A Disulfide-bridged Mutant of Natriuretic Peptide Receptor-A Displays Constitutive Activity

ROLE OF RECEPTOR DIMERIZATION IN SIGNAL TRANSDUCTIONa

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Natriuretic peptide receptor-A (NPR-A), a particulate guanylyl cyclase receptor, is composed of an extracellular domain (ECD) with a ligand binding site, a transmembrane spanning, a kinase homology domain (KHD), and a guanylyl cyclase domain. Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), the natural agonists, bind and activate the receptor leading to cyclic GMP production. This receptor has been reported to be spontaneously dimeric or oligomeric. In response to agonists, the KHD-mediated guanylate cyclase repression is removed, and it is assumed that ATP binds to the KHD. Since NPR-A displays a pair of juxtamembrane cysteines separated by 8 residues, we hypothesized that the removal of one of those cysteines would leave the other unpaired and reactive, thus susceptible to form an interchain disulfide bridge and to favor the dimeric interactions. Here we show that NPR-A C423S mutant, expressed mainly as a covalent dimer, increases the affinity of pBNP for this receptor by enhancing a high affinity binding component. Dimerization primarily depends on ECD since a secreted NPR-A C423S soluble ectodomain (ECD C423S) also documents a covalent dimer. ANP binding to the unmutated ECD yields up to 80-fold affinity loss as compared with the membrane receptor. However, the ECD C423S mutation restores a high binding affinity. Furthermore, C423S mutation leads to cellular constitutive activation (20–40-fold) of basal catalytic production of cyclic GMP by the full-length mutant. In vitro particulate guanylyl cyclase assays demonstrate that NPR-A C423S displays an increased sensitivity to ATP treatment alone and that the effect of ANP + ATP joint treatment is cumulative instead of synergistic. Finally, the cellular and particulate guanylyl cyclase assays indicate that the receptor is desensitized to agonist stimulation. We conclude the following: 1) dimers are functional units of NPR-A guanylyl cyclase activation; and 2) agonists are inducing dimeric contact of the juxtamembranous region leading to the removal of the KHD-mediated guanylyl cyclase repression, hence allowing catalytic activation.

Particulate guanylyl cyclases are an expanding family of single transmembrane domain signaling receptors of which the natriuretic peptide receptors (NPRs)1 are the best studied examples (1). Three different subtypes of natriuretic peptide receptors have been identified. Two of these receptors, NPR-A and NPR-B, represent fully functional particulate guanylyl cyclases. They respond to natriuretic peptides (ANP, BNP, and CNP) by catalyzing the intracellular production of cGMP. NPR-A is stimulated by both ANP and BNP, whereas NPR-B is stimulated by CNP (1–3). cGMP is mediating the effects of NPRs on diuresis, vasorelaxation, and inhibition of the renin-angiotensin-aldosterone system (1, 3). The third receptor, called NPR-C or the clearance receptor, has only a small intracellular domain lacking the guanylyl cyclase function. NPR-C is a disulfide-bridged dimer that has nearly equal binding affinity for all natriuretic peptides (4, 5). This receptor internalizes natriuretic peptides through a fast intracellular cycle process (6) and might also be involved in signal transduction (7).

NPR-A is a ~130-kDa protein that displays a typical particulate guanylyl cyclase structure with four structural domains as follows: an extracellular domain (ECD) with a ligand-binding site, a transmembrane domain, a kinase homology domain (KHD), and a guanylyl cyclase domain (GC) (1). The current model for ligand activation of NPRs includes concerted natriuretic peptide and ATP-dependent regulation of guanylyl cyclase activity (8). According to this model, the signal transduction occurs through five sequential steps. 1) The binding of the natriuretic peptide to ECD induces a conformational change. 2) This modification corresponds to a signal that migrates through the transmembrane domain. 3) The KHD responds to this signal by adopting a conformation that allows ATP binding. 4) ATP binding has two major effects in derepressing the guanylyl cyclase activity and increasing the off-rate of ANP from the receptor. 5) Subsequent desensitization results from reduction in phosphorylation state of the KHD (9).

Several authors (10–12) initially hypothesized that the guanylyl cyclase activity of NPRs must require at least receptor dimerization. This anticipation was based on the observation that adenyllyl and guanylyl cyclases require two catalytic subunits for activity. In addition, x-ray crystallographic studies and modeling have established that both subunits contribute to the catalytic domain of these cyclases (13). Another important

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The abbreviations used are: NPR, natriuretic peptide receptor; rANP, rat atrial natriuretic peptide-1 (1–28) or natriuretic peptide A; pBNP, porcine brain natriuretic peptide-1 (1–32); CNP, C-type natriuretic peptide; PAGE, polyacrylamide gel electrophoresis; GC, guanylyl cyclase; h, human; KHD, kinase homology domain; NTA, nitrilotriacetic acid; IBMX, 3-isobutyl-1-methylxanthine; ECD, extracellular domain; GH, growth hormone; GHR, growth hormone receptor; HT, hexahistidine-tagged.

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observation was provided by cross-linking studies of ANP on NPR-A which demonstrated that high affinity binding is associated with receptor dimers (14). Hence, one can reasonably think that NPR-A dimer constitutes a basic functional unit for guanylyl cyclase activity.

Other studies have demonstrated the occurrence of spontaneous (ligand-independent) NPR-A non-covalent dimers or oligomers. For instance, co-immunoprecipitation studies of hNPR-A constructs tagged with different epitopes led to identification of spontaneously formed dimers (15). On the other hand, two studies have described ligand-independent disulfide-bridged NPR-A tetramers (16, 17). Iwata et al. (16) detected the presence of cross-linked tetramers in membrane preparations from bovine adrenal cortex. It was argued, however, that such disulfide linkage may arise from an artifact of preparation (15). Another study identified covalently linked tetramers in HEK 293 cells stably expressing hNPR-A and determined that the intracellular part of the molecule was essential for the linkage (17).

Another subject of active research has been on the involvement of the intracellular and/or the extracellular portions of the receptor in the dimerization process. One group demonstrated by co-immunoprecipitation, using full-length and truncated hNPR-A, that the extracellular domain is directly implicated in receptor dimerization process (15). Other studies using gel filtration instead of co-immunoprecipitation concluded that the intracellular portion is also involved in receptor dimerization (18). Furthermore, it was shown that this interaction is mediated by a hinge region located between the KHD and GC regions (18, 19).

Within the natriuretic peptide receptor family, NPR-C represents an exception in different ways as follows: it has a singular structure; it lacks catalytic activity and ligand specificity; and it acts as a clearance receptor (1, 4, 6). An interesting feature of NPR-C resides in its disulfide-linked homodimeric structure. The five NPR-C cysteines, all lying in the extracellular domain, have been well studied (4, 20). Mutational studies of these cysteines indicated that the first four cysteines are joined sequentially, forming the Cys104-Cys132 and the Cys209-Cys257 loops and that the fifth cysteine Cys469 (juxtamembraneous) is absent. From these observations, it is easily conceivable that these two conserved juxtamembranous cysteine also contributing to interchain disulfide bridge (21).

Interestingly, considerable sequence homologies are present in the extracellular domain between the NPRs, and the conservation of spacing between the cysteine residues is remarkable. Following these observations, we studied alignment of the juxtamembranous regions of the NPRs with all the known particulate guanylyl cyclases and of natriuretic peptide receptors. Amino acid sequences obtained from NCBI Data base for rat GCA, -B, -D, -E, -F, and NPR-C were aligned with Pileup (GCG software) followed by manual alignment. The sequence of the juxtamembrane region of all known GC and NPR from varying species were then added and manually aligned. The juxtamembrane region shown corresponds to amino acids 411–435 of NPR-A at the end of exon 6 and six residues prior to the postulated transmembrane domain. GC-C (guanylin receptor) sequences that have no juxtamembrane cysteine and that could not be properly aligned with other GC are not shown in this figure. AP, Arbauna punctulata; SP, Strongylocentrotus purpuratus; HP, Hemicentrotus pulcherrimus.

Experimental Procedures

Construction of NPR-A Mutants—rNPR-A mutants were engineered in the expression vector pBK-Neo (Stratagene). The rNPR-A HT-ECD mutant was constructed by inserting a SalI site at codon positions 437–439 using the mutagenesis kit from CLONTECH with the mutagenic primer 5'-CCAACACCTGTGACACCTGGAGTT-3'. Transmembrane and intracellular corresponding regions of this mutant were eliminated by a SalI/ApaI digestion. A synthetic linker (complementary oligonucleotides 5'-TCAGAGTTGACGTAATTAAGGC-3') was ligated to complete the construction. This linker, including SalI and ApaI cohesive ends, was composed of a BglII site followed by a hexa-histidine coding sequence and a stop codon. The SalI mutation resulted in a codon change of Phe437 to Leu, thus the Phe437 codon was restored by mutagenesis using the oligonucleotide 5'-CCAAACACCTGTGACACCTGGAGTT-3'. The nucleotide sequences of the functional primer 5'-CGTTCCCTACCTGACGAGAGT-3' were ligated to complete the construction. The linker, including SalI and ApaI cohesive ends, was composed of a BglII site followed by a hexa-histidine coding sequence and a stop codon. The SalI mutation resulted in a codon change of Phe437 to Leu, thus the Phe437 codon was restored by mutagenesis using the oligonucleotide 5'-CCAAACACCTGTGACACCTGGAGTT-3'. This construct was confirmed by sequencing on the two strands using the Sequenase kit from U.S. Biochemical Corp.

Cell Culture—The human embryonal kidney cell line 293 (American Type Culture Collection) was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 units of penicillin/streptomycin in a 5% CO2 incubator at 37 °C. For the cyclic GMP stimulation experiments, cells of the NPR-A, NPR-A C423S, and rNPR-A were transfected into HT-1080 cells using the TransFast reagent (Promega Corp.). The culture medium was then supplemented with 500 units of heparin and 10 nM ANP. The cells were stimulated for 15 min and then assayed for cGMP production by RIA. The results were expressed as picomoles of cGMP produced per mg of protein.

Transient and Stable Expression in HEK 293 Cells—Transient expression of the HT-ECD, HT-ECDD423S, rNPR-A and rNPR-A C423S was
obtained by transfection using the CaHPO4 precipitation as described elsewhere (22). For stable expression of the NPR-A, NPR-A-C(423)S and HT-ECD, clones were selected in 600 μg/ml G-418 (Geneticin, Boehringer Mannheim) in culture medium. For the control, clones were selected after transfection with pBR322 Vector DNA or DMEM. Membranes for the binding studies were prepared as follow. 72 h post-transfection, the cells were rinsed twice with phosphate-buffered saline and lysed in ice-cold homogenization buffer (10 mM Tris, pH 7.4, 2 mM EDTA containing 10 mM aprotinin, 10 mM pepstatin, 10 mM leupeptin, 10 mM peflabloc). Cells were first homogenized with a Polytron homogenizer followed by further homogenization in a Triton glass Potter. The pellet was washed twice with buffer for 30 min at 35,000 × g. The pellet was resuspended and washed twice in the same buffer. Finally, membranes were resuspended in ice-cold freezing buffer (50 mM Tris, pH 7.4, 0.1 mM EDTA, 250 mM sucrose, 1 mM MgCl2, and the proteases inhibitors), frozen in liquid nitrogen, and stored at −80 °C. For the particulate guanylyl cyclase studies, membranes were prepared from the stable clones (rNPR-A, rNPR-A-C(423)S, and Neo) according to a similar procedure. In that case, homogenization and freezing were performed in 50 mM HEPES, pH 7.4, containing 20% glycerol, 50 mM NaCl, 10 mM NaPO4, 0.1 mM NaF, 1 mM Na3VO4 and the proteases inhibitors. The protein concentration was determined using the BCA protein assay kit (Fierce).

**Covalently Dimeric Mutant of NPR-A Is Constitutively Active**—We investigated the possibility that mutation of Cys223 to Ser would allow intermolecular linkage between two receptor subunits. Membrane preparations from HEK 293 cells expressing wild type rat NPR-A and NPR-A-C(423)S were analyzed on SDS-PAGE under reducing and non-reducing conditions. As shown in Fig. 2 the migration of the C423S mutant under non-reducing conditions indicates that its state of oligomerization is mainly higher than monomeric. The estimated molecular mass is about 260 kDa, compatible with NPR-A dimer. A residual monomeric band is visible and accounts for approximately 15% of the total signal as estimated by densitometry. However, in other experiments this signal was barely detectable indicating that the proportion of residual monomers may vary (not shown). In repeat experiments, no higher molecular weight oligomer above the dimer could be seen neither for the wild type nor in the mutant NPR-A.

Thus, our results demonstrate that disulfide-linkage of rNPR-A can be specifically induced by the C423S mutation and that the wild type receptor does not spontaneously form intermolecular covalent linkage. Inherent to these results is the capacity of rNPR-A to dimerize non-covalently in absence of ligand.

**Interchain Disulfide Bridge Involves Extracellular Domain**—We also assessed the capacity of the C423S mutation to produce disulfide linkage of a soluble NPR-A extracellular domain. His-tagged wild type HT-ECD and C423S mutated (HT-ECD-C(423)S) receptors were produced, purified on Ni-NTA agarose gel, and analyzed for their state of dimerization. As seen in Fig. 3, wild type HT-ECD shows a single band on non-reducing SDS-PAGE corresponding to ECD monomers of ~67 kDa. Interestingly, HT-ECD-C(423)S migrates as two bands corresponding to monomers (~67 kDa) and dimers (~127 kDa). This 127-kDa band mainly accounts for ~60% of the total signal as evaluated by densitometry. It can be concluded from these results that the ECD-C(423)S can spontaneously form disulfide-linkage in the absence of ligand but with significantly less efficiency than the full-length rNPR-A-C(423)S.

**Data Analysis—**Dose-response curves were analyzed with the program AllFit for Windows based on the four-parameter logistic equation (25). Radioligand binding data were analyzed with the same program based on a model for the law of mass action (26).
tides were chosen because our recent work demonstrated that rANP displays only high affinity binding on membranes prepared from COS-P-expressing rNPR-A, whereas pBNP shows two classes of binding components corresponding to high and low affinities (27). As shown in Fig. 4, using receptor expressed in HEK 293, competition studies on membranes using rANP are still showing a unique high affinity binding component with rNPR-A (pK10.7 ± 0.06) as well as with rNPR-A C423S (pK 10.37 ± 0.17) (Table I). pBNP competition curve on wild type rNPR-A yields two components corresponding to high (pK 9.56 ± 0.06) and low (pK 7.91 ± 0.05) affinities, which is similar to the values obtained in our previous work for intact rNPR-A (27). Interestingly, competition binding of pBNP on rNPR-A C423S yields a curve that is globally shifted to the left (Fig. 4). Analysis of this curve still discriminates two binding components with pK values of 10.11 ± 0.16 and 8.2 ± 0.19.

**FIG. 2.** SDS-PAGE analysis of the inter-chain disulfide linkage of the mutant (HT-ECD C423S) and wild type (HT-ECD) rNPR-A secreted ectodomains. Membranes prepared from HEK 293 cells expressing HT-ECD or HT-ECD C423S were subjected to SDS-PAGE (7.5% gels) under non-reducing (NR) and reducing (R) conditions. The receptor was revealed by Western blotting using a commercial anti-tetra-histidine antibody. The molecular mass standards (in kDa) were myosin (200), β-galactosidase (116.3), phosphorylase b (97.4), and bovine serum albumin (66.2). The positions of monomers (M) and disulfide linked dimers (D) are indicated. SDS-PAGE under reducing conditions confirms the disulfide nature of the linkage.
HEK 293 cell transfection, membrane preparation, and competitive ligand binding were performed as described under “Experimental Procedures.” Results, expressed as pK\(_{A} (−\log K_{A})\), were obtained by competitive binding of nonradioactive peptides against \(^{125}\)I-rANP binding on membranes from cells expressing the wild type or the mutant receptor. The competition curves from which these values were derived are shown in Fig. 4. Values are the mean ± S.E. of three experiments.

### Table I

Affinities of rANP and pBNP for the wild type rat NPR-A and for the C423S mutant

| Peptides   | rANP\(_{28}\) | Wild type rNPR-A | High affinity component | pK | % |
|------------|---------------|------------------|-------------------------|----|---|
| rANP\(_{28}\) | 10.71 ± 0.06  | 100              |                         | 10.37 ± 0.17 | 100 |
| pBNP\(_{32}\) | 9.56 ± 0.06   | 35.5 ± 4.5       |                         | 10.11 ± 0.16 | 67.4 ± 5.9 |
|            | 7.91 ± 0.05   |                  |                         | 8.20 ± 0.19  |     |

Covalently Dimeric Mutant of NPR-A Is Constitutively Active

Expression of (His)_\(_n\)-tagged NPR-A ectodomains in HEK 293 cells and their purification from the culture medium on Ni-NTA agarose gel are described under “Experimental Procedures.” Results, expressed as pK\(_{A} (−\log K_{A})\), were obtained by competition of nonradioactive peptides against \(^{125}\)I-rANP binding on purified HT-ECD or HT-ECD\(_{C423S}\). The competition curves from which these values were derived are shown in Fig. 5. Values are the mean ± S.E. of two experiments.

### Table II

Affinities of rANP and pBNP for the soluble extracellular domains of wild type rNPR-A (HT-ECD) and HT-ECD\(_{C423S}\) mutant

| Peptides   | Wild type rNPR-A ECD, pK | rNPR-A\(_{C423S}\) ECD\(_{C423S}\), pK |
|------------|----------------------------|---------------------------------|
| rANP\(_{28}\) | 8.82 ± 0.22               | 10.17 ± 0.01                    |
| pBNP\(_{32}\) | 7.35 ± 0.06               | 8.70 ± 0.05                     |

Dimerization Leads to Constitutive Activation of rNPR-A—Taking into account that disulfide linkage fulfills one of the steps leading to a high affinity binding. It can also be reasoned that rANP would induce sufficient further stabilization or conformational change. On the contrary pBNP might not be as efficient to induce by itself further stabilization. Finally, considering that pBNP can induce a high affinity component in the full-length receptor, it is reasonable to think that the intracellular and/or the transmembrane domain participates in pBNP high affinity binding.

![Fig. 5. Competition binding analysis on (HT-ECD) and mutant (HT-ECD\(_{C423S}\)) rNPR-A ectodomains.](image)

From these results it can be hypothesized that the dimerization of the soluble ECD by disulfide linkage fulfills one of the steps leading to a high affinity binding. It can also be reasoned that rANP would induce sufficient further stabilization or conformational change. On the contrary pBNP might not be as efficient to induce by itself further stabilization. Finally, considering that pBNP can induce a high affinity component in the full-length receptor, it is reasonable to think that the intracellular and/or the transmembrane domain participates in pBNP high affinity binding.

![LOG CONC PEPTIDE (M)](image)
wild type receptor (52-fold).

At this point, it was important to assess the capacity of the mutant to reach the cell surface since its very low level of stimulation in response to ANP might be caused by an alteration of maturation, resulting in intracellular trapping. We have checked this hypothesis by comparing the cellular localization of the wild type and mutant NPR-A. Receptor localization was measured through quantitative study of C423S-rANP binding on intact cells as compared with total binding on solubilized cell membrane preparation (data not shown). The results indicate that wild type NPR-A is almost entirely localized to the cell surface, whereas almost (80%) of NPR-A C423S mutant is at the cell surface. Therefore, the C423S mutation does not lead to major maturation defects and intracellular trapping.

In conclusion, the C423S mutation induces a constitutive activation of the receptor in cells and also alters directly or indirectly its maximal response to ANP.

Constitutively Active Dimeric Mutant Is Desensitized—In view of the latter characteristics of rNPR-A C423S, it was of interest to biochemically test the guanylyl cyclase activity of the mutant. Maximal guanylyl cyclase activity is traditionally determined with a Triton X-100/Mn2+ treatment that is assumed to artifically stimulate guanylyl cyclases to their maximal level (28). Membrane preparations were tested through different conditions using GTP alone (basal), ATP, ANP, ANP + ATP, and Triton/Mn2+ (maximal stimulation). Surprisingly, with Triton/Mn2+ treatment, the maximal activation of the C423S mutant is altered showing a 50% reduction as compared with the wild type rNPR-A. We thus expressed the results as percentage of maximal activation (Triton/Mn2+) (Fig. 7).

Based on these results, several observations can be made. First, the C423S mutant displays a basal constitutive activity that accounts for 5.6 times the wild type value. Also, ATP alone induces a 3-fold activation on rNPR-A C423S catalytic activity, whereas ATP activation is barely detectable for the wild type receptor. On the other hand, ANP treatment produces a 10.4-fold stimulation for rNPR-A but only of 1.7-fold for the mutant. Moreover, the stimulation of the mutant with ANP alone is about twice less than with ATP. Finally, ANP + ATP treatment gives a strong synergistic effect (35% of maximal level) for wild type NPR-A but only a cumulative effect for the mutant result-

**FIG. 6. Whole cell guanylyl cyclase stimulation of wild type rNPR-A and mutant rNPR-A C423S.** Dose-dependent stimulation of cyclic GMP production by rANP in HEK 293 clones stably expressing wild type rNPR-A (■), rNPR-A C423S mutant (○), or in the Neo (○) clones. As described under “Experimental Procedures,” confluent cells on 24-well cluster plates were incubated (1 h, 37 °C) with increasing concentrations of rANP in the presence of IBMX. Cyclic GMP was measured in the extracellular medium by radioimmunoassay. Note that the ANP stimulation curve is merged with the abscissa due to its negligible level as compared with the other curves. Each data point represents the mean ± S.D. of duplicate determinations.

**FIG. 7. Guanylyl cyclase assays with membranes of cells expressing wild type rNPR-A and mutant rNPR-A C423S.** Stimulation of the particulate guanylyl cyclase of wild type rNPR-A and mutant rNPR-A C423S with different treatments. Membranes were prepared as described under “Experimental Procedures” and were incubated (5 min) for 10 min at 37 °C in the presence of theophylline, IBMX, creatine phosphate, creatine kinase, GTP, and MgCl2. Different conditions were tested by adding 1 μM rANP, 1 mM ATP, rANP and ATP together or 1% Triton X-100 with 4 mM MnCl2 instead of MgCl2. The produced cyclic GMP was purified by chromatography on alumina and evaluated by radioimmunoassay. Even though about the same level of receptor is expressed in the two clones, the maximal activation in Triton/Mn2+ is 50% less for rNPR-A C423S as compared with the wild type rNPR-A (see “Results”). Thus, for clarity, the results were normalized as percentage of maximal activation in Triton/Mn2+.

**DISCUSSION**

In this work, we have shown that the C423S mutation of rat rNPR-A yields a disulfide-bridged receptor dimer, conceivably through the juxtamembranous Cys432. This receptor dimer displays an improved binding for pBNP and is constitutively activated. We also studied the properties of rNPR-A soluble ECD. As previously reported (8), this soluble receptor exhibits low affinity binding for rANP. However, the same C423S mutation applied on ECD yields disulfide-bridged dimers upon which rANP displays a binding affinity comparable to that for the full-length receptor. This indicates that receptor dimers represent basic units involved in high affinity ligand-receptor recognition.

A study from Lowe (17) has described the occurrence of disulfide-bridged human NPR-A. In this case, spontaneous forms of tetramers, dimers, and monomers were detected. In another work, Iwata et al. (16) detected disulfide-bridged NPR-A tetramers from bovine membranes preparations (16). However, since they used 1 mM dithiothreitol throughout membrane preparation, artifactual disulfide bridge shuffling may have been favored in this latter case. Also, the number of cysteines in the extracellular domain of the bovine receptor is not known making difficult any further interpretation. Noteworthy, this group also suggested that in all probability the two juxtamembrane cysteine residues in NPR-A may be involved in inter-chain tetrameric linkage (4). This hypothesis may be theoretically conceivable, but direct proof of such organization has not been provided. Also, some of our results are not compatible with that concept. For instance, as we are showing here, the
disulfide linkage probably through the juxtamembrane cysteine 432 of rNPR-A\textsuperscript{C423S} leads to constitutive activation. Thus, hypothetical disulfide-linked tetramers involving this same cysteine would be susceptible to cause constitutive activation of NPR-A, which is obviously not what is found naturally. Different explanations might be possible, for example that covalent tetramerization, unlike disulfide-linked dimerization, may not favor constitutive activation. Hence, alternate pairing of the two juxtamembranous cysteines involved in a hypothetical tetramerization might prevent the constitutive activation. Finally, disulfide-linked tetramers may arise from some kind of artifact and are not relevant in NPR-A normal function. These questions are opened.

In the present work, by inducing disulfide-bridged dimerization with the C423S mutation, we are confirming that covalent oligomer would be easily detectable if they were present. Furthermore, since no covalent oligomers are observed for wild type rNPR-A, we could analyze the biochemical effects of the rNPR-A\textsuperscript{C423S} mutation or covalent dimerization. The substantial increase of the pBNP high affinity binding observed with rNPR-A\textsuperscript{C423S} indicates that the disulfide linkage favors high affinity ligand-receptor interaction. Furthermore, the constitutive activation of rNPR-A\textsuperscript{C423S} demonstrates that the disulfide linkage also mimics the agonistic effect. However, it is difficult to evaluate the activation induced by the mutation in reason of the desensitization of the receptor. Nevertheless, the constitutive activity indicates that a stabilized dimer represents a basic functional unit for rNPR-A cyclase activity. This goes along with the results obtained by Rondeau \textit{et al.} (29) who demonstrated that the ligand/receptor stoichiometry of bovine NPR-A (L:1/R:2) is in agreement with ligand-stabilized dimer. That further non-covalent oligomerization events could follow (i.e. leading to tetramers) cannot be excluded. However, such putative large aggregates might not be required for receptor activation.

Several studies have demonstrated that hNPR-A spontaneously forms non-covalent dimers (15, 18). Thus, the disulfide bridge in rNPR-A\textsuperscript{C423S} is probably not inducing dimerization but may rather modify the interactions between already assembled dimers. From this, a model based on “loose” and “tight” conformations can be imagined, the tight state corresponding to high ligand binding affinity and guanylyl cyclase activation. Thus, rANP might efficiently contact both receptor subunits and tighten their interaction. This effect might be less efficiently induced by pBNP, and this would be reflected by the high (tight?) and low (loose?) affinity components. Finally, covalent dimerization of the C423S mutant would trap a state closely related to the tight conformation.

According to our results, it is likely that a dimeric interaction of the domain surrounding the disulfide bridge is important for the activation process. This can be discussed in the perspective of other single transmembrane domain receptors. For instance, in the case of the growth hormone receptor (GHR), it has been shown that the growth hormone (GH) is stabilizing receptor dimer through simultaneous contact of the ligand with two receptor subunits. Interestingly, the crystal structure of the GHR-GH complex indicates that, in addition to these hormone-stabilized receptor interfaces, there is also an important dimeric contact of the extracytoplasmic domain close to the transmembrane spanning domain (30). Furthermore, it has been shown that the mutation D152H underlying the Laron syndrome (familial GH resistance) acts by disturbing the folding of this contact region, hence abolishing the GHR response to GH (31). Thus, dimeric contact involving this juxtamembrane region is important in the activation process of GHR.

On the contrary, the crystal structure of the EpoR complexed with a synthetic agonist shows no direct interaction of the corresponding juxtamembranous region (32). Interestingly, however, mutagenesis studies have shown that the introduction of a cysteine residue through the R129C mutation yields a disulfide-bridged, constitutively activated receptor (33). As judged from the crystal structure, this mutated residue is located at the bottom of the juxtamembranous domain (32). Thus, in order to allow the bridge formation, this specific region of Epo-R must transiently dimerize in the absence of ligand.

This approach of introducing cysteine residues has been used to study the oligomerization of other membrane receptors. In particular, it has been used for an exhaustive study on the interactions between the protomers of the bacterial aspartate receptor (34). Since no cysteine is naturally found in this receptor, introduction of cysteines at specific points of the molecule allowed the study of spatial proximity between the subunits. However, in this case, the use of oxidative reagents was necessary to catalyze formation of the intermolecular bridges. Once again, productive covalent dimerization was found to arise from the juxtamembranous region.

In another study, the introduction of a cysteine residue in the juxtamembranous region of the EGF-R resulted in a disulfide-bridged receptor dimerization that was ligand-dependent (35). On the other hand, some mutations producing disulfide-bridged dimer and constitutive activation are naturally occurring and are underlying genetic diseases. For example, several mutations associated with Cruzon syndrome are causing spontaneous dimerization and activation of the fibroblast growth factor receptor (36, 37). These mutations, all involving the change of a wild type residue to Cys, are occurring either in the juxtamembranous domain or in the linker region between the Ig2 and Ig3 immunoglobulin-like domains. It is noteworthy that the Ig3 domain plays an important role in ligand binding and is, thus, probably part of a natural interface between the receptor subunits. Finally, a series of mutations (MEN 2A) occurring in the juxtamembranous domain of the \textit{RET} proto-oncogene are leading to covalent dimerization and constitutive activation (38). They all involve the loss of one cysteine residue. It is assumed that the intermolecular disulfide bound is due to another cysteine that is left unpaired and reactive.

Considering our results and these observations, it is likely that the region surrounding the inter-molecular disulfide bridge of rNPR-A\textsuperscript{C423S} is a natural interface involved in activation. Supporting this idea, this region is well conserved among the various guanylyl cyclases, indicating its functional importance (Fig. 1). As we proposed above, it is likely that upon agonist binding this juxtamembranous region establishes a dimeric contact. This tightening, which is likely mimicked by the disulfide linkage of the mutant, might induce some conformational changes in the intracellular domain. However it should be mentioned that, even if the constitutive activation of the C423S mutant can be easily attributed to the interchain bridge, we cannot definitively exclude the contribution of an eventual conformational change induced by the mutation independently of the linkage.

An important player in the regulation of particulate guanylyl cyclase activity is the kinase homology domain (KHD). Hypothetically, a contact of the juxtamembranous domain might modify the intracellular interactions between the subunits, with the initial consequence of removing the repressor effect of KHD. Also, according to the current model of sequential activation of NPRs (8), ATP binding to the KHD precedes and/or is concomitant with guanylyl cyclase derepression. These events must be associated with some modifications in the KHD molecular interactions within the monomer and/or the dimer. Strikingly, our results are showing a dramatic increase of the
ATP effect on GC activity of rNPR-A^{C423S} as compared with the wild type. This may further indicate that the KHD is affected by the mutation and is “trapped” in a conformation corresponding to the activated state. However, such increased sensitivity to ATP has also been observed with rNPR-A when desensitized through ANP pretreatment (9). Thus, this ATP effect on rNPR-A^{C423S} may also be caused by a desensitization arising from the constitutively activated state.

In order to be catalytically active, the GC subunits must establish reciprocal adequate positioning (13). In this respect, the KHD-GC hinge might play an important function as judged from the work of Wilson and Chinkers (18). Furthermore, KHD deletion mutagenesis studies have demonstrated the constitutive activation of rNPR-A (39). Thus, in the absence of KHD the productive positioning of the GC subunits seems to be spontaneous. The intracellular portion of the receptor probably possesses a certain level of autonomous dimerization potential, but the accessibility of the interfaces may be transitory. The net result might depend on the mutual influence of the ECD, the KHD, the hinge, and the GC domains. Such contribution of the intracellular domains may explain the difference on the level of dimerization that we observed between the full length and the ECD^{C423S}.

Finally, another constitutively active rNPR-A mutant has been described. Garbers and colleagues (40) generated a constitutively activated rNPR-A via a mutation within the guanylyl cyclase catalytic domain. Guanylyl cyclase activity of this mutant was 7 times higher than the wild type but was unresponsive to any treatment, including ATP stimulation. Indeed, the guanylyl cyclase of this mutant behaved as autonomously activated without any influence of the receptor context. Noteworthy, this mutant seems not to be desensitized via dephosphorylation. On the contrary, the constitutive activation of rNPR-A^{C423S} basically stems from the receptor context. Furthermore, it is likely to be closely related to a step corresponding to the normal agonist-induced activation. Finally, it has been shown that ligand-induced desensitization of NPR-A is not due to feedback phenomena arising from cGMP accumulation in the cell but probably through dephosphorylation of the KHD during the activation process (41). Hence, the KHD of the C423S mutant might show the same dephosphorylation, an aspect we are currently investigating.

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