Agonistic Induction of a Covalent Dimer in a Mutant of Natriuretic Peptide Receptor-A Documents a Juxtamembrane Interaction That Accompanies Receptor Activation*

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The natriuretic peptide receptor-A (NPR-A) is composed of an extracellular domain with a ligand binding site, a transmembrane-spanning domain, a kinase homology domain, and a guanylyl cyclase domain. In response to agonists (atrial natriuretic peptide (ANP) and brain natriuretic peptide), the kinase homology domain-mediated guanylate cyclase repression is removed, which allows the production of cyclic GMP. Previous work from our laboratory strongly indicated that agonists are exerting their effects through the induction of a juxtamembrane dimeric contact. However, a direct demonstration of this mechanism remains to be provided. As a tool, we are now using the properties of a new mutation, D435C. It introduces a cysteine at a position in NPR-A corresponding to a supplementary cysteine found in NPR-C6, another receptor of this family (a disulfide-linked dimer). Although this D435C mutation only leads to trace levels of NPR-A disulfide-linked dimer at basal state, covalent dimerization can be induced by a treatment with rat ANP or with other agonists. The NPR-A^KD435C mutant has not been subjected to significant structural alterations, since it shares with the wild type receptor a similar dose-response pattern of cellular guanylyl cyclase activation. However, a persistent activation accompanies NPR-A^KD435C dimer formation after the removal of the inducer agonist. On the other hand, a construction where the intracellular domain of NPR-A^KD435C has been truncated (ΔKC^{D435C}) displays a spontaneous and complete covalent dimerization. In addition, the elimination of the intracellular domain in wild type ΔKC and ΔKC^{D435C} is associated with an increase of agonist binding affinity, this effect being more pronounced with the weak agonist pBNP. Also, a D435C secreted extracellular domain remains unlinked even after incubation with rat ANP. In summary, these results demonstrate, in a dynamic fashion, the agonistic induction of a dimeric contact in the juxtamembrane domain of NPR-A. In addition, this process seems to require membrane attachment of the receptor. Finally, the intracellular domain represses this contact at the basal state, showing its potent influence on the outer juxtamembrane domain.

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The natriuretic peptide receptors (NPRs)

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1 The abbreviations used are: NPR, natriuretic peptide receptor; rNPR, rat NPR; ANP, atrial natriuretic peptide; rANP, rat ANP (residues 1–28) or natriuretic peptide A; pBNP, porcine brain natriuretic peptide (residues 1–32); CNP, C-type natriuretic peptide (residues 1–22); API, atriopeptin I (rANP residues 5–25); C-ANF, des-[Gln^{13},Ser^{14},Gly^{6},Leu^{6},Gly^{14}]rANP 4–23–NH₂ (rat); PAG, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; IBMX, 1-methyl-3-isobutylxanthine; ECD, extracellular domain; TM, transmembrane domain; KHD, kinase homology domain; GC, guanylyl cyclase domain; HT-ECD, His-tagged ECD; DMEM, Dulbecco’s modified Eagle’s medium; EpoR, erythropoietin receptor.
More precisely, we took as a basic observation the presence two invariant cysteines, spaced by 6–8 residues, which are present in nearly all of the juxtamembrane domains of NPRs (Fig. 1). These two cysteines have been shown to be linked through an intrachain disulfide bond in rat NPR-A (16). On the other hand, we also pointed out a noticeable exception found in the NPR-C5 receptor, where the first juxtamembrane cysteine is absent (15) (Fig. 1). Consequently, the only juxtamembrane cysteine (Cys469) in NPR-C5 is free to form an interchain disulfide bridge. Hence, this receptor is found as a covalent homodimer (6).

By analogy to the cysteine distribution of NPR-C5, we previously designed the mutation C423S in NPR-A, which eliminates its first juxtamembrane cysteine (15). The expectation was that, in the absence of this cysteine (equivalent to Cys423 in NPR-A), it would permit interchain linkage of the second cysteine (Cys1432 in NPR-A, equivalent to Cys469 of NPR-C5). Indeed, this mutation led to a spontaneously disulfide-bridged NPR-A[C423S] dimer.

This NPR-A[C423S] mutant was also found to be constitutively activated, and it displayed an important increase in the binding affinity of pBNP, a weak agonist (15). Using these observations, we proposed a model where agonists are inducing a dimeric “tightening” in the juxtamembrane region of NPR-A, hence allowing catalytic activation of the guanylyl cyclase. However, we indicated at the time that we could not exclude the contribution of a conformational change induced by the mutation independently of the interchain disulfide linkage (15). For instance, it was not known if the disruption of the Cys1432–Cys469 bond might by itself take part in the constitutive activation of NPR-A[C423S].

In the current study, our objective is to definitively demonstrate that a juxtamembrane dimerization event is associated with NPR-A activation. To limit eventual structural alterations, we chose to avoid the disruption of the Cys423–Cys469 internal bond. For this, we referred to a minor splicing isoform of NPR-C (NPR-C6) that displays a supplementary juxtamembrane cysteine also forming an accessory interchain disulfide bridge (17). By comparing the juxtamembrane regions of NPR-C6 and NPR-A, this supplementary cysteine in NPR-C6 aligns with the aspartate 435 in NPR-A (Fig. 1). We thought that the addition of a cysteine at position 435 might lead again to a covalently dimerized NPR-AC435S. If so, the contribution of a conformational change induced by the mutation would be minimal (15). For instance, it was not known if the disruption of the Cys432–Cys423 bond might by itself take part in the constitutive activation of NPR-A[C423S].

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Conformational Change of NPR-A Juxtamembrane Domain

CNP, or C-ANF were included in the incubation mixture. The agonist-induced dissociation was allowed to proceed for 22 h at 4 °C. Following incubation, the samples were centrifuged in microcentrifuge at 10,000 × g for 10 min. The pellets were carefully resuspended in ice-cold deionized water, and 2× SDS-PAGE sample buffer (without β-mercaptoethanol) was added immediately. The samples were then immediately boiled for 5 min. The covalent dimerization was assessed by Western blotting after the separation of membrane proteins on a 5% SDS-PAGE. The induction of HT-ECDD435C was tested at 4 °C or at 22 °C, with 1 μM rANP, for 22 h in 0.1 ml of binding buffer (50 mM sodium phosphate buffer, pH 7.4, 0.3 mM NaCl, 1 mM EDTA, 0.1% BSA, 0.05% β-mercaptoethanol). The presence of covalent dimer was assessed by Western blotting after the separation of proteins (nonreducing conditions) on a 7.5% SDS-PAGE.

Receptor Binding Assay—125I-rANP was prepared using the lactoperoxidase method as described elsewhere (15). Binding to membranes was performed at 4 °C for 22 h in 0.1 ml of binding buffer (50 mM Tris, pH 7.4, 0.1 mM EDTA, 5 mM MnCl₂, and 0.5% BSA). Competition experiments were done by incubation of 3–5 μg of HEK 293 membrane expressing rNPR-A, rNPR-A D435C, ΔKC, or ΔKC D435C with 10 fmol of 125I-rANP and increasing concentrations of nonradioactive peptides. Bound 125I-rANP was separated from free ligand by filtration on GF/C filters precoated with 1% polyethyleneimine.

Whole Cell Guanylyl Cyclase Stimulation—Cells stably expressing rNPR-A D435C were allowed to grow to subconfluence on 24-well cluster plates. The wells were washed twice with serum-free DMEM and were incubated in a final volume of 1 ml of the same medium containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 0.5% BSA, and varying concentrations (10⁻¹ to 10⁻⁷ M) of rANP. After 1 h of incubation, the medium was collected, and extracellular cyclic GMP was determined by radioimmunoassay as described elsewhere (19). After the assay, 1× SDS-PAGE sample buffer (95 °C) was added to several wells. The wild type and mutant receptor levels were estimated by Western blotting, which was used to normalize their relative cGMP production.

Induction and Measurement of Persistent Guanylyl Cyclase Activity—Stable clones expressing NPR-A and NPR-A D435C were allowed to grow to subconfluence on 10-cm plates. After having washed the cells twice with serum-free DMEM, 6 ml of DMEM (37 °C) containing 0.5% BSA and 10⁻⁷ M rANP was added to the plates. The incubation was allowed to proceed for 30 min in a 5% CO₂ incubator at 37 °C. The cells were then carefully washed twice with DMEM, 0.5% BSA (37 °C) and incubated for another 30 min. After this postincubation, the cells were washed again twice with phosphate-buffered saline (37 °C). Membrane preparation was done as described above except that homogenization, washings (three times), and freezing were realized 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 protease inhibitors. The protein concentration was determined, and these membranes were used for guanylyl cyclase assays as described in other studies (13, 20). 5 μg of membrane proteins were incubated during 10 min at 37 °C in 50 mM Tris-HCl, pH 7.6, with 10 mM MgCl₂, 10 mM IBMX, 1.1 mM manganese phosphate, 1 mM creatine phosphate, 10 units of creatine kinase, 1 mM GTP, and 4 mM MgCl₂. Different conditions were tested using GTP alone (basal) or by adding 1 μM rANP, 1 mM ATP, rANP and ATP together or adding 1% Triton X-100 with 4 mM MnCl₂ instead of MgCl₂. Cyclic GMP was separated from GTP by chromatography on alumina and evaluated by radioimmunoassay as previously reported (19).

Western Blotting and Immunodetection—Membrane proteins were separated on SDS-PAGE in the presence or absence of 5% β-mercaptoethanol in the loading buffer. The proteins were transferred to a nitrocellulose membrane (Bio-Rad) using the liquid Mini Trans-Blot System (Bio-Rad). Detection of NPR-A and ΔKC was achieved using a rabbit polyclonal antiserum raised against the sequence YGERGSSTRG and purified by affinity chromatography. This sequence corresponds to human NPR-A carboxyl terminus preceded by a tyrosine for radioiodination purposes. The rat NPR-A differs from this epitope at a single position; however, both receptors are recognized. Specific signal was probed with an HRP-coupled anti-rabbit polyclonal antibody according to the ECL Western Blotting Analysis System (Amersham Pharmacia Biotech). For the His-tagged HT-ECDD435C, purified aliquots based on the four-parameter logistic equation (21). Radioligand binding data were analyzed with the same program on a model for the law of mass action (22). For simplicity, the same binding data were also analyzed as dose-response curves, and ED₅₀ values are mentioned in the discussion instead of Kᵢ values.

RESULTS

Cellular Induction of a Disulfide-bridged Dimer of NPR-A D435C by rANP—We considered the possibility that the introduction of a cysteine at position 435 could cause a covalent dimerization of rNPR-A subunits. This site was chosen because of its linear alignment with an accessory cysteine involved in the linkage of NPRC-C6 dimers (Fig. 1).

Initial studies with rNPR-A D435C expressed in HEK293 showed, however, only low levels of spontaneous dimerization. At this point, we proceeded to further testing and explored the possibility that some dimeric linkage might be induced by rANP, the main agonist of NPR-A. This hypothesis turned out to be right, since dimers of rNPR-A D435C could be detected after a cellular incubation with 1 μM rANP (30 min, 37 °C). We further detailed this induction through a dose-effect study using a clone stably expressing NPR-A D435C (Fig. 2). A clear signal of ~380 kDa corresponding to NPR-A D435C dimers is detected on nonreducing SDS-PAGE. It is noteworthy that such an induction is not seen in cells expressing wild type NPR-A. A densitometric analysis of the dimeric induction gave an approximate ED₅₀ of ~900 pM, which is higher than the ED₅₀ obtained in cellular guanylyl cyclase stimulation (87 pM). The exact explanation for this difference is not known. However, the reaction between cysteines leading to disulfide formation could be a limiting element of the process and might affect the apparent dose-effect curve. Therefore, considering this supplementary element, an ED₅₀ of ~900 pM constitutes an acceptable value.

These results are showing that ANP induces a particular dimeric contact in the juxtamembrane domain of NPR-A D435C. This conclusion is in accordance with our previous hypothesis based on the observation of constitutively dimerized NPR-
mitted to several conditions using GTP alone (basal) or to-
though extensive washing of cells, followed by postincubation
level of costimulation with ATP plus ANP tends to diminish in
reference of enzyme activity.

thermore, the near absence of NPR-A D435C covalent dimer-
expressing NPR-AWT and NPR-AD435C were treated for 30 min
preparations obtained from ANP-treated cells. Stable clones
in Fig. 3, the ED50 obtained for the wild type (71 ± 12.8 pm)
and the mutant (87 ± 4.6 pm) are essentially comparable, and their
maximal levels of stimulation are similar. It should be noted
that we reproducibly observed a very slight increase in the
basal activity of NPR-A D435C as compared with that of NPR-
AWT (Fig. 3). This almost negligible increase might indicate
that the D435C mutation has very slightly modified the inter-
action as a result of ANP pretreatment. This latter
phenomenon has been previously observed following receptor
desensitization of NPR-A (13). However, this increase in ATP
response is significantly higher in the D435C mutant than in
the wild type receptor (Fig. 4). These results indicate a more
pronounced tendency of NPR-A D435C toward persistent activa-
tion as a result of ANP pretreatment.

A Persistent Activation Accompanies NPR-A D435C Dimer For-
We investigated if the induction of NPR-A D435C dimer forma-
tion does not appear to significantly modify the interaction
in the juxtamembrane region. Alternatively, it may be
attributed to a trace level of NPR-A D435C dimer sometimes
detectable on Western blot through signal overexposure (not
shown).

From this result, it can be reasonably concluded that NPR-
A D435C displays a response to rANP that is very close to that
for wild type NPR-A. Therefore, the conclusions based on the
results obtained with this mutant are likely to be applicable to
the wild type receptor.

A Persistent Activation Accompanies NPR-A D435C Dimer For-
We investigated if the induction of NPR-A D435C covalent
dimerization goes along with persistent activation. Gua-
yl cyclase activation was tested in vitro with membrane
preparations obtained from ANP-treated cells. Stable clones
expressing NPR-A WT and NPR-A D435C were treated for 30 min
(37 °C) with 10 nM ANP. Removal of the ligand was realized
through extensive washing of cells, followed by postincubation
without ligand and washings during membrane preparation
(see “Experimental Procedures”). The membranes were sub-
mitted to several conditions using GTP alone (basal) or

Several observations can be made from the results. First, the
level of costimulation with ATP plus ANP tends to diminish in
membranes obtained from ANP-treated cells (NPR-A: non-
treated 38.9 ± 4.5%, treated 29.8 ± 5.9%; NPR-A D435C: non-
treated 39.8 ± 6.9%, treated 29.6 ± 2.6%). This may be attributed
to some desensitization of the receptor. Also, as shown in
Fig. 4, cellular ANP treatment results in an increase of basal
activity that is 3-fold higher for NPR-A D435C than for the wild
type. Finally, ATP stimulation is appreciably increased for both
receptors as a result of cellular ANP pretreatment. This latter
phenomenon has been previously observed following receptor
desensitization of NPR-A (13). However, this increase in ATP
response is significantly higher in the D435C mutant than in
the wild type receptor (Fig. 4). These results indicate a more
pronounced tendency of NPR-A D435C toward persistent activa-
tion as a result of ANP pretreatment.

The Covalent Dimerization of NPR-A D435C Is Specifically
Induced by Agonists in Vitro—We also tested if the agonist-
duced dimerization could occur in vitro in membrane prepa-
ratations. A clear dose-dependent induction of a ∼260-kDa dimer
can be seen when membranes are incubated with rANP for 22 h
at 4 °C (Fig. 5). Such induction is not seen in the wild type
control. Although dimerization is not complete at the highest
ANP concentration used, an ED50 of approximately 52 nM can
be estimated. This value is far from what is obtained in cellular
activation (Fig. 2). However, at 10−6 M the dimerization is more
complete than what is seen in cells. Considering what has
already been mentioned for the cellular dimeric induction, it is
possible that the lower temperature of incubation (used to
minimize protein degradation) might directly or indirectly
affect the rate of formation of the disulfide bond, which may be
limiting in these conditions.

Nevertheless, to assess the specificity of the process, we
tested the induction using the main selective ligands of the
natriuretic peptide receptors (rANP, API, pBNP, CNP, and
C-ANF). A concentration of 1 nM of the different peptides was
chosen, since, due to the high ED50 of the ANP-induced
dimerization in these conditions, this concentration was likely
to discriminate the relative potency of the agonists. As shown in
Fig. 6, the level of induction correlated well with agonists
specificity on NPR-A (rANP > pBNP > API; CNP and C-ANF
no induction).
In vitro dose-dependent induction by rANP of disulfide-linked rNPR-A(D435C) dimers. Membranes obtained from clones stably expressing rNPR-A or rNPR-A(D435C) were treated with or without 10⁻⁵ M rANP for 30 min at 37 °C. After having washed off ANP, the cells were further incubated for 30 min without ANP. Membranes were prepared as described under "Experimental Procedures." For the in vitro guanylate cyclase assay, membranes were incubated (5 μg) during 10 min at 37 °C in presence of theophylline, IBMX, creatine phosphate, creatine kinase, GTP, and MgCl₂. Different conditions were tested using GTP alone (basal) or by adding 1 μM rANP, 1 mM ATP, rANP, and ATP together or adding 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. The results were normalized as a percentage of maximal activation in Triton/Mn²⁺ (nPR-A: nontreated 456 ± 2 pmol/10 min, treated 413 ± 4.3 pmol/10 min; nPR-A(D435C): nontreated 450 ± 3.0 pmol/10 min, treated 356 ± 0.84 pmol/10 min). Each column represents the mean ± S.D. of three determinations. The experiment was repeated twice with similar results.

### FIG. 4. Effect of a cellular rANP pretreatment on the membrane guanylyl cyclase activity of cells expressing rNPR-A and rNPR-A(D435C). Cells stably expressing wild type rNPR-A or rNPR-A(D435C) were treated with or without 10⁻⁵ M rANP for 30 min at 37 °C. After having washed off ANP, the cells were further incubated for 30 min without ANP. Membranes were prepared as described under "Experimental Procedures." For the in vitro guanylate cyclase assay, membranes were incubated (5 μg) during 10 min at 37 °C in presence of theophylline, IBMX, creatine phosphate, creatine kinase, GTP, and MgCl₂. Different conditions were tested using GTP alone (basal) or by adding 1 μM rANP, 1 mM ATP, rANP, and ATP together or adding 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. The results were normalized as a percentage of maximal activation in Triton/Mn²⁺ (nPR-A: nontreated 456 ± 2 pmol/10 min, treated 413 ± 4.3 pmol/10 min; nPR-A(D435C): nontreated 450 ± 3.0 pmol/10 min, treated 356 ± 0.84 pmol/10 min). Each column represents the mean ± S.D. of three determinations. The experiment was repeated twice with similar results.

### FIG. 5. In vitro dose-dependent induction by rANP of disulfide-linked rNPR-A(D435C) dimers. Membranes obtained from clones stably expressing rNPR-A or wild type rNPR-A were incubated (22 h, 4 °C) with increasing doses of rANP (see "Experimental Procedures"). 25 μg of membrane proteins were present in the incubation. After incubation, the membrane proteins (20 μg) were separated SDS-PAGE (5% gels) under nonreducing conditions. Western blotting using an anti-carboxyl terminus antibody revealed the receptor. The mass standards were as follows: myosine (200 kDa), β-galactosidase (116.3 kDa), phosphorylase b (97.4 kDa). The positions of monomers (M) and disulfide-linked dimers (D) are indicated.

ΔKC-NPR-A WT Is a Disulfide-bridged Dimer—At this point, we wanted to assess the potential role of the intracellular domain in the source of the constraint preventing NPR-A WT ΔKCD435C.

covalent dimerization at the basal state. We thus realized constructions with truncations of the intracellular domain on wild type NPR-A (ΔKC WT) and on NPR-A(D435C) (ΔKC D435C). These were analyzed for their level of covalent dimerization.

Strikingly, ΔKC D435C covalent dimerization appears complete on nonreducing SDS-PAGE (~130 kDa), whereas ΔKC WT remains monomeric (~70 kDa) (Fig. 7). Therefore, the intracellular domain is at the origin of a constraint that represses Cys435 interaction at basal state. On the other hand, ΔKC D435C spontaneously reaches the dimeric state, allowing Cys435 disulfide linkage. These observations indicate that a balance between the antagonistic constraints of the extracellular and the intracellular domains modulates the juxtamembrane dimeric interactions.

Intracellular Constraint Represses High Affinity Binding—We wanted to further define the effects of this constraint originating from the intracellular domain on the functions of the extracellular domain. As a tool, we used the binding properties of rANP and pBNP, which are respectively strong and weak agonists of rNPR-A. As we have shown earlier, the competition binding of pBNP against 125I-rANP is biphasic and can be modeled into high and low affinity components (15, 23). The molecular events associated with this complex binding are not fully understood. However, since the proportion of high affinity component is increased in NPR-A(C423S), we have previously associated the high affinity state with a tight juxtamembrane conformation corresponding to the activated state (15). We also formerly hypothesized that the binding of pBNP was more influenced by the intracellular domain than that of rANP.

Binding studies were performed on NPR-A, NPR-A WT ΔKCD435C, ΔKC WT, and ΔKC D435C. As shown in Fig. 8, the respective binding characteristics of rANP and pBNP on NPR-A WT and NPR-A D435C are similar. Also, the binding of pBNP on NPR-A and NPR-A D435C can be modeled with similar biphasic curves. For simplicity, we will provide here the ED₅₀ values obtained from simple dose-effect analysis instead of Kᵣ values. The ED₅₀ values corresponding to pBNP binding on NPR-A WT (22.9 nM) and NPR-A D435C (28.5 nM) are comparable. However, the pBNP binding curves on the truncated receptors are showing pronounced shifts to the left and are becoming nearly monophasic. The corresponding ED₅₀ values for ΔKC WT and ΔKC D435C are
of also tested for induction of dimerization with rANP (10^9, only a trace amount of spontaneous dimer can be seen. We
test on gel filtration if rANP is inducing noncovalent dimer-
ization and found that the dimerization was complete after an
overnight incubation (22 °C) with 10^6 M rANP (not shown).

These results indicate that, in response to ligand induction,
the juxtamembrane dimeric interactions are probably different
in the ECD receptor mutant as compared with its membrane
counterpart. On the other hand, it is possible that the trans-
membrane domain of the receptor might influence the struc-
ture of the outer juxtamembrane region, and, thus, its presence
might be essential for ligand- induced disulfide linkage of
Cys435.

DISCUSSION

In this work, we have shown that agonists are inducing a
particular dimeric contact in the juxtamembrane domain of
rNPR-A. Our results also indicate that the intracellular do-
main sterically hinders the juxtamembrane tightening associ-
ated with receptor activation. At basal state, this negative
constraint presumably overcomes the ectodomain positive con-
straint. Upon agonist binding, the balance is switched toward
juxtamembrane dimerization and, consequently, receptor ac-
tivation (Fig. 10). In that respect, the induction of the C435
dimeric linkage in the full-length NPR-A^D435C represents a
direct tracer of these molecular events. Finally, membrane
localization of this mutant has proven to be essential for the
covalent dimeric linkage.
Conformational Change of NPR-A Juxtamembrane Domain

FIG. 9. Nonreducing SDS-PAGE of the wild type (HT-ECD) and
mutant (HT-ECDΔD435C) rNPR-A secreted ectodomains. Ecto-
domains were purified on Ni²⁺-nitrilotriacetic acid-agarose gel from
supernatants of HEK 293 cells expressing HT-ECD or HT-ECDΔD435C
as described under “Experimental Procedures.” An aliquot of each prepa-
rated was incubated for 22 h at 4 °C in the presence of 1 μM rANP. The purified
ectodomains and the incubated samples were subjected to
SDS-PAGE (7.5% gels) under nonreducing (NR) and reducing (R) con-
ditions. Western blotting using a commercial anti-tetrahistidine anti-
body revealed the receptor. The mass standards were as follows: myo-
sine (200 kDa), β-galactosidase (116.3 kDa), phosphorylase b (97.4
kDa), bovine serum albumin (66.2 kDa), and ovalbumin (45 kDa).

tamembrane dimerization. On the other hand, our results show a
striking increase of pBNP binding affinity for both ΔKCΔC435C
and ΔKCWT as compared with the full-length receptor, whereas
the affinity of ANP is not affected. Taken together, these ele-
ments strongly indicate that the ΔKC spontaneously reaches a
“tight” dimeric state, probably closely related to the ectodomain
dimeric positioning that occurs in the activated full-length
NPR-A. Since the presence of the intracellular domain affects
more deeply pBNP binding, one can think of the existence of a
“threshold level” of juxtamembrane dimeric tightening beyond
which the intracellular negative constraint might be mainly
overcome by the positive influence of the ectodomain. Hence,
ANP would be much more efficient than pBNP to overcome this
threshold level. Therefore, the observed affinity of an agonist
might result from a combination of its “pure” affinity for the
membrane-anchored ectodomain together with its capacity to
overcome the intracellular negative constraint. According to
this model, the binding results observed with the ΔKC are
providing the “pure affinities” of rANP and pBNP for the mem-
brane-anchored ectodomain. Indeed, the difference of affinity
between rANP and pBNP is much less for the ΔKC (~6–10-
fold) as compared with the full-length receptor (~500-fold).

One may wonder which part of the cytoplasmic domain of
NPR-A is particularly involved in this steric hindrance. One
can suspect a potent influence of the KHD. Of note, it has been
shown that the removal of the KHD leads to a constitutive
activation of the GC (24, 25). Therefore, since the KHD seems
to exert a potent regulatory role on GC activity, it might as well
influence the ectodomain properties.

Agnostic induction of disulfide linkage in a mutated receptor
has been described in one other case (26). In this study, Sorokin
et al. have shown that EGF was stimulating such linkage in a
mutant of the EGF receptor having a supplementary cysteine
in the juxtamembrane domain. The induced dimer was found to
possess persistent tyrosine kinase activity and also displayed
increased high affinity binding. The authors concluded that the
disulfide bridge is stabilizing a particular dimeric arrangement
of the two protomers corresponding to the activated state.

Interestingly also, a recent study by Tanner et al. has shown
that the intracellular domain of the EGF receptor sterically
hinders its EGF-induced dimerization rate (27). Moreover, they
found that when the EGF is completely removed from the
receptor, the activation persists for a long period of time (28).
They hypothesized that this phenomenon might be due to in-
teractions between subunits of the cytoplasmic domains, which
impair significant stabilization of the dimeric state of the ac-
tivated enzyme. A similar phenomenon might explain the resi-
dual persistent activation that we have seen, in the presence
of ATP, with the ANP-pretreated wild type NPR-A (Fig. 4).
Since this persistent activation was significantly higher in the
case of NPR-AΔD435C, it is possible that the formation of the
disulfide bridge might provide further stabilization of the ac-
tivated dimeric complex.

Interesting studies have been recently made on molecular
aspects of the erythropoietin receptor (EpoR) activation (29–
31). Indeed, a relative correlation between agonist potency and
juxtamembrane orientation was deduced from the crystal
structures of EpoR complexed with erythropoietin or with less
potent synthetic agonists. The authors have proposed a model
where the juxtamembrane domains of two subunits are
brought into a closer proximity in response to agonist induction
(32). This mechanism has been supported by in vivo studies
using a protein fragment complementation assay (33). It should
be noted that these studies are showing the intracellular do-
main as passively responding to the agonistic stimulation of
the ectodomain. In addition, truncation of the intracellular
domain of the EpoR is presumably not leading to spontaneous
juxtamembrane dimerization (33). Therefore, the balance of
constraints present in the EpoR seems to differ from that in
NPR-A. Indeed, as we are showing here, the intracellular do-
main of NPR-A exerts a potent constraint on its ectodomain.

One of the main advantages of the current NPR-A mutant is
that ligand induction of covalent dimerization occurs without
the addition of any cross-linking reagent. Notably, the distance
range between the ω-carbons participating in a disulfide bond
is ≤7 Å (34, 35). The disulfide linkage of cysteines separated by
more than 7 Å necessitates motion of the protein backbone (35).
The rate of disulfide formation might be influenced by the
inherent reactivity of cysteines, including accessibility of the
sulphydryl pair and the frequency of structural fluctuations
that cause collisions between the reactive residues (35). This
latter parameter might have influenced the rate of dimer for-
mation in our in vitro assay at 4 °C. It is noteworthy that
studies with the EGF receptor have shown that a reduction of
temperature diminishes the rate of EGF-induced receptor
dimerization, which might be related to reduced structural
transition rate to the active state (27). Nevertheless, the cellu-
lar induction of NPR-AΔD435C dimerization is detectable after a
30-min incubation at 37 °C, indicating a good reactivity of
Cys435 under these conditions.

In our previous work, we mutated the Cys423 of NPR-A into
serine, which left the other cysteine 432 free to form an inter-
chain disulfide bridge. This NPR-AΔC423S dimer displayed an
elevated constitutive activity (15). We then hypothesized that
agonists, during the activation process, are inducing a dimeric
tightening of the juxtamembrane domain of NPR-A. However,
we mentioned then that we could not exclude the possibility
that the mutation had induced a conformational change that
activates the receptor independently of the disulfide bridge.
Following our study, Hao et al. (36) realized the double mutant
NPR-AΔC423S,C432S, which eliminated both juxtamembrane
cysteines. Since this mutant also displayed constitutive activity,
they indicated that the disulfide bridge in NPR-A\(^{423\text{C}}\) was not responsible for the constitutive activation. Unfortunately, the reciprocal NPR-A\(^{423\text{S}}\) mutant was not provided in their study, which would have definitively completed this structure-function analysis and enabled them to unambiguously support their conclusion. They hypothesized that the disruption of the Cys\(^{432}\)–Cys\(^{423}\) linkage had altered the structure of the receptor in this region, which is essential for receptor signaling. In view of the results that we are presenting here, it is possible that this structural modification resulted in the increase of the spontaneous activating “positive” constraint of the ectodomain.

Recently, van den Akker et al. (37) have provided the crystal structure of the NPR-A extracellular domain. This structure is documented up to Asp\(^{435}\) and shows a ligand-free dimer. Indeed, spontaneous noncovalent dimerization of NPR-A ECD has been shown to occur at very high protein concentrations typical of crystallization conditions (38). The C-terminal region (residues 423–435) forms a protruding irregular structure, which shows residue 435 as nonburied, as expected from our results. To complete the dimeric structure, the authors extrapolated the unresolved region 426–435 of one of the monomers from the known structure of the other. From this reconstitution, they calculated a distance of \(~14\) Å between the C-α of the two chains at position 435 and concluded that their structure corresponded to an active dimer.

Our results complement these structural data. As already mentioned, in response to agonist binding, the C-α of residue 435 of the full-length receptor reaches a distance of \(~7\) Å. On the other hand, the 14 Å provided by the structural data is not close enough to mediate the disulfide linkage at this position. This, taken together with the absence of ligand-induced covalent dimerization in the ECD\(^{435\text{SC}}\), suggests that the conformation of the liganded ECD does not exactly correspond to the agonist-activated state of the full-length receptor. Furthermore, according to our results, it is more likely that the ΔKC spontaneously approaches the “activated” conformation. This suggests a significant influence of the transmembrane helix and/or membrane proximity on the tertiary and quaternary structure of the juxtamembrane region.

In conclusion, this study with NPR-A\(^{435\text{C}}\) allowed us to define several fundamental constraints that occur in this receptor. Also, it has provided us with the opportunity to define an activation model supported by the detection of a precise dimeric molecular interaction. Furthermore, this kind of information constitutes a significant asset to interpret the crystallographic data of NPR-A. In summary, these results definitively demonstrate that agonists induce a tight dimeric interaction in the juxtamembrane domain of NPR-A, an event that is closely related to its activation process.

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