 cells according to the method described previously (*40*).

Measurement of GCase Activity in Adrenal Membranes. GCase activity was assayed by incubating 5 μg of the bovine adrenal membranes with 1 mM GTP at 37 °C for 10 min in 100 μL of 50 mM Tris-HCl buffer, pH 7.6, containing 1 mM 3-isobutyl-1-methylxanthine, 10 mM 1,3-dimethylxanthine (theophylline), 0.1 mg/mL creatine phosphokinase, 5 mM creatine phosphate, and 2.5 mM $MnCl_2$. At the end of incubation, 1 mL of 0.1 M sodium acetate buffer, pH 4.0, containing 2 mM EDTA was added, and the mixture was boiled for 10 min. After centrifugation, cGMP in the supernatant was measured by radioimmunoassay using a cGMP radioimmunoassay kit from Amersham (Arlington, IL).

Measurement of cGMP Production in Transfected COS-1 Cells. The cGMP level in transfected COS-1 cells was determined according to Leitman et al. (*46*). Briefly, the transfected COS-1 cells in 6-well plates were incubated in 1 mL of Dulbecco's modified Eagle's medium with 0.5 mM isobutylmethylxanthine at 37 °C for 30 min. ANP was then added to a final concentration of 1 μM, and the cells were incubated for an additional 15 min. The medium was removed, and the cells were disrupted by two rounds of rapid freezing and thawing. The cGMP was extracted with 0.75 mL of 4 mM EDTA. The extract was boiled for 10 min and centrifuged at 20000*g* for 20 min. Aliquots of the supernatant were used to determine the cGMP concentration by radioimmunoassay.

RESULTS

Ligand-Induced Proteolytic Cleavage of the ANP Receptor in Bovine Adrenal Membranes. Photoaffinity labeling of the ANP receptor in partially purified bovine adrenal cortex plasma membranes using $N_{4\alpha}$-azidobenzoyl-^{125}I-ANP(4−28) (N_3Bz-^{125}I-ANP) gave a dominant band at the position corresponding to a molecular mass of 130 kDa, indicating that the membranes contain mostly the intact full-length ANP receptor. When an aliquot of the same membrane preparation was incubated at room temperature for 24 h, subsequent photoaffinity labeling yielded a predominant 130-kDa band (result not shown), indicating that the receptor remained intact under such incubation conditions. In marked contrast, when the receptor in the adrenal membranes was allowed to bind with N_3Bz-^{125}I-ANP and then the membranes were incubated at room temperature, we observed rapid disappearance of the 130-kDa band and generation of a 65-kDa band, as detected by photoaffinity labeling (Figure 1A). This result suggests that the 130-kDa ANP receptor is cleaved by a membrane-associated protease to yield a 65-kDa ANP-binding polypeptide fragment. On the other hand, in the control experiment (Figure 1B), in which the membranes

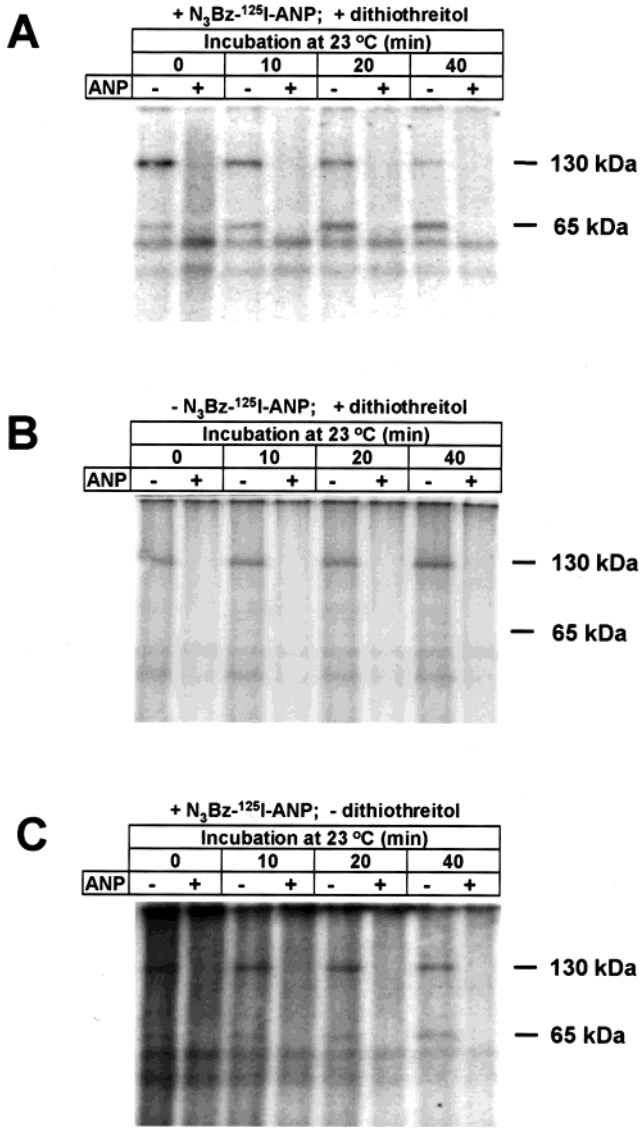

FIGURE 1: Ligand-induced proteolytic cleavage of the ANP receptor in bovine adrenal membranes. (A) The membranes were incubated with 0.5 nM N_3Bz-^{125}I-ANP at 0 °C for 1 h to allow binding [lanes indicated with minus (−) signs]. The membranes were then incubated at 23 °C for varying periods of time (0, 10, 20, and 40 min; shown boxed above the gel), followed by additional 1-h incubation at 0 °C and photolysis. The materials were separated by SDS−PAGE after reduction with dithiothreitol, and radiolabeled bands were detected by autoradiography. In control experiments [lanes indicated by plus (+) signs], N_3Bz-^{125}I-ANP was added with 0.1 μM ANP(4−28). These experiments control where to detect nonspecific background photolabeling. (B) Experiments were carried out in the same manner as in panel A, except that the initial 1-h incubation at 0 °C and subsequent incubation at 23 °C were carried out without ligands. N_3Bz-^{125}I-ANP was added without (−) or with 0.1 μM ANP(4−28) (+) immediately before the last 1-h incubation at 0 °C. (C) The same set of materials as in panel A were separated without reduction with dithiothreitol.

were incubated without N_3Bz-^{125}I-ANP, the 130-kDa band remained unchanged and no 65-kDa band was generated. These results suggest that the ANP receptor is readily cleaved by a protease when it is occupied by the ligand N_3Bz-^{125}I-ANP, but is resistant to the protease when it is not bound with the ligand.

When the aliquots of the membranes containing the cleaved receptor were separated by SDS−PAGE without reduction, the 130-kDa band remained unchanged (Figure 1C), suggesting that the 65-kDa fragment was still linked with the remaining part of the receptor molecule by one or more disulfide bonds. This ligand-induced receptor cleavage was suppressed by addition of diisopropyl fluorophosphate, aprotinin, and chymostatin, but not by EDTA, pepstatin, or *N*-ethylmaleimide (data not shown), suggesting proteolysis catalyzed by a serine protease.

The effect of receptor cleavage on GCase activity was examined by the following experiments. Aliquots of the adrenal membranes were first incubated with or without a saturating concentration of ANP (0.1 μM) for 1 h at 23 °C. After washing, the two fractions were incubated with 0.1 μM ANP at 0 °C for 1 h to allow maximum binding. Aliquots of the membrane incubations were then used to measure GCase activity. The membranes incubated without ANP during the first stage of incubation at 23 °C showed a GCase activity of 2.1 pmol min^{-1} (mg of protein)$^{-1}$. On the other hand, the membranes incubated with ANP at 23 °C showed a GCase activity of 0.8 pmol min^{-1} mg^{-1}. The decreased GCase activity in the latter membrane preparation suggests that the ligand-induced cleavage of the receptor abolished GCase activation by ANP.

Together, these results suggest that the ANP receptor contains a sequence region that becomes susceptible to proteolysis when the receptor is bound with ANP and that the cleavage in that region abolishes GCase activation by ANP. We therefore postulated that the region containing the cleavage site forms an intramolecular hinge structure that undergoes a conformational change upon ANP binding and further that this conformational change plays a role in signal transduction. To test this hypothesis, we examined whether the ligand-dependent cleavage also occurs with recombinant ANP receptor expressed in COS cells. We also generated a series of site-directed mutations in the hinge region and examined their effects on GCase activation by ANP.

Ligand-Induced Cleavage of Rat ANP Receptor Expressed in COS-1 Cells. The ligand-dependent cleavage of recombinant rat ANP receptor in COS cell membranes was examined in the same manner as with the adrenal membranes. Cleavage occurred much more slowly, proceeding at a rate roughly 50-fold slower than that observed with the bovine adrenal membranes. For this reason, thermolysin was added to the incubation at a protein-to-enzyme ratio of 100:1 to facilitate the reaction. Under these conditions, the ANP receptor was rapidly cleaved when occupied with the ligand N_3Bz-^{125}I-ANP and incubated at 23 °C, yielding a 65-kDa ANP-binding fragment (Figure 2). On the other hand, in the absence of the ligand, the receptor was resistant to thermolysin. The 65-kDa band generated by the cleavage gradually disappeared, but no smaller fragment bands were seen. The disappearance of the 65-kDa band is likely due to elimination of the ^{125}I-Tyr residue from the carboxyl terminus of the receptor-bound ANP photoligand by the action of thermolysin which was present in solution, rather than in a membrane-bound state, in the incubation medium. The persistence of the 65-kDa fragment observed with the adrenal membranes may have resulted from the inability of the endogenous membrane-bound protease to reach and cleave the receptor-bound ^{125}I-ANP photoligand. As was the case with the adrenal membranes, SDS−PAGE of the cleaved receptor without reduction gave only the 130-kDa band (Figure 2B),

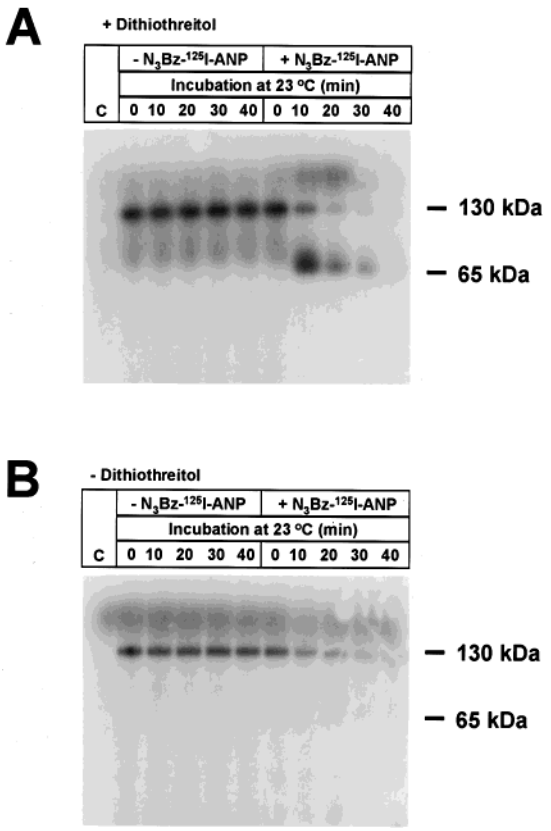

FIGURE 2: Ligand-induced proteolytic cleavage of the ANP receptor expressed in COS-1 cell membranes. (A) The membranes were incubated with N_3Bz-^{125}I-ANP (+) at 0 °C for 1 h to allow binding. Thermolysin was added to the membranes, and the suspension was incubated at 23 °C for varying periods of time (0, 10, 20, 30, and 40 min; shown boxed above the gel). After addition of EDTA, the membranes were incubated for an additional 1 h at 0 °C and photolabeled. The control experiments were carried out in the same manner as above, except that the membranes were incubated without N_3Bz-^{125}I-ANP (−) during the initial 1-h incubation at 0 °C and subsequent incubation at 23 °C (0, 10, 20, 30, and 40 min). N_3-Bz-^{125}I-ANP was added at the beginning of the last 1-h incubation at 0 °C. Lane C: untreated membranes photoaffinity-labeled in the presence of 0.1 μM ANP(4−25). (B) The same set of sample aliquots was separated by SDS−PAGE without reduction.

suggesting that the 65-kDa polypeptide is disulfide-linked with the remainder of the receptor molecule. The ligand-induced receptor cleavage was also examined for the receptors containing Pro and Cys residue mutations in the hinge region (see below), which showed no induction of cleavage by the ligand (data not shown).

The ANP-induced cleavage was also examined using protease K and chymotrypsin, which produced 65-kDa and 70-kDa fragments, respectively (data not shown). Generation of a 70-kDa fragment has also been observed previously by Liu et al. (*47*) by limited proteolysis of the ANP receptor in bovine adrenal membranes by trypsin. It appears, therefore, that ANP binding induces significant conformational changes in both juxtamembrane regions at extracellular and intracellular domains.

Cleavage Site and Pro-Rich Hinge Structure. The primary structure of rat ANP receptor deduced from the cDNA sequence (*13*) contains Cys residues at positions 60, 86, 164, 213, 423, 432, 569, 576, 582, 623, 633, 750, 874, 948, 960, and 1025 (Figure 3A). In separate studies, using purified NPR-ECD, we determined the disulfide bonds in the extracellular domain to be Cys_{60}−Cys_{86}, Cys_{164}−Cys_{213}, and Cys_{423}−Cys_{432}.[2] No intermolecular disulfide bond was found. This is in contrast to the disulfide structure of ANP clearance receptor which has been shown to contain two intrachain disulfide bonds, and one or two interchain disulfide bonds (*48*, *49*). Using NPR-ECD, we also determined the combined mass of oligosaccharide moieties attached to the polypeptide to be approximately 10 kDa (*38*). As described above, ligand-induced cleavage of both bovine adrenal ANP receptor and recombinant rat brain ANP receptor expressed in COS cells each yielded a 65-kDa ANP-binding fragment that was disulfide-linked to the remainder of the receptor polypeptide. Based on these findings, it was evident that the cleavage occurred within the peptide loop formed by the disulfide bond Cys_{423}−Cys_{432}. The sequence containing this putative cleavage site occurs in the extracellular domain near the transmembrane sequence (Figure 3A). Amino acid sequence alignment shown in Figure 3B reveals that the hinge region sequence is highly conserved among A-type natriuretic receptors. The sequence contains closely spaced Pro residues and the disulfide bond Cys_{423}−Cys_{432}. These unique structural features are also found in the corresponding regions in B-type receptors. However, C-receptors, which are not coupled to GCase, lack such structures. These facts also suggest a important role of the hinge structure in signal transduction.

Site-Directed Mutagenesis of Pro and Cys Residues in the Hinge Structure. To examine the possible role of the hinge structure in transmembrane signaling, we produced a series of point mutations of the Pro and Cys residues in the hinge region, and examined their effects on cGMP stimulation by ANP. The effects of Pro mutations to Ala at positions 412, 416, 417, 418, 421, and 430 are summarized in Table 1. ANP binding assays with intact COS-1 cells expressing the wild-type receptor gave a K_d value of 0.43 nM. The cells expressing mutant receptors gave K_d values in the range of 0.2−1.0 nM, indicating that the mutations had minimal effect on binding of the physiological ligand ANP. Photoaffinity labeling by N_3Bz-^{125}I-ANP gave single 130-kDa bands for both the wild-type and mutant receptors (not shown), suggesting that the expressed proteins were properly processed and inserted into the cell membrane. To allow comparison of cGMP responses, the levels of the receptors expressed at the cell surface were measured with the intact COS-1 cells and used to normalize the values of cGMP production. The density of the wild-type receptor on the transfected cells determined using the anti-ANP receptor extracellular domain antibodies was approximately 50 000 sites per cell. The densities of the mutant receptors were at comparable levels, ranging from approximately 40 000 to 60 000 sites per cell. These values were consistent with the binding-site densities (B_{max} values) obtained by saturation binding assays performed with intact cells in culture dishes at 4 °C using ^{125}I-ANP as the trace ligand (data not shown).

The basal level of cGMP production in the cells expressing the wild-type receptor was 0.47 ± 0.06 pmol of cGMP per 10 fmol equivalent of the ANP binding sites on the cell surface (normalized by cell surface receptor density) (Table 1). The cells expressing mutant receptors showed basal levels of cGMP production ranging approximately from 0.2 to 1.6 pmol/10 fmol binding site, indicating that the mutations did

[2] M. Miyagi and K. S. Misono, submitted for publication.

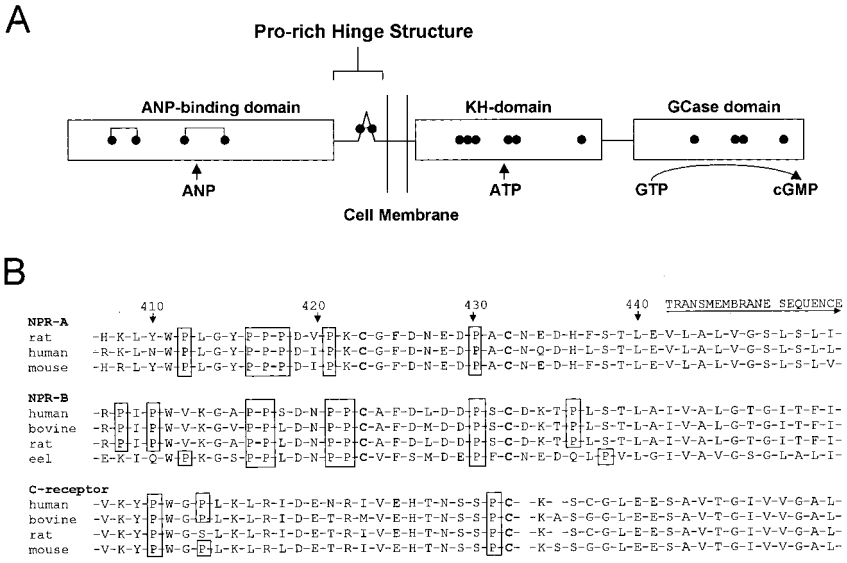

FIGURE 3: ANP receptor domain structure and amino acid sequence of the hinge region. (A) Schematic presentation of the domain structure of the ANP receptor. The extracellular ligand-binding domain (441 amino acid residues in rat NPR-A) is connected through the Pro-rich hinge structure to the transmembrane sequence and the intracellular domain (567 amino acid residues) containing the kinase-homologous (KH) domain and the GCase domain. Solid circles indicate the approximate positions of Cys residues in the primary structure, while the hinge structure region is expanded for clarity. (B) Alignment of the amino acid sequences of natriuretic peptide receptors in the hinge region containing the putative ligand-induced proteolytic cleavage site. Pro residues are shown boxed, and Cys residues shown in boldface type. Cys_{423} and Cys_{432} in rat ANP receptor are disulfide-linked (M. Miyagi and K. S. Misono, unpublished data). The putative transmembrane sequence regions are indicated by the arrow above the sequences.

Table 1: Stimulation of cGMP Production by ANP in COS-1 Cells Expressing the Wild-Type ANP Receptor and Mutant Receptors in Which Pro Residues in the Hinge Region Are Mutated to Ala Residues

receptor expressed	receptor density[a] ($\times 10^3$ sites/cell)	K_d (nM) for ANP(1−28)	cGMP produced[b] (pmol/10 fmol binding site) −ANP	cGMP produced[b] (pmol/10 fmol binding site) +ANP	*n*-fold stimulation
wild-type	50.1 ± 2.8	0.43	0.47 ± 0.06	109 ± 7.8	230
mutant receptors					
P412A[c]	45.7 ± 3.0	0.17	0.24 ± 0.18	55.0 ± 2.7	220
P416A	46.2 ± 1.3	0.54	0.44 ± 0.16	130 ± 11	300
P417A	41.9 ± 1.7	1.04	0.64 ± 0.22	2.6 ± 0.6	4.2
P418A	57.8 ± 3.1	0.84	0.96 ± 0.04	104 ± 9.5	110
P421A	52.5 ± 1.9	0.41	0.29 ± 0.11	63.8 ± 9.2	220
P430A	55.9 ± 1.6	0.52	1.05 ± 0.10	102 ± 1.1	97
P416A/P417A	47.9 ± 1.3	0.76	1.17 ± 0.08	15.8 ± 3.2	14
P417A/P418A	51.3 ± 3.5	0.49	1.31 ± 0.51	9.0 ± 4.2	6.8
P416A/P417A/P418A	58.7 ± 2.5	0.62	1.65 ± 0.26	5.0 ± 1.5	3.0

[a] The density was measured for the receptors at the cell surface of intact cells (see text). [b] The values are normalized as the amount of cGMP (pmol) produced per 10 fmol equiv of ANP binding sites found on the transfected COS-1 cells. [c] The mutation is indicated for the amino acid residue by single-letter notation and its residue number followed by the residue with which it was replaced. For example, P412A denotes mutation of Pro-412 to an Ala residue.

not cause substantial constitutive activation or inhibition of the basal GCase activity.

In the cells expressing the wild-type ANP receptor, addition of a saturating concentration of ANP (0.1 μM) resulted in an increase of cGMP production from the basal level of 0.47 ± 0.06 pmol of cGMP to 109 ± 7.8 pmol of cGMP per 10 fmol binding site, representing a stimulation of approximately 230-fold (Table 1). In the cells expressing the receptor with Pro_{417} to Ala mutation (P417A), on the other hand, the stimulated level of cGMP production was only 2.6 ± 0.6 pmol/10 fmol receptor sites, a 4.2-fold stimulation over its basal level at 0.64 ± 0.22 pmol/10 fmol receptor sites. This result indicated that the P417A mutation caused uncoupling of the ANP receptor.

In the cells expressing other single-point mutants (P412A, P416A, P418A, P421A, and P430A), the levels of ANP-stimulated cGMP production ranged from 55 to 130 pmol/10 fmol binding site, which were generally comparable to that observed with the cells expressing the wild-type (109 ± 7.8 pmol/10 fmol binding site). The extent of stimulation ranged from approximately 100-fold to 300-fold compared to 230-fold with the wild-type (Table 1). In cells expressing receptors with the double mutations P416A/P417A and P417A/P418A and the triple mutation P416A/P417A/P418A, cGMP stimulation by ANP was reduced to a level similar to that found in cells expressing the P417A mutant receptor.

Elimination of the disulfide bond Cys_{423}−Cys_{432} by mutating both Cys residues to Ser resulted in a marked elevation of the basal level of cGMP production (Table 2). The basal level increased to 35.2 ± 7.5 pmol/10 fmol receptor sites in the cells expressing the C423S/C432S mutant receptor as compared to 0.84 ± 0.06 pmol/10 fmol

Table 2: Stimulation of cGMP Production by ANP in COS-1 Cells Expressing the Wild-Type ANP Receptor and the Mutant Receptor in Which the Disulfide-Linkage in the Hinge Region Is Eliminated by Cys to Ser Mutations

receptor expressed	receptor density[a] ($\times 10^3$ sites/cell)	K_d (nM) for ANP(1−28)	cGMP produced[b] (pmol/10 fmol binding site)		*n*-fold stimulation
			−ANP	+ANP	
wild-type	34.4 ± 1.7	0.43	0.84 ± 0.06	101 ± 17	120
C423S/C432S[c]	35.2 ± 5.2	0.09	35.2 ± 7.5	70.9 ± 18	2.0

[a−c] As defined in Table 1.

receptor sites in those expressing the wild-type receptor, an increase of approximately 40-fold. There was a slight decrease in the K_d for ANP from 0.43 nM for the wild-type to 0.1 nM for the mutant receptor. ANP stimulated cGMP production by only 2-fold but to a level (70.9 ± 18 pmol/10 fmol receptor sites) that was comparable to that found in the cells expressing the wild-type receptor (101 ± 17 pmol/10 fmol receptor sites). These results indicate that the removal of the disulfide bond by the C423S/C432S double mutation causes constitutive activation of the ANP receptor without significantly affecting the maximal level of stimulation.

Binding Specificity of the Wild-Type and Mutant Receptors. Binding specificity of the wild-type and mutant receptors expressed in the COS cell membranes was characterized by competitive binding assays using cell membrane preparations. Membranes from the cells expressing the wild-type receptor showed binding specificity toward natriuretic peptide isoforms in the order ANP > BNP ≫ CNP, the specificity consistent for NPR-A (Figure 4A). AP-I, a truncated ANP peptide with weak biological activity, showed a low binding affinity. In contrast, the P417A mutant receptor, in which cGMP stimulation by ANP was abolished by the mutation, showed high-affinity binding to all the natriuretic peptide isoforms (ANP, BNP, and CNP; Figure 4B). In addition, AP-I also bound with a high affinity. On the other hand, the P416A and P418A mutants, in which cGMP stimulation by ANP was not abolished by the mutations, retained the binding specificity toward natriuretic peptide isoforms similar to that of the wild-type, exhibiting selectivity in the order ANP > BNP ≫ CNP [Figure 4C; binding data shown are for the P416A mutant; the P418A mutant gave similar binding data (not shown)]. AP-I, on the other hand, bound with a high affinity at the level comparable to that of ANP and BNP.

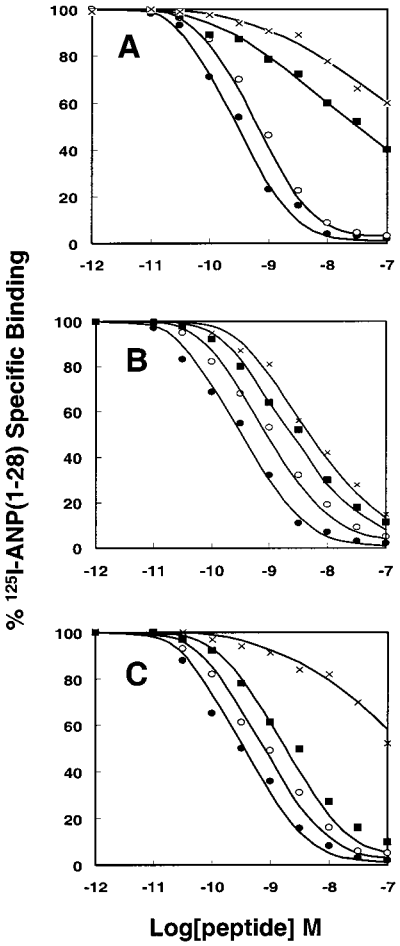

FIGURE 4: Binding specificity of the wild-type and mutant ANP receptors. Competitive binding assay of the wild-type (A), P417A mutant (B), and P416A mutant (C) of rat ANP receptor expressed in COS-1 cell membranes. The membranes were incubated with ^{125}I-ANP as the tracer and varying concentrations of ANP (●), BNP (○), CNP (×), and AP-I (■).

DISCUSSION

In the present study, we observed rapid proteolytic cleavage of the ANP receptor in bovine adrenal cortex membranes when the receptor was bound with the photoligand N_3Bz-^{125}I-ANP. The 130-kDa ANP receptor was cleaved to yield a 65-kDa ANP-binding fragment by an endogenous, membrane-bound protease. In contrast, no cleavage was observed in the absence of the ligand. This ligand binding-induced limited proteolysis was also observed with the recombinant rat brain ANP receptor expressed in COS-1 cell membranes. The marked change in the susceptibility to proteolysis suggests that the cleaved region may form an intramolecular hinge structure connecting the extracellular domain and the transmembrane helix that undergoes a significant conformational change responding to ANP binding. Furthermore, we have found that this ligand-induced cleavage abolished GCase activation by ANP. Based on these findings, we hypothesize that the hinge region forms a specific structure whose conformational change in response to ANP binding mediates transmembrane signaling. This view was strongly supported by the results of mutagenesis studies of several highly conserved Pro and Cys residues in the hinge region. Mutation of Pro_{417} to Ala yielded an uncoupled receptor, a receptor that bound ANP but did not stimulate cGMP production in response to ANP binding. Mutation of other Pro residues in the region did not cause uncoupling. Elimination of a disulfide bond by Cys to Ser mutations yielded a receptor that was constitutively active. Neither of these mutant receptors showed ANP-induced cleavage. These results together support our hypothesis that the hinge structure plays a critical role in transmembrane signaling.

The ligand-induced proteolytic cleavage in both the naturally occurring ANP receptor in bovine adrenal membranes and recombinant rat brain ANP receptor expressed in COS-1 cell membranes yielded an ANP-binding 65-kDa polypeptide fragment that was disulfide-linked to the remaining part of the receptor molecule. Based on the receptor sequence, the positions of disulfide bonds, and the total mass of the extracellular domain, it is evident that the proteolytic cleavage occurred within the peptide loop formed by the disulfide bond Cys_{423}−Cys_{432} near the membrane (Figure 3A). Notably, this region contains a cluster of closely spaced Pro residues and a number of relatively hydrophilic amino acid residues, consistent with our notion that the region forms an interdomain hinge structure. Sequence comparison with other natriuretic peptide receptors (Figure 3B) shows that the unique structural features, including the cluster of Pro residues and a disulfide-linked eight-residue peptide loop, are highly conserved in both NPR-A and NPR-B receptors. Such structures are not shared by C-receptors which are not coupled to GCase. These facts are also consistent with our hypothesis that the hinge structure is critical for transmembrane signaling.

The potential role of the hinge structure was examined by a series of mutagenesis studies directed to the Pro residues and the disulfide bond. These residues were selected because their mutations are likely to influence the polypeptide backbone conformation rather than merely the chemical nature of a specific amino acid side chain. We observed that mutation of Pro_{417}, but not other Pro residues, resulted in uncoupling of the receptor. Thus, the structure contributed by Pro_{417} is critical in maintaining the conformation necessary for signaling. On the other hand, mutations of both Cys_{423} and Cys_{432} to Ser yielded a receptor that was constitutively active, suggesting that the disulfide bond between the two Cys residues is necessary for maintaining the basal state. These findings suggest that the hinge region forms a specific structure required for proper signaling and that the residue Pro_{417} and the disulfide bond Cys_{423}−Cys_{432} are critical for maintaining such a structure. Remarkably, sequence comparison among different families of receptor/GCases from various species (Figure 5) reveals that Pro_{417}, Cys_{423}, and Cys_{432} are conserved while other amino acid residues in the region are largely variable. The only exception to the above is the enterotoxin receptor which has a Pro residue corresponding to Pro_{417} but lacks Cys residues. It has been shown by sequence comparison that the enterotoxin receptors diverge first in evolution from all other members of the receptor/GCase family known to date (*50*). Nevertheless, strong conservation of Pro_{417}, Cys_{423}, and Cys_{432} is consistent with our notion that the structure maintained by Pro_{417} and the disulfide bond Cys_{423}−Cys_{432} are critical for the receptor's ability to mediate transmembrane signaling.

We searched Protein Information Resource-International Protein Sequence Database at the National Biomedical Research Foundation for the sequence pattern $P(X)_nC(X)_mC$ (in which Pro and the two Cys residues are several amino acid residues apart from each other and the two Cys residues are disulfide-bonded) among transmembrane receptors. We found no other receptors that contain a similar structure in the membrane-proximal region (within ∼50 amino acid residues to the transmembrane sequence) in the extracellular domain. Nearest analogous sequences were found in the EGF receptor family and insulin receptor family of proteins. Human EGF receptor, for example, has the sequence -L-**C**-H-**P**-N-**C**-T-Y-G-**C**-T-G-P-G-L-E-G-**C**-P-T-N-G-P-K-I-P-S- adjacent to the transmembrane sequence. However, disulfide bonds in this sequence occur in a 1−3, 2−4 pairing pattern (*51*), which is different from that occurring in the ANP receptor. These disulfide bonds constitute part of a 1−3, 2−4, 5−6, 7−8 disulfide-pairing pattern which repeats several times in the cysteine-rich regions in these classes of receptors. Thus, the sequence containing residues Pro_{417} and the disulfide-bonded Cys_{423} and Cys_{432} appears to be unique to GCase-coupled receptors. This structure may represent a signature motif for the unique juxtamembrane hinge structure that undergoes a marked conformational change responding to ligand binding and mediates transmembrane signaling.

GC-A	SEHKLYWPLGYP**P**PDVPK-**C**GFDNEDPA**C**NQDHF
GC-B	PNVEIHWPSGSV**P**IDNPP-**C**VFETDIAS**C**NQATF
RetGC	GGTPIHFPGGRP**P**RADAK-**C**WFAEGKI-**C**HGGID
GC-D	LGTAVHFPGGSP**P**AHDAS-**C**WFDPNTL-**C**IRGVQ
GC-E	GTPVHFPRGAPA**P**GPDPS-**C**WFDPDVI-**C**NGGVE
GC-F	FRGTPIHFPGGR**P**TSADAK**C**WFAQGKI-**C**QGGID
GC-G	DDLNASGPHGSH**P**EYKPD-**C**GFHED-L-**C**RTKPP
GC-SU	DLNPPVWHNRDD**P**PLDMPV**C**GFHGE-L-**C**TNWGL
DrGC	NGSIDWPSGGEK**P**ADEPM-**C**GFANE-L-**C**KKDDT
GCY-X_1	TVGQLHWVGGKP**P**TDLPI-**C**GYDKSK--**C**PGYPL

FIGURE 5: Alignment of amino acid sequences corresponding to the Pro-rich, juxtamembrane hinge structure in different subtypes of membrane-bound receptor/guanylate cyclases from various species. Sequences of rat A-type natriuretic peptide receptor (GC-A) (*10*); shark B-type natriuretic peptide receptor (GC-B)(*14*); particulate GCase cloned from human retina (RetGC-1) (*22*); receptor/GCases from rat olfactory organ (GC-D) (*25*), the eye (GC-E, GC-F) (*23*), and small intestinal mucosa (GC-G) (*44*); sea urchin speract receptor (GC-SU) (*17*); a fruit fly (*Drosophila melanogaster*) GCase (DrGC) (*28, 29*); and a *C. elegance* receptor/GCase (GCY-X_1) (*26*) are shown. In rat GC-A extracellular domain, Cys_{423} and Cys_{432} are disulfide-linked.[2] Pro_{417}, and disulfide-forming Cys_{423} and Cys_{432} residues (in boldface), that were shown to be critical for signal transduction in GC-A by this study, are strictly conserved among all the receptors, while other residues in the region are largely variable.

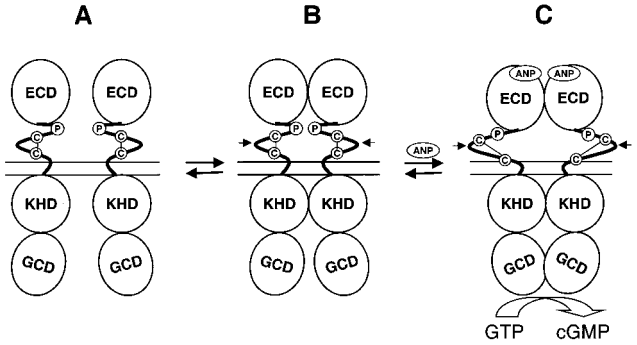

FIGURE 6: Possible involvement of ANP-induced conformational change in the Pro-rich hinge structure in transmembrane signal transduction. In the absence of ANP, the receptor is in equilibrium between its monomer (A) and dimer states (B). Dimerization may involve both the extracellular domain (*37*) and intracellular domain (*58*). The hinge region contains Pro_{417} and disulfide-bonded Cys_{423} and Cys_{432} (thin lines) that are essential for the receptor's ability to activate GCase responding to ANP binding. (C) Binding of ANP (small open ellipses) facilitates dimerization and, at the same time, causes a significant conformational change in the hinge structure (arrows) among other possible conformational changes. The effect then may reorient the intracellular domains of the dimer such that the GCase domains, which are a distance apart in the unliganded state, come together resulting in GCase activation.

Recently, Labrecque et al. (*52*) have reported that a mutation of Cys_{423} to Ala in rat NPR-A produced a receptor that was constitutively active. This C423A mutant receptor occurred mostly as a covalently linked dimer formed by an intermolecular disulfide bond presumably between the unpaired Cys_{432} by air oxidation. Only a small fraction (15%) of the mutant receptor was found as a monomer. These results were taken to suggest that formation of receptor dimer lead to constitutive activation. It is necessary to note, however, that the constitutive activation found in this study with the C423S/C432S double mutant cannot be due to disulfide-bonded dimer formation because both Cys_{423} and Cys_{432} are changed to Ser residues. Thus, the constitutive activation in the C423S/C432S mutant is due to an alteration in the structure by the mutation. Our findings then suggest that the constitutively active C423A mutant (*52*) may have resulted in part from a conformational alteration by the mutation rather than solely by disulfide-linked dimer formation.

Earlier, we have expressed the extracellular domain of rat NPR-A in soluble form (NPR-ECD) and purified it by ANP-affinity chromatography (*38*). Using this material, we have shown that binding of ANP causes dimerization of NPR-ECD. This finding suggests that the dimeric structure is involved in ANP receptor signaling. However, we also observed that, at high concentrations, NPR-ECD alone in the absence of ANP undergoes spontaneous self-association to a homodimer. Therefore, although ANP binding facilitates receptor dimerization, simply bringing two receptor molecules together to a dimer is not sufficient to cause receptor activation because activation is known to require ANP. These findings then suggest that a certain effect, presumably an intramolecular conformational effect, needs to occur upon ANP binding and be transduced across the cell membrane to the intracellular domain. The rapid proteolytic cleavage occurring only in the presence of bound ANP suggests that the conformational effect caused by ANP binding is prominently reflected in the conformation of the hinge region. The inability of the cleaved receptor to respond to ANP stimulation, uncoupling by the P417A mutation, and constitutive activation upon elimination of the disulfide bond by the C423S/C432S double mutation together suggest that a specific conformational change in the hinge structure caused by ANP binding may mediate transmembrane signaling.

A link between the ANP binding and the conformation of the hinge structure is also evident in the changes in ligand specificity caused by the Pro mutations. These mutations caused subtle yet distinct changes in ligand-binding specificity, while their affinity to the native ligand ANP was essentially unaltered. Thus, through an as yet unknown intramolecular effect, the ANP-binding site and the hinge structure appear to be functionally and conformationally related to each other.

Previously, we found that the ANP receptor in partially purified bovine and rat adrenal membranes was cleaved by an endogenous, membrane-associated metalloprotease when the membranes were exposed to acidic conditions (pH 4−5.6) (*53*). This cleavage also yielded a disulfide-linked 65-kDa polypeptide, suggesting that the cleavage occurred at a similar site. In contrast to the cleavage at the neutral pH, which was dependent on ligand binding, the acidic pH-induced cleavage occurred irrespective of the presence of ANP. This observation suggests that acidic pH may cause a conformational change in the hinge region similar to that induced by ANP binding. Additionally, acidic pH-induced cleavage caused a loss of membrane-associated GCase activity, again suggesting the functional importance of the hinge region in receptor activity. It is not clear whether the ligand-induced cleavage at neutral pH or ligand-independent cleavage at acidic pH observed in vitro reflects any physiological processes. It is tempting to speculate that the receptor cleavage and resulting annulment of receptor activity may be involved in desensitization or down-regulation of the ANP receptor under, for example, certain disease conditions. Further studies are necessary to discern such a possibility.

By photoaffinity labeling, we and others have shown previously that ANP receptors with apparent molecular sizes of 120−130 kDa are the predominant ANP receptor species present in the plasma membranes from the adrenal gland (*40*), kidney (*54*), and aortic smooth muscle (*19*). However, a number of reports using the affinity cross-linking technique have described low molecular mass (50−70 kDa) proteins as the dominant receptor forms in these organs (*55, 56*). Such low molecular mass receptor species are often assumed to represent C-receptors (*57*). However, it is necessary to note here that, in the affinity cross-linking experiments reported in the latter studies, the membranes were first allowed to bind ^{125}I-labeled ANP and then were incubated with a bifunctional reagent at an ambient temperature to effect cross-linking. The observation presented in this report suggests that such incubation would lead to spontaneous and rapid cleavage of the 130-kDa ANP receptor to yield a low molecular mass (∼65 kDa) receptor species by the action of endogenous proteases. Thus, the low molecular mass ANP receptors described in those studies are likely to represent products of artificial proteolytic cleavage during the affinity cross-linking experiments, but not C-receptors. This notion is consistent with the fact that membranes from aorta, kidney, and adrenal gland display binding specificity of NPR-A but not that of the C-receptor (*11, 53, 58*) and that Northern blot analyses generally failed to detect C-receptor mRNA in aorta, kidney, and adrenal tissues (*19, 59, 60*). Additionally, our findings suggest that the ligand-induced cleavage of the ANP receptor in membranes in vitro abolishes GCase activation by ANP. This phenomenon may explain why the stimulation of cGMP production by ANP measured with membrane preparations (2−3-fold) is generally much lower than that observed with the intact cells, which often exceeds 100-fold over the control levels (*61*).

The rapid cleavage of the receptor upon ANP binding reflects an increased steric accessibility of the hinge-region sequence. Neither the nature nor the extent of this conformational change in the hinge structure is known. Recently, the 3-dimensional structures of ligand-bound (*62*) and unbound forms of erythropoietin receptor (*63*) have been determined by X-ray crystallography. The structures suggest that agonist binding causes the transmembrane regions of a receptor dimer, which are a distance apart in the unliganded state, to come close to each other, thus bringing the intracellular domains together and allowing autophosphorylation of associated JAK kinases to occur. The ANP-induced conformational change in the hinge region may be involved in a similar topological reorientation in the ANP receptor dimer that brings the intracellular GCase domains juxtaposed

to each other to effect GCase activation (Figure 6). Further studies are necessary to understand what conformational effect is caused in the hinge structure by ANP binding and how such an effect is transferred into the intracellular domains to activate the GCase domain.

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