Reduced Activity of the NPR-A Kinase Triggers Dephosphorylation and Homologous Desensitization of the Receptor†

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ABSTRACT: NPR-A, the receptor for the atrial natriuretic peptide (ANP), is a 130-kDa protein presenting an extracellular ANP-binding domain, a single transmembrane domain, an intracellular regulatory kinase homology domain (KHD), and a guanylyl cyclase catalytic domain. Upon stimulation, NPR-A receptors are activated to produce cyclic guanosine monophosphate (cGMP) and are subsequently desensitized through dephosphorylation of residues at their KHD. We used wild-type rat (r) NPR-A (WT) and a disulfide-bridged mutant (C423S) expressed in human embryonic kidney (HEK) 293 cells to study receptor phosphorylation. We have previously characterized the C423S receptor as constitutively active and desensitized. At basal state, ^{32}P incorporation in the rNPR-A^{C423S} covalent dimer is about 24 times less efficient than incorporation in the WT rNPR-A. When membranes from WT and rNPR-A^{C423S} are incubated with [^{35}S]ATPγS, the mutant dimer receptor displays 3.5% of the thiophosphate incorporation found for WT rNPR-A. Since the rNPR-A^{C423S} dimer is already extensively dephosphorylated, we then used the WT rNPR-A to study dephosphorylation. As previously documented, adding ANP globally induces time-dependent dephosphorylation of the receptor. However, in pulse−chase experiments with the WT rNPR-A, adding ANP during the chase does not lead to a significant effect on receptor dephosphorylation. On the other hand, thiophosphorylation of the WT rNPR-A previously desensitized with ANP is reduced to 8.3% of the incorporation for untreated receptor, similar to results found with the rNPR-A^{C423S} at basal state. These results demonstrate that ANP-induced rNPR-A desensitization is modulated by a significant reduction in the activity or affinity of the rNPR-A kinase that contributes to the low phosphorylation level after induction. Moreover, we further document a close relationship between tight dimerization, dephosphorylation, and desensitization.

Guanylyl cyclase receptors exist in both soluble and membrane forms in many tissues (*1*−*3*). In mammals, eight different forms have been described, but half of these are orphan receptors (*4*). Three of the remaining four forms are membrane-bound receptors for known ligands (*4*). Cell surface guanylyl cyclase receptors are a unique family of receptors that mediate distinct functions and are also highly conserved among different species. Only two of them, NPR-A[1] and NPR-B, have endogenous catalytic activity that produces a second messenger, cGMP (*5, 6*), in response to binding of different natriuretic peptides (ANP, BNP, CNP). The other form, the NPR-C dimer, acts as a natriuretic peptides scavenger (*7, 8*). Interactions between the signal transduction pathways of the NPRs and the G protein-coupled receptors have recently been found. Interestingly, the NPR-C pathway has been shown to inhibit the adenylyl cyclase/cAMP signal transduction system (*9, 10*), and the NPR-A pathway is possibly implicated in inhibition of G-protein activation (*11*).

The guanylyl cyclase receptor type A, or natriuretic peptide receptor A (NPR-A), is known for modulating vasodilation, natriuresis, and diuresis and acts as counterbalance to the renin−angiotensin−aldosterone system (*12*−*14*). Following the extracellular domain (ECD) of this receptor are the single transmembrane domain, the kinase homology regulatory domain (KHD), and the catalytic guanylyl cyclase domain (GC), which responds to ANP (atrial natriuretic peptide) binding to ECD by producing cyclic GMP (*15*). The ligand to receptor binding stoichiometry is still controversial, but the preformed NPR-A dimer is thought to be activated by an agonist-induced tight dimerization (*16*−*18*). At basal state, the KHD is phosphorylated on four serine and two threonine residues (*19*). This domain also requires ATP binding for the receptor to display maximal catalytic activity (*20*−*22*). Like many cell surface receptors, endogenous or exogenous overstimulation of NPR-A leads to a desensitization of the receptor (*23, 24*). For NPR-A, this process is mediated by the dephosphorylation of the KHD (*25, 26*). Maintenance

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[1] Abbreviations: NPR-A, natriuretic peptide receptor type A; rANP, rat atrial natriuretic peptide-(1−28); KHD, kinase homology domain; HEK, human embryonic kidney; PAGE, polyacrylamide gel electrophoresis; ATPγS, adenosine 5′-*O*-(thiotriphosphate); DMEM, Dulbecco's modified Eagle medium; GR, glucocorticoid receptor; CDKs, cyclin-dependent protein kinases; JNK, c-Jun N-terminal kinase; PSTKs, protein serine/threonine kinases; PSTPs, protein serine/threonine phosphatases.

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of heart failure and hypertension are possibly consequences of the shutdown by desensitization. Recent studies showed an autocrine action of the endogenous ligand ANP on the atrium, and this affected the heart hypertrophy mechanisms in hypertension pathology (*27*−*29*). Genetic alterations of the N-terminal ANP binding domain of human NPR-A or NPR-A knockout in mice have also been correlated with hypertension and cardiac hypertrophy (*30, 31*).

Although some pathways affected by the rise in cGMP and leading to physiological response are known, the pathways directly acting on NPR-A desensitization are still unknown. In turn, cGMP modulates signaling cascades through close downstream effectors, namely, cGMP-dependent protein kinases (PKGs), cyclic nucleotide-gated ion channels (CNG channels), and cGMP-regulated phosphodiesterases (*32*). In the vascular system, natriuretic peptides produce vasodilation through increases in intracellular cGMP. Many effectors have been found to be controlled by the cGMP/PKG system. These effectors are the L-type Ca^{2+} channels, the Ca^{2+}-dependent K^+ channels, the Na^+/Ca^{2+} exchanger, the Na^+/K^+-ATPase, the Ca^{2+}/Mg^{2+}-ATPase of the plasma membrane and of the sarcoplasmic reticulum membrane (associated with phospholamban), the IP_3 receptor, and myosin light-chain phosphatase (*32*). None of those effector pathways has been shown to modulate NPR-A desensitization by short-term feedback inhibition. Thus, the presence of a cGMP-independent pathway closely associated with the KHD domain of the NPR-A is not excluded. However, ANP autoregulates the NPR-A level through suppression of receptor gene expression and receptor synthesis. This long-term suppression is suspected to act through a cGMP-dependent element located upstream from the gene's transcription starting point (*33*).

Studies on NPR-A or NPR-B $^{32}PO_4$ content and guanylyl cyclase activity after ANP treatment showed that the decrease in activity accompanied decreased ^{32}P content in a time-dependent fashion (*25, 34*). These results echoed similar findings with sea urchin guanylate cyclase, which had indicated time- and agonist-dependent loss of 15 phosphates/mol of protein resulting in desensitization and a dramatic 10 kDa gel shift (*35*). The experiments with the NPRs looked at the overall desensitization process without detailing the molecular mechanisms involved in receptor dephosphorylation. Not much is known about the protein kinases and protein phosphatases acting on the phosphorylation state of the NPR-A. When individual protein kinases (PKA, PKC, PKG) were added to the purified NPR-A in vitro, each enzyme was able to phosphorylate the receptor equally (*36*). Also, treatment of whole cells with phorbol esters, known to activate PKCs, leads to the dephosphorylation of one residue on the NPR-A (*37*). Regarding the phosphatase that dephosphorylates the NPR-A, evidence for an association with protein phosphatase 5 (PP5) has been shown (*38*), and a recent investigation showed that this association is mediated by the formation of a multiprotein complex with HSP 90 and $p50^{cdc37}$ (*39*).

We previously described a NPR-A^{C423S} mutant receptor that is a disulfide-bridged covalent dimer (*17*). This mutant displayed high-affinity binding to natriuretic peptides, was responsive to ATP, and showed constitutive activation and desensitization (*17*). The term "constitutive activation and desensitization" means that the receptor has high basal activity but is unresponsive to agonist. These observations suggested a maintained receptor conformation possibly similar to the desensitized form of the WT NPR-A. The desensitization observed also suggested a dephosphorylation of the mutant NPR-A at basal state. Since we did not have to add agonist to the C423S receptor to obtain a desensitized receptor, we used this mutant at first to investigate phosphorylation events. Thus, studies were undertaken to determine the phosphorylation state of the NPR-A^{C423S} and its phosphorylation/dephosphorylation kinetics compared to those of wild-type receptor. Here, we show that the dimerization event following the C423S mutation is the event that triggers dephosphorylation and constitutive activity of the NPR-A^{C423S}. Also, the NPR-A-phosphorylating kinase has markedly reduced activity for the NPR-A^{C423S} desensitized form and for the ANP-treated WT receptor, whereas the NPR-A-dephosphorylating phosphatase has no significantly increased activity for the ANP-activated WT receptor. Thus, the unidentified KHD-phosphorylating kinase represents a short-term regulator of NPR-A desensitization.

EXPERIMENTAL PROCEDURES

Cell Culture. The NPR-A^{C423S} mutant (previously described; *17*) and the wild-type receptor were stably expressed in HEK 293 cells (*17*). The cell line was grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 units of penicillin/streptomycin, and 600 μg/mL G-418 (Geneticin, Boehringer Mannheim), in a 5% CO_2 and 37 °C incubator. Geneticin was removed one step before any experiment with the cells.

Membrane Preparations. Stable cell lines expressing WT and C423S NPR-A receptors were rinsed with phosphate-buffered saline and scraped in ice-cold homogenization buffer (50 mM Hepes, pH 7.4, 50 mM NaCl, 20% glycerol, 1 mM Na_3VO_4, 10 mM NaH_2PO_4, and 10 mM NaF containing 10^{-5} M Pefabloc, 10^{-6} M leupeptin, 10^{-6} M pepstatin, and 10^{-7} M aprotinin). Cells were homogenized with a Polytron homogenizer and centrifuged 30 min at 30500*g* in a Sorvall SS-34 rotor. The pellet was resuspended in 2 mL total volume of the same buffer and homogenized again by aspiration through a needle. Finally, membranes were aliquoted, frozen in liquid nitrogen, and stored at −80 °C. The protein concentration was determined by use of the BCA protein assay kit (Pierce) and ranged from 800 to 2000 μg/mL.

Western blotting, Immunodetection, and Immunoprecipitation. Membrane proteins were separated on SDS-containing 5% or 7.5% polyacrylamide gels under nonreducing or reducing conditions (5% β-mercaptoethanol) and transferred to a nitrocellulose membrane (Trans-Blot, 0.45 μm, Bio-Rad) by use of the mini or full-size Trans-Blot transfer system (Bio-Rad). Detection of NPR-A was achieved with an affinity chromatography-purified rabbit polyclonal antiserum raised against the sequence YGERGSSTRG that is the sequence of the C-terminal portion of human NPR-A. Specific signal was detected with a horseradish peroxidase-coupled anti-rabbit polyclonal antibody according to the ECL Western blotting analysis system (Amersham Pharmacia Biotech). Immunoprecipitation was carried out by addition of the anti-NPR-A antibody to the receptor solubilized in solubilization buffer (50 mM Hepes, pH 7.4, 10% glycerol, 100 mM NaCl,

0.1 mM EDTA, 1 mM Na_3VO_4, 10 mM NaH_2PO_4, 10 mM NaF, 1% Triton X-100, and protease inhibitors) to get 2% final antibody concentration. Incubation was pursued 18 h at 4 °C, and a 2-fold volume excess of protein A−Sepharose CL-4B gel (Amersham Pharmacia Biotech) prewashed in solubilization buffer was added and incubated with agitation by rotation 4 h at 4 °C. The gel was washed 5−7 times by centrifugation with 10 volumes of solubilization buffer and once with solubilization buffer without detergent. The gel was then eluted with hot sample buffer (0.25 M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 10% β-mercaptoethanol, and 0.0025% bromophenol blue), and the eluate was loaded on SDS−PAGE. In cases where whole cells were the starting material, a preclearing was accomplished by addition of normal rabbit serum and the protein A−Sepharose to the solubilized membrane preparation, as mentioned, and the supernatant was submitted to specific immunoprecipitation. In the case of SDS−PAGE separation under nonreducing conditions, 6 M urea was added in both the gel and sample buffer, and 20 mM iodoacetamide was added to homogeneization buffer in order to avoid cysteine shuffling of the mutant monomer receptor (see Figures 1, 2, and 5B).

ANP-Induced Dephosphorylation Experiment. This experiment was based on the protocol of Potter et al. (*25*). Briefly, duplicate plates of HEK 293 cells expressing WT NPR-A were incubated 4 h in DMEM without sodium phosphate containing 1 mCi/mL [^{32}P]orthophosphate. After the addition of 1 μM ANP for the periods of time indicated, cells were solubilized with solubilization buffer containing 0.2 μM okadaic acid and receptor was purified by immunoprecipitation as described above. Proteins were then submitted to SDS−PAGE and the ^{32}P signal associated with the NPR-A in the dried gel was visualized with a PhosphorImager (Molecular Dynamics).

^{32}P Incorporation and Pulse−Chase Experiments. Approximately 5×10^5 cells expressing WT or C423S rNPR-A were plated in 3 mL of DMEM onto 3.5 × 1.0 cm wells in Linbro multiwell plates. After 24 h, cells were washed with 3 mL of the same medium without sodium phosphate and incubated 4 h in 1 mL of DMEM without sodium phosphate containing 0.5−1.0 mCi of [^{32}P]orthophosphate (Amersham Pharmacia Biotech). The excess radioactivity was removed, and the cells were washed with 3 mL of DMEM and then incubated in 3 mL of DMEM for different times. The medium was removed, and the cells were washed with 3 mL of ice-cold phosphate-buffered saline and solubilized twice with 0.5 mL of cold solubilization buffer containing 0.2 μM okadaic acid. For maximal incorporation determination or in the case of a simple ^{32}P incorporation study, labeled cells were washed and homogenized after the 4 h incorporation period. In all experiments, two wells were used in parallel in Western blot analysis to evaluate receptor quantity between the cell types or samples.

[^{35}S]ATPγS Incorporation Studies. Each membrane preparation (WT and C423S) was diluted in incubation buffer (25 mM Hepes, pH 7.4, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM Na_3VO_4, 10 mM NaH_2PO_4, 10 mM NaF, and protease inhibitors) to obtain a final concentration of 100 ng/μL in eppendorf tubes containing 0.2 μM okadaic acid final. [^{35}S]ATPγS (specific activity > 1000 Ci/mmol) was added to have an activity of 20 nCi/μL in a final volume of 100 μL. Nonspecific incorporation was determined by addition of 10^{-4} M ATPγS. Tubes were placed on ice, and then membranes were added to the mixture and incubated at 37 °C for different times. To end the incorporation, tubes were placed on ice and 1 mL of ice-cold stop buffer (50 mM Hepes, pH 7.4, 10% glycerol, 100 mM NaCl, 5 mM EDTA, 0.2 μM okadaic acid, and protease inhibitors) was added to each tube. Tubes were centrifuged at 4 °C for 30 min at 12000*g*, and the pellets were resuspended in 100 μL of solubilization buffer. The receptor was purified by immunoprecipitation and SDS−PAGE as described previously, and radioactivity was measured in a PhosphorImager (Molecular Dynamics).

Statistical Analysis. Comparison of treatment groups was done by unpaired Student's *t* tests. In the case of time course of dephosphorylation, curves for control and ANP treatment groups were first analyzed by nonlinear regression according to a sum of two (fast and slow) exponential components by use of the MS Excel function solver. Comparison of control and treatment curves was then performed by stepwise ANOVA testing of a second curve fitting by use of a common formula based on the same sum of two exponentials for both curves.

RESULTS

WT and NPR-A^{C423S} Receptors Detection by Western Blot Analysis. As previously shown, NPR-A^{C423S} is a disulfide-bridged covalent dimer (*17*). Membrane preparations from HEK 293 cells expressing wild-type NPR-A and NPR-A^{C423S} were loaded on SDS−PAGE under reducing and nonreducing conditions. As shown in Figure 1A, the migration under reducing conditions (left panel) points to a 130 kDa band corresponding to the molecular weight of the WT rNPR-A. When cleavage of the disulfide bridge is avoided by removal of the reducing agent β-mercaptoethanol, the migration of the C423S mutant indicates a molecular weight of about 260 kDa, consistent with the presence of a rNPR-A dimer (Figure 1A, right panel). It is noteworthy that a 130 kDa band remains and accounts for about 10−15% of the total signal as determined by densitometry. The signal for the monomer varied depending on the experiment but always remained between 10% and 20% of the total immunodetection signal. In Figure 1B, qualitative dephosphorylation was investigated by gel shift in Western blot analysis. A gradual shift in molecular weight, consistent with dephosphorylation, can be observed, independent of receptor amount. The receptors, ranked by weight, are found in the following order: NPR-A^{C423S} < ANP-activated NPR-A < NPR-A. Thus the NPR-A^{C423S} appears to be more dephosphorylated than ANP-treated WT NPR-A. However, we cannot exclude that part of the shift seen for the NPR-A^{C423S} may be the consequence of a slight change in receptor conformation induced by the mutation.

Thus, the disulfide-bridged rNPR-A formed by the C423S mutation revealed itself as a good system to study the desensitization process. Also, as already reported, we reproducibly found that NPR-A does not spontaneously form covalent higher-ordered receptor oligomers. This allows for a direct comparison between the inactive monomeric state and the NPR-A^{C423S} covalent dimer, the latter presumably corresponding to the activated form of the receptor. On the other hand, because we could not conclude that the gradual

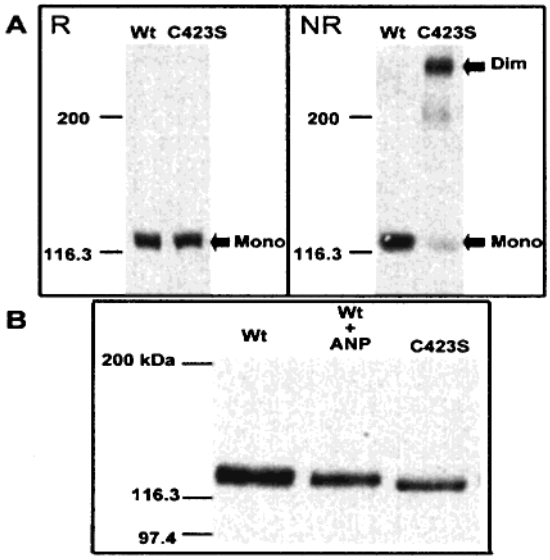

FIGURE 1: Western blot analysis of WT rNPR-A and rNPR-A^{C423S}. (A) Analysis showing the intermolecular disulfide bridge in the mutant receptor. Membranes prepared from HEK 293 cells expressing wild-type (WT) rNPR-A and mutant rNPR-A^{C423S} were purified by SDS−PAGE (5% gels) under reducing (R) and nonreducing (NR) conditions. In each lane, 20 μg of membrane proteins was loaded. The receptor was revealed by Western blotting with an anti-carboxyl terminus as described under Experimental Procedures. (B) Analysis showing the gel shift pointing to dephosphorylation. Membranes of WT NPR-A (WT), ANP-treated NPR-A (WT + ANP), and NPR-A^{C423S} (C423S) were purified also by SDS−PAGE under reducing conditions and detected as described. The molecular mass standards (in kilodaltons) were myosin (200), β-galactosidase (116.3), and phosphorylase b (97.4). The positions of monomers (Mono) and disulfide-linked dimers (Dim) are indicated ($n = 3$).

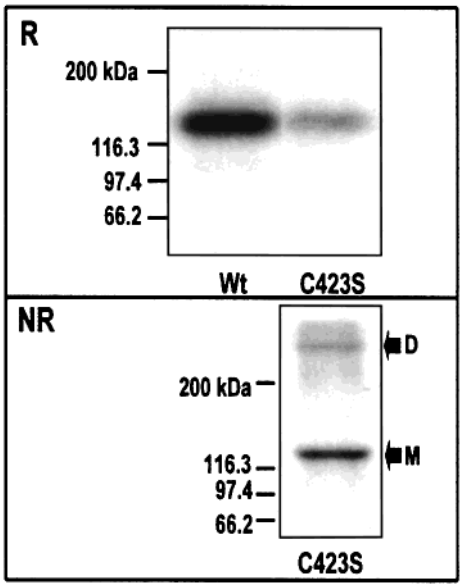

FIGURE 2: Basal state ^{32}P incorporation in WT and C423S rNPR-A. Upper panel (R): rNPR-A^{C423S} mutant receptor is dephosphorylated. In two separate experiments, ^{32}P-labeled HEK 293 cells expressing WT rNPR-A (WT) and rNPR-A^{C423S} (C423S) were solubilized, immunoprecipitated, and fractionated by SDS−PAGE under reducing conditions. The gel was dried and exposed to PhosphorImager (Molecular Dynamics) for visualization. Lower panel (NR): the ^{32}P signal is mostly contained in the minor rNPR-A^{C423S} monomeric form. The same experiment as in the upper panel was accomplished in duplicate with rNPR-A^{C423S}, only this time under nonreducing conditions. The molecular mass standards (in kilodaltons) 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.

shift observed in Western blot analysis was generated only by dephosphorylation, we sought to determine the phosphorylation state of each receptor form and the phosphorylation/dephosphorylation kinetics leading to each state.

NPR-A^{C423S} Is Dephosphorylated at Basal State in Whole Cells. NPR-A^{C423S} was previously shown to be a desensitized receptor, approximately 37-fold less activated by maximal treatment with ANP than the WT receptor (*17*). Since dephosphorylation has been shown to be the modulating event of desensitization (*25, 26*), we investigated the phosphorylation state of NPR-A^{C423S}. Figure 2 represents the ^{32}P content of both WT and C423S receptors at basal state, as purified on SDS−PAGE. Under reducing conditions (upper panel), WT NPR-A has approximately 9.5 times the ^{32}P content of the NPR-A^{C423S}. But under these reducing conditions, results were suspected to be biased. In fact, as shown by Western blot analysis, the NPR-A^{C423S} heterogeneous population of receptors is composed of monomer and dimer. Hence, the possibility remained that these two components were distinct maturation products, each possibly having a distinct phosphorylation state. In turn, the same ^{32}P incorporation studies were repeated with NPR-A^{C423S} but under nonreducing conditions. As shown in Figure 2 (lower panel), most of the radioactive signal is found for the band representing the NPR-A^{C423S} monomer, even though this form is present in smaller amount than the dimer form as indicated by Western blot analysis. Thus, the ^{32}P radioactivity signal values were corrected for receptor quantities (Table 1). The corresponding value for WT receptor was based on the fact that the sum of the radioactive signals of NPR-A^{C423S} is approximately 9.5 times less than WT NPR-A as evaluated before. In turn, the WT NPR-A/NPR-A^{C423S} dimer signal ratio gives a value of ~24, indicating a large decrease in ^{32}P content for the mutant C423S dimer. On the other hand, the WT NPR-A/NPR-A^{C423S} monomer signal ratio is around 1.4, indicating a similarity in ^{32}P content between the monomer form of the mutant and the WT NPR-A. These data show a drastic reduction in ^{32}P content when the mutant receptor is found in dimeric form, showing a close relationship between dimerization and the phosphorylation state.

Inhibition of rNPR-A Phosphorylation by Amiloride. To segregate the dephosphorylation process associated with the WT rNPR-A receptor, we initiated pulse−chase experiments. These experiments are useful for proteins that are phosphorylated at basal state and for which there is a constant phosphate turnover. The C423S mutant is extensively dephosphorylated at basal state. In turn, use of the WT receptor was necessary in such studies on dephosphorylation. The pulse−chase experiment was validated with the use of the diuretic drug amiloride. This molecule is known to be a nonspecific protein kinase inhibitor, acting as an ATP analogue ($K_i = 350$ μM) (*40*). It acts on intact cell systems and is 3-fold concentrated intracellularly (*40*). On the other hand, it has no effect on protein phosphatases (*40*). Also, it has been shown to increase the number of high-affinity binding sites on the NPR-A and to inhibit the hormone-dependent cyclase activity of NPR-A (*22, 41*). We used amiloride to investigate its effect on NPR-A phosphorylation and to show that this experiment efficaciously isolates the activity of the NPR-A phosphatase. Thus, we labeled HEK

Table 1: ^{32}P Content of Dimeric and Monomeric rNPR-A^{C423S} Relative to WT rNPR-A[a]

receptor	quantity[b] (arbitrary units)	radioactive signal (pixels)	corrected radioactive signal[c] (pixels)	WT/mutant signal ratio[d]
rNPR-A WT	1000	34 510 ± 2390[e]	34 510 ± 2390	----
rNPR-A^{C423S} monomer	94	2340 ± 120	24 880 ± 1230	1.39 ± 0.07
rNPR-A^{C423S} dimer	906	1300 ± 140	1430 ± 150	24 ± 2

[a] HEK 293 cells expressing WT and rNPR-A^{C423S} were incubated 4 h with ^{32}P, receptors were purified, and radioactivity was measured by PhosphorImager, as described under Experimental Procedures. Values are the mean ± SEM of two experiments. [b] Receptor quantity as determined by densitometry of results of Western blot analysis (Figure 1A). [c] Radioactive signal calculated when all receptor quantities are set at 1000 arbitrary units. [d] The WT/mutant signal ratio represents the WT receptor radioactive signal value divided by the radioactive signal found for each mutant receptor form. [e] Radioactive signal values for WT rNPR-A were calculated knowing that the sum of the signals for rNPR-A^{C423S} is 9.5-fold less than for WT receptor (Figure 2, upper panel).

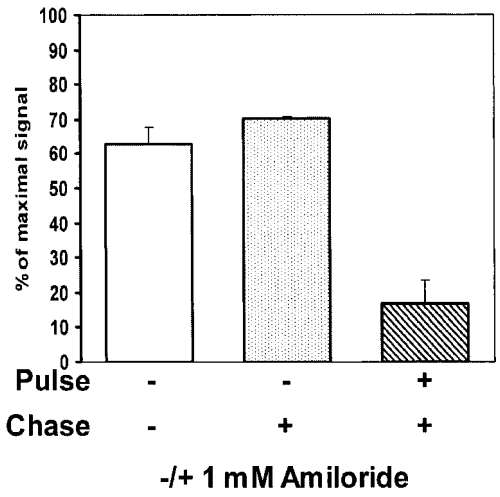

FIGURE 3: Effect of amiloride on WT rNPR-A ^{32}P incorporation. Validation of the pulse−chase method was accomplished by use of amiloride. Duplicate plates containing HEK 293 cells expressing WT NPR-A were incubated in DMEM without sodium phosphate for 4 h with 1 mCi/mL [^{32}P]orthophosphate (pulse). After that period, cells were washed with regular medium, and incubation was resumed for 1 h (chase) in phosphate-replete medium. Where indicated, 1 mM amiloride was added only during the chase or during both the pulse and the chase. The cells were washed and solubilized in homogeneization buffer, and the NPR-A was immunoprecipitated and purified by SDS−PAGE as described under Experimental Procedures. Phosphorylation was quantitated by PhosphorImager analysis. Results are shown as percentage of the maximal incorporation found after the 4 h of pulse ($n = 3$, mean ± SEM).

293 cells expressing WT rNPR-A with ^{32}P for 4 h (pulse), and after that period of time, the radioactive medium was replaced with regular medium (chase) for 1 h (see Experimental Procedures). In some pulse−chase experiments 1 mM amiloride was added either during the chase (1 h), to assess the efficiency of the chase step in blocking further ^{32}P incorporation, or during both the pulse and the chase, to test the ability of amiloride to inhibit protein kinases. As shown in Figure 3, the 1 h chase period led to a 35% reduction in ^{32}P content of the receptor. Adding amiloride during the chase did not significantly reduce WT rNPR-A ^{32}P content beyond the level obtained in the sample containing regular medium only. However, the presence of amiloride during both the pulse and the chase led to an 80% reduction in NPR-A ^{32}P content. This shows that amiloride inhibits the NPR-A-phosphorylating enzyme activity and that protein kinases have no impact on the NPR-A ^{32}P content during the chase period.

ANP-Induced NPR-A Dephosphorylation Is Not Mediated by an Increase in the Activity of the NPR-A Phosphatase. First, as a control, we needed to show the global effect of ANP on receptor phosphorylation at basal state. The time-dependent effect of adding 1 μM ANP to ^{32}P-labeled HEK 293 cells expressing WT rNPR-A is shown in Figure 4A. The ^{32}P content is reduced by ~40% after 1 h and by ~60% after 2 h. These results are consistent with those of Potter et al. (*25*), showing ANP-induced dephosphorylation of NPR-A. Second, to investigate the effect of ANP specifically on the NPR-A phosphatase, we repeated pulse−chase studies with the addition of 1 μM ANP during the chase for different times. Chase with phosphate-replete medium efficiently stops de novo ^{32}P incorporation into NPR-A. Figure 4B shows the effect of rANP on the time-dependent decrease in ^{32}P content of WT rNPR-A during chase. Addition of rANP slightly but nonsignificantly ($F = 1.1$, NS) alters the kinetics of dephosphorylation of the receptor. Thus, ANP produces no significant effect on receptor-dephosphorylating protein phosphatase activity. Therefore, the inducing effect of ANP on NPR-A dephosphorylation shown in Figure 4A must be due to reduced activity or affinity of the NPR-A kinase, since ANP has no effect on the activity or affinity of the NPR-A phosphatase (Figure 4B). We then proceeded to directly document the effect of ANP on NPR-A phosphorylation under conditions where receptor dephosphorylation is prevented.

NPR-A Kinase Is Unable To Phosphorylate the NPR-A^{C423S} Dimer in Vitro. The rNPR-A^{C423S} was first used as a substrate for the receptor-phosphorylating protein kinase, because this mutant was permanently desensitized and because investigating this mutant's phosphorylation kinetics could lead to information on WT receptor mechanistic events. To isolate the receptor-phosphorylating protein kinase activity, we used [^{35}S]ATPγS as a substrate for the receptor-phosphorylating kinase. In fact, protein kinases are unable to discriminate between this substrate and regular ATP. However, thiophosphates are poor substrates for protein phosphatases. Thiophosphorylation is thus partially protected against dephosphorylation. Okadaic acid, a protein phosphatase inhibitor, was also added to further reduce protein phosphatase activity (*42*). Figure 5A represents the time-dependent arbitrary units of thiophosphorylation of each receptor form under reducing conditions. Again each signal was corrected for receptor number as determined by Western blot analysis. The mutant receptor has a blunted signal compared to WT rNPR-A, overall representing 19.3% ±

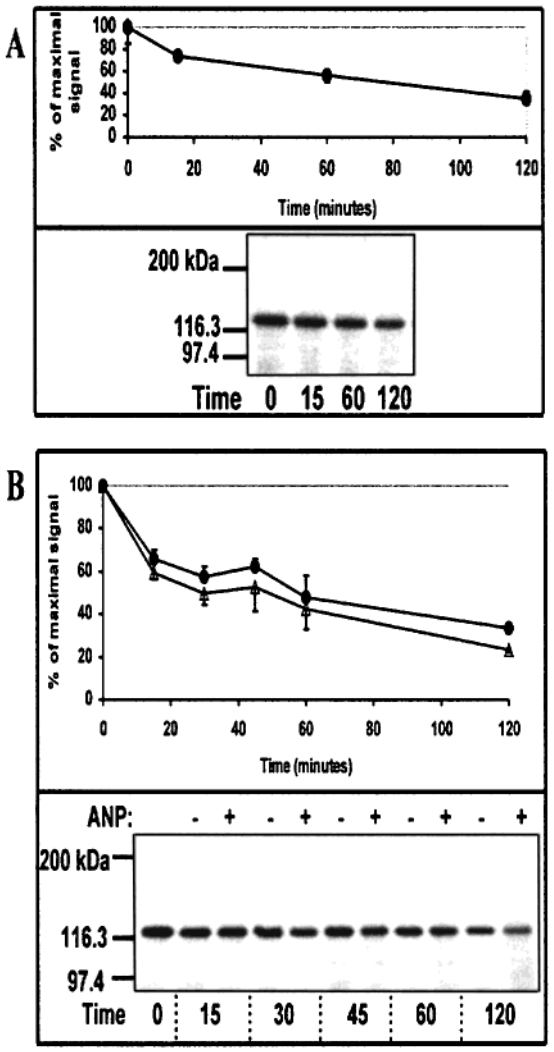

FIGURE 4: Effect of rANP on rNPR-A dephosphorylation. (A) Effect of rANP on global rNPR-A phosphorylation. Duplicate plates of HEK 293 cells expressing WT NPR-A were incubated 4 h in DMEM without sodium phosphate containing 1 mCi/mL [^{32}P]-orthophosphate. After that period, 1 μM ANP was added for the periods of time indicated, followed by solubilization of the cells for purification of the NPR-A by immunoprecipitation and SDS−PAGE. The vertical bars within each symbol represent the range of values obtained from two separate experiments. (B) Effect of rANP on the isolated dephosphorylation component. Exposure of HEK 293 cells expressing WT rNPR-A to ANP, after ^{32}P incorporation, does not increase the rate of ^{32}P removal from the receptor. Duplicate plates of HEK 293 cells expressing WT receptor were incubated 4 h with ^{32}P. Radioactivity was then removed and replaced with regular medium (DMEM) (●) or medium containing 1 mM rANP (△) for different times, followed by preparation of whole-cell detergent extracts for purification of NPR-A by immunoprecipitation and SDS−PAGE under reducing conditions as described under Experimental Procedures. The vertical bars within each symbol represent the range of values obtained from two separate experiments, which were assayed in duplicate. Results are shown as percentage of the maximal incorporation found after the 4 h of pulse. The images under graphs depict results seen by PhosphorImager of a representative time-dependent ^{32}P content experiment.

6.6% of the signal for WT receptor (Table 2). Under nonreducing conditions (Figure 5B), the NPR-A^{C423S} dimeric form shows an even more decreased incorporation representing 3.5% ± 3.5% (Table 2) of the thiophosphorylation signal of the WT NPR-A after 30−60 min (some experiments showed no significant signal). This implies that the mutant

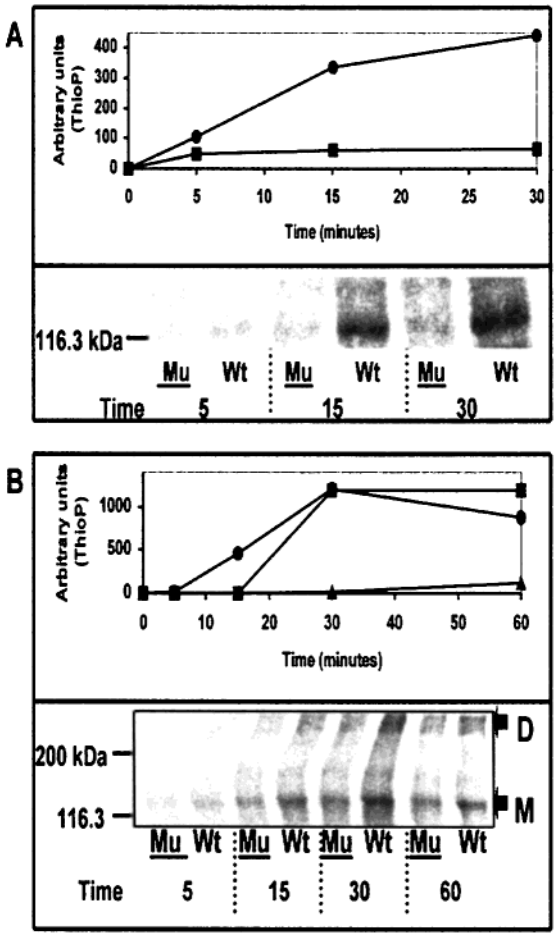

FIGURE 5: Thiophosphorylation of the rNPR-A^{C423S} (Mu). (A) Membrane preparations of HEK 293 cells expressing either WT rNPR-A (●) or rNPR-A^{C423S} (■) were incubated at 37 °C for different times with [^{35}S]ATPγS in buffer containing protein phosphatase inhibitors (NaF, Na_3VO_4, NaH_2PO_4, okadaic acid), solubilized in detergent, and purified by immunoprecipitation and SDS−PAGE under reducing conditions. Proteins were blotted on a nitrocellulose membrane and radioactivity was revealed by PhosphorImager. Receptors are in equal amounts, as determined by Western blot analysis. (B) Same experiment as in panel A was accomplished with purification under nonreducing conditions. Signals for WT rNPR-A (●), monomeric rNPR-A^{C423S} (■), and dimeric rNPR-A^{C423S} (▲) are depicted. Receptor quantities were adjusted to obtain equal monomeric receptor phosphorylation since WT receptor was expressed 5 times more than the mutant receptor. The results in the graphs are representative of one experiment of two and have been corrected for receptor quantity, background signal, and nonspecific ^{35}S signal. The images under graphs represent time-dependent PhosphorImager signals seen for one representative experiment.

receptor dimeric form, although possessing potential phosphorylation sites, is inaccessible to the receptor-phosphorylating protein kinase. Yet, the rNPR-A^{C423S} monomeric form has a phosphorylation profile similar to that of WT rNPR-A (Figure 5B). It represents 93.4% ± 6.6% (Table 2) of the WT receptor thiophosphorylation in the same time range. It seems that the mutant receptor's transition from monomer to covalent dimer changed the activity of the kinase for the KHD domain, presumably through a dimerization-induced conformational change of this domain. Also, a close regulation of the guanylyl cyclase domain by the KHD has been documented (*43*, *44*). Hence, it seems unlikely that the phosphorylated form of the rNPR-A^{C423S} (monomer) has the same guanylyl cyclase activity patterns as the dephospho-

Table 2: ^{35}S Content of Each Mutant Receptor Form as Percentage of the Content of WT rNPR-A[a]

conditions	mutant receptor form	WT rNPR-A ^{35}S content[b] (%)
reducing	rNPR-AC423S	19.3 ± 6.6
nonreducing	rNPR-AC423S monomer	93.4 ± 6.6
	rNPR-AC423S dimer	3.5 ± 3.5

[a] Membrane preparations of HEK 293 cells expressing WT or NPR-A^{C423S} were incubated with ATP-γ-^{35}S for different times, receptors were purified, and radioactivity was measured in PhosphorImager, as described under Experimental Procedures. [b] Results were first corrected for receptor quantity (Figure 5), and then the values at different times for the mutant receptor were expressed as percentage of the signal for WT rNPR-A. Values are the mean ± SEM of four experiments.

rylated form (dimer). This could explain why we measured a 1.4-fold induction for the C423S mutant in guanylate cyclase experiments (*17*); i.e., the residual monomer is not desensitized.

rANP-Desensitized rNPR-A Is Not Phosphorylated by the NPR-A Kinase. The results of both the pulse−chase experiments and the rNPR-A^{C423S} thiophosphate incorporation studies indirectly proved that the desensitization process is mainly due to blunting of the NPR-A-phosphorylating kinase activity, with minimal changes in phosphatase activity. The objective was now to show it directly by studying the phosphorylation of the native receptor. Thus, we repeated [^{35}S]ATPγS incorporation studies, using membrane preparations of WT receptor desensitized with ANP. The membrane preparations were done with HEK 293 cells expressing WT rNPR-A that had been treated or not with 1 μM rANP for 1 h. Our hypothesis is that the KHD domain of the covalent dimeric form of the C423S mutant has a conformation very similar to the KHD domain of the WT desensitized receptor. Thus, we suspect that the agonist-desensitized WT receptor will have a blunted thiophosphate incorporation profile compared to untreated WT receptor. Figure 6 shows the time-dependent arbitrary units of thiophosphorylation for WT rNPR-A treated or not with rANP. Okadaic acid was again added to the assay to inhibit protein phosphatase activity. The ANP-desensitized receptor incorporates less thiophosphates than the WT receptor at all times measured. The activated NPR-A has 8.3% ± 4.6% of the thiophosphate signal found for inactivated NPR-A after 30 min, a value comparable (t = 1.3, NS) to the reduction found for the dimeric NPR-A^{C423S} (3.4% ± 3.2%). Also, in Figure 6, there seems to be a time-dependent significant reduction in thiophosphate content in the agonist-activated rNPR-A. This interesting pattern could be explained by gradual and residual in vitro desensitization induced by ANP maintained in the in vitro assay to ensure constant presence of the agonist.

It is noteworthy that attempts to observe differences in thiophosphate content when ANP was added in vitro to membrane preparations of WT NPR-A were unsuccessful, suggesting that the complete molecular machinery necessary for initiating NPR-A desensitization is altered with cell breakage (data not shown).

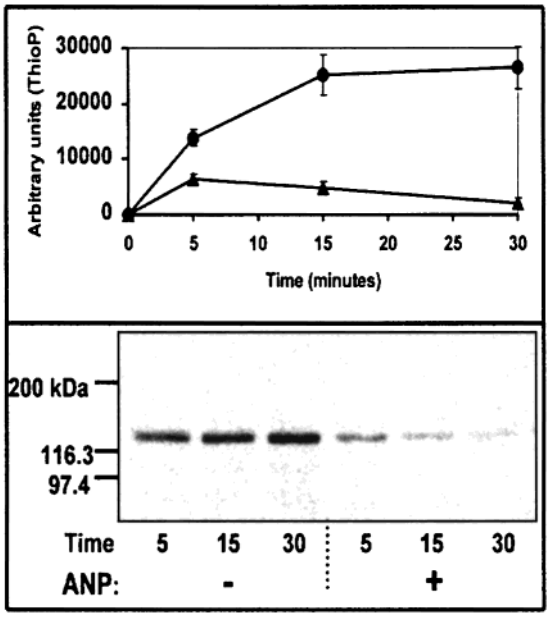

FIGURE 6: Thiophosphorylation of the rANP-treated WT rNPR-A. HEK 293 cells expressing WT rNPR-A were incubated 1 h in medium containing (▲) or lacking (●) 1 μM rANP. Membrane preparations of these cells were then made and incubated at 37 °C for different times with [^{35}S]ATPγS in buffer containing protein phosphatase inhibitors (NaF, Na_3VO_4, NaH_2PO_4, okadaic acid), immunoprecipitated, and separated in SDS−PAGE under reducing conditions as described under Experimental Procedures. The graph depicts time-dependent thiophosphorylation in four experiments (mean ± SEM). The image under the graph shows PhosphorImager results of one representative experiment.

DISCUSSION

In this work, we have shown that the C423S mutation leads to a disulfide-bridged dephosphorylated receptor, already known to be unresponsive to ANP (*17*). This receptor dimer displays a significantly blunted capacity to be thiophosphorylated in membrane preparations. On the other hand, a residual noncovalently bound form of this mutant has thiophosphate and ^{32}P content similar to that of WT rNPR-A. The diuretic drug amiloride was found to efficiently inhibit the NPR-A kinase. We also studied the effect of excess ANP on WT receptor dephosphorylation in whole cells, and found this effect to be nonsignificant after 2 h (F = 1.1, NS). Moreover, WT receptor desensitized with ANP has reduced thiophosphorylation compared with untreated receptor. The incorporation seen in the WT is not significantly different from what is found for the covalent dimeric C423S mutant (t = 1.3, NS). This further demonstrates that the KHD conformation in the dimeric rNPR-A^{C423S} mimics the conformation of this domain in the agonist-stimulated rNPR-A. Overall, this shows that receptor desensitization by dephosphorylation is carried out by abrogated kinase activity and relatively maintained phosphatase activity. This might be due to an inhibition of the protein kinase involved or to reduction in the accessibility of the kinase to the NPR-A receptor. Moreover, this process seems to be associated with receptor dimerization.

Studies from Potter and Hunter (*19*, *45*) previously reported direct evidence supporting NPR-A desensitization by dephosphorylation. Mutation of all the normally phosphorylated serine and/or threonine residues (S497, T500, S502, S506, S510, and T513) to alanine, except T513, abolished the hormone-dependent guanylyl cyclase activity of NPR-A. Also, mutation of all known phosphorylation sites to glutamate yielded a receptor still 4 times less active than the normal NPR-A. They concluded the presence of an

unknown dephosphorylation-independent process, although they did not investigate the binding affinities of their mutant receptors for ANP. Furthermore, suggestion was made that activation of the NPR-A-phosphorylating protein phosphatase might mediate dephosphorylation. This was based on results showing a protective effect of the phosphatase inhibitor microcystin on the guanylyl cyclase activity of the WT rNPR-A in membrane preparations. Nevertheless, phosphorylation and dephosphorylation are dynamic events in whole cells. It remains possible that the receptor-associated kinase shows a bigger drop in activity for the activated receptor than the increase in phosphatase-related activity, as shown in the present study.

Besides membrane guanylyl cyclases, very few examples of signal transduction interruption mechanisms by protein dephosphorylation of Ser/Thr residues have been described. The only receptor with similar regulation is the nuclear glucocorticoid receptor (GR). All steroid hormone receptors are phosphorylated and go through hormone-induced hyperphosphorylation of three to eight serines in the N-terminal domain (*46*). Hyperphosphorylation is specific for glucocorticoid agonists and is due to accelerated phosphorylation (*47*). With GRs (eight suspected phosphorylation sites), alanine mutants are up to 75% less active in hormone-induced transactivation (*47*). Moreover, Zuo et al. (*48*) found that Ser/Thr protein phosphatase type 5 (PP5) is closely associated with the GR. In fact, PP5 activation leads to inhibition of $p21^{WAF1/Cip1}$ transcription, mediated by dephosphorylation of GR and p53 transcription activators. In turn, $p21^{WAF1/Cip1}$, a general cyclin-dependent protein kinase (CDKs) inhibitor, cannot repress these cell growth mediators, and cell growth ensues. The GR-associated kinase has not been identified, but in vitro studies point to CDK kinases and MAPK kinases (*49*). This system differs from the NPR-A dephosphorylation system in the activation sequence. Both receptors are phosphorylated at basal state, but GR probably undergoes conformational change due to ligand binding, which permits phosphorylation, and thus activation, by an associated protein kinase. In turn it possibly possesses a broader "dephosphorylation range" compared to rNPR-A. Indeed, as suggested in the literature, steroid hormone receptor phosphorylation serves not as an on−off switch but modulates function more subtly.

Another example is the c-Jun/JNK complex. The regulation of c-Jun by the c-Jun N-terminal kinase (JNK) modulates apoptosis, cell cycle arrest, and DNA repair processes (*50*−*52*). c-Jun is regulated via heterodimerization with Fos or through phosphorylation of clusters of Ser/Thr residues in the C- or N-terminus that either inhibit DNA binding or activate transcriptional activity, respectively (*53*). Phosphorylation of Ser^{63}/Ser^{73} by JNK enhances transcriptional activity and stability by activating the p300/CBP coactivator recruitment (*54*, *55*). May et al. (*56*) described the interaction of the c-Jun/JNK complex. Their results suggest that JNK can be tethered to c-Jun through multiple interacting regions (δ docking site, P−Q region, and bZIP domain) and, upon activation, can induce c-Jun phosphorylation without need of dissociation from the substrate. No c-Jun-associated phosphatase has been identified so far, but evidence for the activation of JNK by phosphorylation and the presence of a JNK-inactivating phosphatase activated by Hsp72 has been found (*57*). In this case, regulation seems to stem more from three-dimensional interactions than regulation by phosphorylation. This modulation may be important in rNPR-A, since enzyme activity experiments have shown that the elements required for sensitization and desensitization of rNPR-A likely reside in the membrane itself (*42*), and because phosphorylation of rNPR-A is suspected to be an "all or nothing" event (*19*).

Specificity of phosphorylation/dephosphorylation events is often achieved through subcellular targeting of distinct modules of kinase or phosphatase toward substrates at specific subcellular locations (*58*−*60*). Based solely on general substrate specificity, no assumptions can be made as to which enzyme could be responsible for NPR-A desensitization, since both types of enzyme families have been described as potentially highly specific in vivo (*58*, *59*). Ser/Thr kinases (PSTKs) and phosphatases (PSTPs) differ in the means of creating structural and functional diversity. Compared to kinases, the structural complexity of PSTPs in vivo is created as a result of a combination of associated regulatory domains and subunits (*58*, *59*). In turn, care should be taken in the interpretation of results of dephosphorylation processes following cell disruption, since accessory subunits may be released or bound with cell breakage.

Recently, Huo et al. (*61*) investigated the double mutant $NPR\text{-}A^{C423S,C432S}$. These mutations eliminate the first intramolecular disulfide bridge aside of the membrane in the extracellular domain. In consequence, no covalent interchain link can be made in this receptor. The authors found that this mutant also displays constitutive activity and concluded that the constitutive activity we found for our $NPR\text{-}A^{C423S}$ mutant was not linked to the dimerization process but rather to mutation-induced alteration of the receptor structure. These interesting results bring other possibilities. It remains possible that the C432S mutation alone creates this phenotype. Also, our hypothesis is that dimerization is the activation inducer. Obviously, this process does not need to be a covalent dimerization. Namely, any event or mutation that will bring two ectodomains together will, if correctly oriented, have a chance to activate the receptor. We gathered strong evidence showing that one of the intrinsic receptor structures that seems to be required in order to block this event in absence of agonist is the extracellular juxtamembrane region (*18*). It is likely that completely removing this structure, as was done with $NPR\text{-}A^{C423S,C432S}$, creates a receptor that can noncovalently dimerize in the absence of agonist in whole cells and demonstrate constitutive activity. We must specify that the terms monomer and dimer are used to discriminate noncovalently linked $NPR\text{-}A^{C423S}$ from the disulfide-bridged dimer. Noncovalent dimerization of the monomeric $NPR\text{-}A^{C423S}$ is not excluded, consistent with a model of loose or tight conformations (*17*, *18*).

Furthermore, in the current study, we investigated the phosphorylation state of our $NPR\text{-}A^{C423S}$ mutant. We found that the receptor population generated by this mutation is heterogeneous. As explained earlier, 10−15% of NPR-A mutant stays in a WT receptor phosphorylation-like monomeric form, while 85−90% is dimeric and dephosphorylated. The minor form may have been generated if two monomers of mutated receptor failed to interact during the maturation process. As mentioned, the dimeric species showed characteristics inherent to the activated form of regular rNPR-A, namely, ATP modulation and dephosphorylation. The phos-

phorylated state of the residual monomeric rNPR-A^{C423S} suggests an inactive form in the absence of agonist. In turn, the overall 1.4-fold activation by ANP, seen in our previous study in whole-cell guanylyl cyclase stimulation of mutant rNPR-A^{C423S} (*17*), might be due to the presence of this residual monomer. Thus, the sole C423S mutation does not create as such a constitutively active receptor. Dimerization during the maturation process is the event that triggers this phenotype.

The reasons for the constitutive activity of the NPR-A^{C423S} dimeric form are still unknown, but dephosphorylation of the KHD with tight dimerization is consistent with desensitization. It is possible that the C423S mutation created a "frozen" receptor not flexible enough, in its dimeric state, to enter the next step of rNPR-A desensitization, namely, the guanylyl cyclase activity shutdown. Nevertheless, we show that treatment of WT rNPR-A with ANP creates a strong drop in receptor-phosphorylating kinase activity, as is seen for the dimeric rNPR-A^{C423S}. Also, the WT rNPR-A-dephosphorylating phosphatase seems to have unvarying activity toward the KHD in whole-cell experiments. No unique PSTK consensus sequence was identified for the clustered sites of the KHD. Thus, identification of the agonist-sensitive NPR-A-phosphorylating kinase and of processes regulating the enzyme is of great interest.

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