Activation of many single-transmembrane receptors requires ligand-induced receptor oligomerization. We have examined the oligomerization of the atrial natriuretic peptide receptor, NPR-A, using epitope-tagged receptor in a co-immunoprecipitation assay. Unlike other single-transmembrane receptors, NPR-A oligomerized in a ligand-independent fashion. Extracellular receptor sequences were both necessary and sufficient for oligomer formation. NPR-A was also able to oligomerize with the related natriuretic peptide receptor, NPR-B. A truncated NPR-A lacking most of the cytoplasmic domain blocked activation of the full-length receptor, presumably through formation of an inactive heteromer. These results indicate that oligomerization of this single-transmembrane receptor is important for the transduction of a conformational change across the plasma membrane but are not consistent with models in which natriuretic peptide receptor oligomerization serves merely to bring intracellular domains together.

Several large families of receptors have single transmembrane domains (1, 2). The mechanism by which these receptors transduce an extracellular signal into intracellular conformational changes is controversial. The transmembrane domain connecting extracellular and intracellular domains is thought to be a rigid α-helix. In one model, movement of this helix into the membrane would be the driving force for a conformational change (3). This model does not appear to account for receptor activation in solution, however. A widely accepted model for tyrosine kinase receptors involves receptor activation by ligand-induced dimerization (1). In this model, ligand binds to a receptor's extracellular domain, changing its conformation to favor its dimerization. A resulting association between intracellular domains then leads to their activation. In support of such a model, a number of single-transmembrane receptors form dimers in a ligand-dependent fashion (1, 4). The observation that growth factor receptors with cytoplasmic truncations can form inactive heterodimers with wild-type receptors further supports the importance of associations between intracellular domains (5-8).

A recently described family of single-transmembrane receptors has intrinsic ligand-activated guanylyl cyclase activity (9). The receptor/guanylyl cyclases are similar in overall topology to the growth factor receptors, including a protein kinase-like domain that seems to function as a negative regulator of guanylyl cyclase activity (10, 11). Two members of this family are the natriuretic peptide receptors, NPR-A and NPR-B (10, 12-14). NPR-A is a receptor for the cardiac natriuretic peptides, ANP and BNP, while NPR-B is a receptor for the brain natriuretic peptide, CNP (15). Both ANP and BNP induce natriuresis, diuresis, and vasodilation upon release from the heart, but their secretion is differentially regulated (16, 17). CNP has been suggested to have central effects on fluid homeostasis (18-21). While intravenous administration of these peptides has dramatic physiological effects and the secretion of ANP and BNP is clearly elevated in cardiovascular disease states (22, 23), the normal physiological roles of the natriuretic peptides are unknown (24).

The observation that both adenylyl cyclases and soluble guanylyl cyclases require two catalytic subunits for activity (25-28) has led to suggestions that ligand-induced dimerization of receptor/guanylyl cyclases will lead to their activation by facilitating association of guanylyl cyclase catalytic domains (29, 30). Consistent with this idea, ANP receptors in tissue extracts have been reported to migrate both as monomers and as higher molecular weight complexes when analyzed by gel filtration chromatography (31, 32). Also, a partially purified ANP receptor has been shown to migrate in SDS gels as a high molecular weight disulfide-linked complex (33); the disulfide linkages may be an artifact of purification (cross-linking studies have generally not observed disulfide-linked complexes of NPR-A (34)), but the high molecular weight NPR-A complex could be physiological. Opposing this idea, studies using combinations of gel filtration and sedimentation analysis have suggested that the receptor/guanylyl cyclases are monomeric, even in the presence of ligand (35, 36). These apparently conflicting results have been difficult to interpret because of the problems inherent in attempts to estimate molecular weight for proteins in detergent solution and because high molecular weight receptor complexes formed in crude tissue extracts or partially purified preparations may represent either receptor oligomers or complexes of receptor with other proteins. We have used epitope-tagged NPR-A and a co-immunoprecipitation assay to re-examine this question, and report here that NPR-A does in fact oligomerize, but in a ligand-independent fashion. We also describe the ability of a truncated NPR-A to form an inactive heteromer with the full-length receptor. These results substantiate the importance of receptor oligomerization in signal transduction but challenge the notion that the association of intracellular domains is sufficient for receptor activation. In addition, we describe the formation of a heteromeric receptor by NPR-A and NPR-B.
**Experimental Procedures**

**Construction of Plasmids—**The pSVL vector (Pharmacia LKB Biotechnology Inc.) was used for all expression constructs. Structures of all constructs described below are summarized in Fig. 1. Full-length cDNA clones and deletions of the kinase-like (ΔKIN) or guanylyl cyclase (ΔCYC) domains of rat NPR-A have been described previously (10, 11, 14). To delete all but 5 amino acids of the intracellular domain of NPR-A, a synthetic linker containing a termination codon and Xhol site was inserted into the PvuII site at position 1709 of the rat NPR cDNA. This construct was named ΔKC to denote deletion of kinase-like and cyclase domains. To express the soluble intracellular domain of NPR-A with an amino-terminal epitope "tag," a synthetic linker/adaptor was inserted between the BamHI and PvuII sites at positions 1454 and 1709, replacing wild-type sequences. Digestion with BamHI and BglII excised sequences encoding a methionine followed by the FLAG epitope (DYKDDDDK) and all but the first amino acid of the intracellular domain. This construct was named INT. A construct encoding the FLAG epitope and the guanylyl cyclase catalytic domain, but lacking most of the kinase-related sequences, was prepared by inserting the FLAG linker/adaptor above into the PvuII site at position 2549 of NPR-A. This construct was named CYC. A construct encoding the FLAG epitope and the kinase-like domain, but not the guanylyl cyclase domain, was prepared from the INT construct by inserting a synthetic linker containing a termination codon and Xhol site into the Fgel site at position 2619 of NPR-A. This construct was named KIN. Insertion of FLAG and myc epitopes carboxy to the signal sequences of NPR-A, ΔCYC, and ΔKC involved several steps. The 0.3-kilobase MluII/ApaI fragment of NPR-A was cloned into the corresponding sites of the plasmid pGEM7Zf(+) (Promega), and Sphi and NruI sites were introduced at positions 305 and 315 of NPR-A by oligonucleotide-directed mutagenesis (37). A synthetic linker/adaptor encoding either the FLAG epitope or a myc epitope (EQKLLISEDDL) was then inserted between the Sphi and NruI sites. The resulting MluII/Apal fragment was used to replace the wild-type MluII/Apal fragment in pSIN-NPR-A, pSVL-ΔCYC, or pSVL-ΔKC. To express a secreted extracellular domain (EXT), a termination codon was introduced at position 1635 of NPR-A using the polymerase chain reaction. In all cases, the sequences of regions altered from the wild-type cDNA were confirmed by direct sequencing. Full-length mutations in cDNA fragments synthesized during in vitro polymerase reactions.

**Preparation of a Recombinant Vaccinia Virus Expressing the Extracellular and Transmembrane Domains of NPR-A—**The ΔKC mutant described above was cloned into the XhoI site of vaccinia transfer vector pVZneo, and a vaccinia virus expressing ΔKC (VV-ΔKC) was then prepared by homologous recombination with vaccinia virus strain WR DNA (39). Expression of the truncated NPR-A protein by the plaque-purified recombinant virus was confirmed by measuring specific binding of [3H]-labeled ANP (40) to BSC40 cells (41) infected with wild-type vaccinia virus or VV-ΔKC. Specific binding was approximately 5-fold higher to cells infected with VV-ΔKC than to cells infected with wild-type virus (approximately 50% versus approximately 10% of total counts/min specifically bound).

**Antibodies—**Monoclonal antibodies M1 and M2, recognizing the FLAG peptide sequence, were obtained from ImmuneTex and IBL. Monoclonal antibody 9E10, recognizing a c-myc peptide sequence (42, 43) was obtained from Oncogene Science. Polyclonal antibodies recognizing the extracellular domain of NPR-A were prepared by immunizing female New Zealand White rabbits with 10 plaque-forming units of VV-ΔKC at a single subcutaneous site, followed by a boost 1 month later. Anti-ΔKC sera were collected 10 days after each injection, as well as 3 weeks after the booster injection.

**Cell Culture and Transfections—**COS-7 cells were grown in DMEM/F12 (1:1) containing 20 mM Hepes pH 7.4 and 7% Calf Serum (GIBCO/BRL). Transfections followed a DEAE-dextran method (44). All experiments were performed 2 days after transfection.

**Determination of Cyclic GMP in Intact Cells—**Cyclic GMP concentrations for 5 min at 37 °C in the absence or presence of natriuretic peptides (10, 40) and preparation of cell extracts (45) were performed as described. Cyclic GMP determinations were performed by radioimmunoassay (46). One day after transfections, plates were trypsinized, and cells transfected with the same plasmids were pooled and seeded into 12-well plates. Assays were performed on the following day.

**Metabolic Labeling and Immunoprecipitation—**Metabolic labeling was performed by incubating cells for 4 h at 37 °C in methionine-free Dulbecco's modified Eagle's medium containing [35S]methionine (approximately 30,000 Ci/mmol) with cold 20 mM Hepes pH 7.4, 150 mM NaCl, and lysed in 1 ml of cold buffer A (20 mM Hepes pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 5 μg/ml aprotinin). After 10 min on ice, lysates were clarified by centrifugation at 2 °C for 10 min at 18,500 × g. 0.5 μl of monoclonal antibody or 5 μl of polyclonal antibody were then added to the clarified lysates, followed by incubation on ice for 1 h. Immune complexes were collected by different methods, based on the antibodies' abilities to bind to protein A. We used either an excess of goat anti-mouse IgG beads (Sigma, antibodies M1 and M2, 1-h incubation at 4 ºC with continuous end-over-end mixing) or formalin-fixed Staphylococcus aureus cells (Boehringer Mannheim, anti-ΔKC, 10-min incubation on ice). Immune complexes were then washed 4 × 1 ml with buffer A (M1 or M2) or 3 × 1 ml with buffer A (anti-ΔKC) and eluted by heating in sample buffer (47). For immunoprecipitations with the Ca2+-dependent monoclonal antibody M1, 10 mM CaCl2 was included in lysis and wash buffers. Samples were analyzed by SDS-PAGE in 10 or 11% gels (47), followed by staining with Coomassie Blue, treatment with sodium dodecyl sulfate (48), drying, and fluorography. Molecular weights were determined by comparison with proteins from BSA and standards from Bio-Rad.

**Western Blotting—**To test the immunoreactivity of myc-NPR-A (Fig. 2, center), COS-7 cells in 6-cm plates were lysed in 1 ml of 20 mM Hepes pH 7.4, 40 mM KCl, 1 mM EDTA, 1 mM EGTA, 10 μg/ml aprotinin by passage 10 times through a 22-gauge needle. After centrifugation at 12,000 x g for 20 min at 4 °C, the supernatant was added 100 μl of 20 mM Hepes pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 10 μg/ml aprotinin. After incubation for 20 min on ice, centrifugation was repeated, and an equal volume of 2 × sample buffer (47) was added to the supernatants, followed by heating.
for 5 min at 95 °C. Thirty μl of each detergent extract was analyzed by SDS-PAGE on a 10% minigel followed by blotting to nitrocellulose membranes (49). Rainbow molecular weight markers (Amersham Corp.) were used as size standards. Blots were blocked overnight in 20 mM Tris, 157 mM NaCl, 0.1% Tween 20, pH 7.6 (TBST) supplemented with 5% nonfat dry milk. Blots were then incubated with the primary antibodies in blocking buffer, washed with TBST, and incubated with peroxidase-conjugated anti-mouse IgG in the blocking buffer. After washing with TBST, the bound antibodies were detected by chemiluminescence (Amersham ECL kit).

To examine co-immunoprecipitation of myc-NPR-A with FLAG-NPR-A (Fig. 6), 10-cm cultures of co-transfected COS-7 cells were subjected to immunoprecipitation with 1 μg of M2 and 20 μl of anti-mouse IgG beads as described above, except that cells were not metabolically labeled. Equal aliquots of the immunoprecipitates were analyzed by Western blotting with 9E10 or M2 as described above, except that prestained molecular weight standards were from Bio-Rad.

Materials—FLAG peptide was purchased from Research Genetcs. Rat ANP was from Boehringer Mannheim and Sigma; rat BNP and rat CNP were from Bachem. Synthetic oligonucleotides were from Research Genetics, Midland, and Promega. Anti-mouse IgG conjugated to horseradish peroxidase, chemiluminescence reagents, and ECL nitrocellulose membranes were from Amersham. [35S]Methionine (Trans-35S-labeled) was from ICN, and [3H]-labeled cyclcic GMP tyrosine methyl ester (2500 Ci/mg) was from Biomedical Technologies. Other radiochemicals were from Du Pont-New England Nuclear. Enzymes for restriction and modification of DNA were from Gibco/BRL, Boehringer Mannheim, and New England Biolabs. Vaccinia virus, strain WR, and the pZVneo transfer vector were gifts of Dr. Gary Thomas (Vollum Institute). Reagents for DNA sequencing were from U. S. Biochemical Corp. (Sequenase kit). Other chemicals were of the best grade available from Sigma, VWR, Gibco/BRL, and Boehringer Mannheim.

RESULTS

Characterization of Epitope-tagged NPR-A—We inserted sequences encoding peptide epitopes, recognized by commercially available monoclonal antibodies, into the sequence of NPR-A. The "FLAG" epitope (DYKDDDDK) or a myc epitope (EQKLISEEDL) was positioned such that it would be present at the amino terminus of NPR-A after cleavage of the signal peptide. Addition of either epitope did not interfere with the ability of the receptor to bind or be activated by ANP, as judged by ANP-dependent formation of cyclic GMP in intact COS-7 cells transfected with wild-type and epitope-tagged NPR-A (Table I).

We tested the abilities of two monoclonal antibodies to the FLAG epitope, M1 and M2, to immunoprecipitate FLAG-NPR-A from COS-7 cells after metabolic labeling with [35S]methionine (Fig. 2, left). M1 recognizes the FLAG sequence only when it is precisely at the amino terminus of a protein. A single residue preceding the FLAG sequence prevents recognition, as does the removal of the amino-terminal residue of the epitope (50). In contrast, M2 can recognize internal or amino-terminal FLAG sequences. Both M1 and M2 immunoprecipitated a protein of the expected size (130 kDa). The 130-kDa protein was not immunoprecipitated in the presence of excess FLAG peptide and was not immunoprecipitated from cells transfected with control vector or cells expressing NPR-A lacking the FLAG epitope (Fig. 2, left). These results confirmed the specificity of the FLAG antibodies and suggested that signal cleavage occurred at the predicted site (10, 51).

We also tested the ability of a monoclonal antibody to the

![Table I](image)

| Transfection | ANP | Cyclic GMP formed |
|--------------|-----|------------------|
| Vector       | 0.06 ± 0.02 |                  |
| Vector (+)   | 2.6 ± 0.3   |                  |
| NPR-A        | 0.17 ± 0.01 | 0.02 ± 0.01      |
| NPR-A (+)    | 84 ± 4      | 84 ± 4           |
| FLAG-NPR-A   | 0.15 ± 0.01 | 0.15 ± 0.01      |
| FLAG-NPR-A (+)| 78 ± 2   | 78 ± 2           |
| myc-NPR-A    | 0.21 ± 0.01 | 0.21 ± 0.01      |
| myc-NPR-A (+)| 110 ± 3    | 110 ± 3          |
myc epitope, 9E10, to react with myc-NPR-A in Western blots (Fig. 2, center). The antibody detected a 130-kDa band in a particular fraction from cells expressing myc-NPR-A but not from cells expressing FLAG-NPR-A. Expression of FLAG-NPR-A was confirmed by Western blotting with M2. Thus, the expressed myc-NPR-A protein reacts specifically with monoclonal antibody 9E10.

To analyze untagged NPR-A, specific antisera were prepared by immunizing rabbits with a recombinant vaccinia virus expressing the extracellular and transmembrane domains of NPR-A (VVAKC). The antisera, but not the preimmune sera, immunoprecipitated metabolically labeled NPR-A (130 kDa) from COS-7 cells transfected with the respective cDNAs but not from cells transfected with control vector (Fig. 2, right). The AKC protein migrates as a doublet on SDS gels; the basis for this heterogeneity has not yet been determined. None of the anti-ΔKC sera cross-reacted with NPR-B expressed in COS-7 cells (data not shown; see Fig. 8).

**Co-immunoprecipitation Assay for Receptor Oligomerization**—We reasoned that if NPR-A normally forms dimers or oligomers, heteromers might form between truncated NPR-A proteins and full-length FLAG-NPR-A. In that case, an antibody to the FLAG epitope would immunoprecipitate a complex containing the untagged truncated protein as well as the FLAG-tagged full-length NPR-A. The truncated protein would be distinguished from FLAG-NPR-A by its faster migration during SDS-PAGE. Structures of the truncated proteins used in this study are shown in Fig. 1. As shown in Fig. 3 (top), M2 immunoprecipitates of cells co-expressing FLAG-NPR-A and various truncated NPR-A proteins lacking portions of the intracellular domain contained the truncated proteins (ACYC, AKIN, ΔKC; 115, 97, and 62 kDa, respectively) as well as FLAG-NPR-A. The truncated proteins were not immunoprecipitated by M2 in the absence of FLAG-NPR-A, indicating that they are immunoprecipitated due to their association with FLAG-NPR-A, and not due to reactivity with M2. Control immunoprecipitations with anti-ΔKC confirmed the presence of the truncated proteins in the lysates lacking FLAG-NPR-A (Fig. 3, bottom). Thus, NPR-A proteins lacking part or all of the cytoplasmic domain can form complexes with full-length NPR-A. No other proteins were detected that were specifically co-immunoprecipitated with FLAG-NPR-A.

In contrast, FLAG-tagged soluble intracellular NPR-A constructs did not form immunoprecipitable complexes with untagged full-length NPR-A; NPR-A was not co-precipitated by M2 from lysates containing FLAG-tagged intracellular proteins (INT, KIN, CYC; 60, 35, and 33 kDa, Fig. 3, top), and the soluble intracellular proteins were not co-precipitated by anti-ΔKC from lysates containing NPR-A (Fig. 3, bottom). Control immunoprecipitations with anti-ΔKC confirmed the presence of untagged NPR-A protein in the co-transfected cells (Fig. 3, bottom). Thus, extracellular and/or transmembrane sequences were necessary for receptor oligomerization.

**Ligand Independence of NPR-A Oligomerization**—Addition of ANP did not affect the formation of receptor complexes (Fig. 4). The amounts of untagged proteins containing cytoplasmic deletions (ΔCYC, ΔKIN, ΔKC) co-immunoprecipitated with FLAG-NPR-A by M2 were similar in the absence or presence of ANP (Fig. 4, left). In this experiment, cells were treated with ANP before lysis, and ANP was included in the lysis buffer. Thus, NPR-A differs from other single-transmembrane receptors that undergo ligand-induced dimerization (1, 4); NPR-A appears to be a constitutive dimer or oligomer.

We had not expected to find that oligomerization required extracellular/transmembrane but not cyclase catalytic domain sequences; others have observed dimerization of the purified catalytic core expressed in bacteria (52). It seemed possible that the cyclase domain of the full-length protein might be unable to dimerize in the absence of ligand but that conformational changes after ANP binding might lead to cyclase domain dimerization and activation. Therefore, we examined the ability of full-length NPR-A and of ΔKIN (which is a constitutively active guanylyl cyclase (11)) to associate with the 33-kDa FLAG-tagged catalytic domain (CYC) in cells treated with ANP (Fig. 4, right). Neither NPR-A nor ΔKIN was co-precipitated by M2 with the FLAG-tagged CYC protein, in the absence or presence of ANP (Fig. 4, right). Based on these experiments, NPR-A oligomerization is ligand-independent and requires extracellular and/or transmembrane sequences.

**Extracellular Sequences Are Sufficient for Oligomerization**—To test whether the extracellular sequences alone were sufficient for NPR-A oligomerization, M2 immunoprecipitates were prepared from lysates of cells expressing both FLAG-NPR-A and the secreted extracellular domain of NPR-A,
FIG. 4. Lack of effect of ANP on co-immunoprecipitation. Co-transfected COS-7 cells were treated as in Fig. 3, except that in some cases lysis was preceded by treatment with 100 nM ANP for 10 min at room temperature in Hanks’ balanced salt solution containing 10 mM Hepes, pH 7.4, and, for the ANP-treated cells, lysis buffer contained 10 nM ANP. Immunoprecipitation with M2, SDS-PAGE, and fluorography was carried out as described under “Experimental Procedures.”

FIG. 5. Co-immunoprecipitation of the secreted extracellular domain of NPR-A with FLAG-NPR-A. COS-7 cells were transfected with the indicated plasmids, and aliquots of lysates were immunoprecipitated with M2 (left) or anti-AKC (right), followed by SDS-PAGE and fluorography as described under “Experimental Procedures.” Positions of FLAG-NPR-A, AKC, and EXT are indicated by bullets.

lacking the transmembrane sequences (EXT). Others have shown that similar constructs are secreted and retain ANP binding activity (51, 53). We reasoned that formation of heteromers between NPR-A and the secreted EXT protein might anchor a fraction of the soluble protein to the cell. As shown in Fig. 5, EXT does associate with FLAG-NPR-A, as evidenced by M2 co-immunoprecipitation of a 55-kDa/57-kDa doublet. The amount of EXT co-precipitated with FLAG-NPR-A is similar to the amount of AKC co-precipitated (Fig. 5). In control immunoprecipitations, EXT was not immunoprecipitated by M2 in the absence of FLAG-NPR-A, but was immunoprecipitated by anti-AKC in the absence or presence of FLAG-NPR-A (Fig. 5). Thus, the extracellular domain of NPR-A is sufficient for receptor oligomerization; transmembrane sequences are not required.

Oligomerization of Full-length NPR-A—Although the above experiments showed that NPR-A could form hetero-oligomeric complexes with truncated NPR-A proteins, we were concerned that the ligand independence of this oligomerization, which is somewhat unusual, could be an artifact of receptor truncation. Therefore, we tested the ability of full-length NPR-A to oligomerize in the absence or presence of ANP. Cells were co-transfected with full-length FLAG-NPR-A and full-length NPR-A tagged with a myc epitope (myc-NPR-A). Lysates of these cells were immunoprecipitated with the anti-FLAG monoclonal antibody M2, and the immunoprecipitates were analyzed by Western blotting with anti-myc monoclonal antibody 9E10 (upper panel) or M2 (lower panel) as described under “Experimental Procedures.” Positions of myc-NPR-A and FLAG-NPR-A are indicated by bullets.

FIG. 6. ANP-independent co-immunoprecipitation of myc-NPR-A with FLAG-NPR-A. COS-7 cells were transfected with the indicated plasmids, incubated in the absence or presence of ANP as described in the legend to Fig. 4, followed by immunoprecipitation with anti-FLAG monoclonal antibody M2 and Western blotting with anti-myc monoclonal antibody 9E10 (upper panel) or M2 (lower panel) as described under “Experimental Procedures.” Positions of myc-NPR-A and FLAG-NPR-A are indicated by bullets.
lysates were mixed before rather than after the 10-min incubation on ice and clarification by centrifugation. Immunoprecipitates of lysates from cells expressing FLAG-NPR-A and/or AKC in place of ACYC (not shown) indicate that the receptor associations we expressed NPR-A and NPR-B—The observation that NPR-A oligomerizes raised the possibility of a heteromeric NPR-A/NPR-B receptor. Such a receptor might have novel ligand specificity or altered responsiveness to the known natriuretic peptides. We tested the ability of untagged NPR-B to associate with an epitope-tagged NPR-A construct in a co-immunoprecipitation assay. COS-7 cells were transfected with NPR-B and a FLAG-tagged truncated NPR-A (FLAG-ΔCYC), followed by metabolic labeling and immunoprecipitation with M2 or anti-ΔKC. As shown in Fig. 8 (left), the 130-kDa NPR-B and NPR-A associate with the 115-kDa FLAG-ΔCYC to similar extents. In addition, anti-ΔKC immunoprecipitated NPR-B from cells co-expressing FLAG-ΔCYC, but not cells expressing NPR-B alone (Fig. 8, right). Thus, NPR-A and NPR-B can form heteromers. Similar results were obtained using FLAG-ΔKC in place of FLAG-ΔCYC (not shown), indicating that intracellular sequences are not required for association of NPR-A and NPR-B.

Inhibition of Signal Transduction by a Truncated NPR-A—Others have shown, using other single-transmembrane receptors, that cytoplasmic deletion mutants that dimerize with wild-type receptor can function as dominant negative mutants, apparently due to formation of inactive heterodimers (5–8). Therefore, we examined whether ΔKC, when co-expressed with NPR-A, could inhibit the ability of the receptor to produce cyclic GMP in response to ANP. Cells were co-transfected with plasmids encoding NPR-A and ΔKC or with a plasmid encoding NPR-A and control vector. As shown in Fig. 9A, co-expression of ΔKC shifted the ANP concentration response to the right. At low concentrations of ANP, a dramatic inhibition of cyclic GMP formation was observed in cells expressing ΔKC. At very high pharmacological concentrations of ANP, the same maximal response was observed in the absence or presence of ΔKC. Therefore, at physiological concentrations of ANP, ΔKC is a potent inhibitor of NPR-A function. This shift of the ANP concentration response was not simply due to ANP binding by ΔKC reducing the effective ANP concentration available to wild-type NPR-A. When cells expressing ΔKC are co-cultured with cells expressing wild-type NPR-A, we have never observed any shift in the ANP concentration response (Fig. 9B, inset). Curiously in three of four such co-culture experiments, we did observe significant decreases in maximal cyclic GMP production (Fig. 9B) that have never been observed in the co-transfection experiments (Fig. 9A).

DISCUSSION

The noncovalent, ligand-independent oligomerization of NPR-A is unusual among single-transmembrane receptors. Receptors with tyrosine kinase activity and growth hormone receptors, which represent another large family of single-transmembrane receptors, both undergo ligand-dependent oligomerization (1, 4). Considering the topological similarity of NPR-A to tyrosine kinase receptors which undergo ligand-dependent dimerization, including the preservation of a tyrosine kinase-like domain, one might have expected to observe similar results with NPR-A. In one model for receptor activation, oligomerization functions merely to bring together intracellular domains which then induce conformational changes in each other (1). In another model, oligomerization produces a protein unit having two or more transmembrane helices that can then transduce a signal through ligand-dependent movements of the helices relative to each other (54, 55). Our data are not consistent with the former model, since oligomerization of NPR-A is constitutive and is clearly not sufficient for receptor activation. We cannot rule out, however, that ligand-induced rotations of oligomerized sub-
in the absence of ANP (Table I, Fig. 9), overexpression/oligomerization has not led to receptor activation. Nevertheless, it will be important to repeat these experiments in stable cell lines expressing more moderate numbers of receptors before concluding that ligand-independent oligomerization of native NPR-A occurs in its natural environment.

The detection of interactions between extracellular, but not intracellular, portions of NPR-A was unexpected. It has been argued that the requirement of adenyllyl and soluble guanylyl cyclases for two apparent catalytic domains (25–28) is likely to extend to the receptor/guanylyl cyclases, implying ligand-induced dimerization involving the cyclase catalytic domain (29, 30). Consistent with that hypothesis, Thorpe et al. (52) described the dimerization of the purified catalytic core of NPR-A expressed in bacteria. This hypothesis has certain weaknesses. It is not clear that both of the cyclase homology domains in the heterodimeric adenyllyl and soluble guanylyl cyclases are catalytically active; one of the domains might play a regulatory role. Also, a direct interaction between these domains has not yet been demonstrated. In the case of the bacterially expressed protein, an amphipathic region upstream of the catalytic domain was retained in the construct and could potentially mediate the observed dimerization. While we failed to observe interactions between intracellular sequences, we suspect that such interactions occur in the cell, but are disrupted by the detergent buffer used in our assays. It is difficult to envision generation of an intracellular conformational change in the absence of interactions between cytoplasmic domains.

The size of the NPR-A oligomer is not yet known. The standard sedimentation and cross-linking approaches used to examine this question with tyrosine kinase receptors have not been successful with NPR-A. 1) The variable and sometimes low amounts of oligomer observed in the co-precipitation assays (e.g. Fig. 8) suggested either a low percentage of oligomeric receptor or instability of the receptor complexes in detergent buffer. In attempts to estimate the sizes of the receptor complexes by density gradient centrifugation, we found that all NPR-A migrated as an apparent monomer after overnight centrifugation (data not shown). This suggested that the receptor complexes might be unstable to long incubations in detergent solution. Meloche et al. (36) also observed only monomers when ANP receptor solubilized from adrenal zona glomerulosa was analyzed by density gradient centrifugation and gel filtration chromatography. 2) In preliminary experiments, we were unable to cross-link the receptor complexes with various bifunctional reagents differing in spacer length and reactive groups (disuccinimidyl suberate, bis(sulfosuccinimidyl) suberate, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, disulfosuccinimidyl tetrarate, ethylene glycolbis(sulfosuccinimidylsuccinate), dimethyl pimelimidate, difluorodinitrobenzene), precluding size determination by this method (data not shown). However, during fast protein liquid gel filtration chromatography, which may be performed rapidly enough to avoid problems with stability of the receptor complexes, NPR-A guanylyl cyclase activity expressed in COS cells migrated with an $M_\text{r}$ of 450,000 in the absence of ANP (data not shown), consistent with NPR-A being tetrameric. Further characterization of NPR-A in the absence and presence of ANP will be necessary to determine an accurate molecular weight for the receptor oligomers.

The fraction of receptor in the oligomeric state is also unknown and is somewhat difficult to address due to the apparent instability of receptor complexes after cell lysis. It is possible that all NPR-A in the plasma membrane is oligomeric or that oligomerization occurs co-translationally. Alter-
natively, only a portion of the receptor population may oligomerize, and modulation of oligomerization by unknown factors may regulate responsiveness to natriuretic peptides. In this case, oligomeric NPR-A could represent the higher affinity form of the receptor seen in Scatchard plots (22).

The formation of heteromeric receptors by NPR-A and NPR-B adds a new level of complexity to the biology of the natriuretic peptides. Based on the similar amounts of NPR-A and NPR-B co-immunoprecipitated with an epitope-tagged, truncated NPR-A protein (Fig. 8), NPR-A and NPR-B may associate with each other as readily as they do with themselves. Koller et al. (15) have postulated that NPR-A represents a receptor for both ANP and BNP, although a 10-fold higher concentration of BNP is required for half-maximal receptor activation (14, 15). They have also suggested that NPR-B is the CNP receptor, although the EC50 they reported for stimulation of NPR-B guanylyl cyclase by CNP (100 nM) (15) was surprisingly high for a physiological receptor. It seemed possible that the heteroreceptor might be more responsive to BNP or CNP than homomeric NPR-A or NPR-B. However, while we were able to reproduce the results of Koller et al., we observed similar cyclic GMP responses to ANP, BNP, and CNP in cells co-expressing NPR-A and NPR-B and in cells expressing either receptor alone (data not shown). This could indicate either that the heteroreceptor responds to the peptides similarly to the homomeric receptor or that altered responses were undetectable due to the background of homomeric receptors in the co-transfected cells. In order to address this question properly, it may be necessary to generate complementary NPR-A and NPR-B mutants that are unable to form functional homo-oligomers but that can form functional heteromers. The observation by Wilcox et al. (56) that NPR-A and NPR-B are co-localized in adrenal medulla, anterior pituitary, and cerebellum is consistent with a biological role for the heteromeric receptor. One could envision that the heteroreceptor might show increased or decreased cyclic GMP formation in response to the known natriuretic peptides, could be desensitized or otherwise regulated differently than the homo-oligomeric receptor, or could be specific for an as yet undiscovered natriuretic peptide. Alternatively, the heteromeric receptor may be a laboratory artifact that does not occur in vivo.

The inhibition of NPR-A activation by ΔKC argues that receptor oligomerization is required for signal transduction. However, some of the details of the experimental observations are puzzling. First, assuming ligand independence of receptor oligomerization, one would expect co-expression of wild-type and truncated receptors to lead to a reduction of maximal ANP-dependent cyclic GMP production, rather than a shift in the concentration response. The observed shift in concentration response, with no change in maximal cyclic GMP production, suggests that ANP can shift the equilibrium to favor formation of wild-type oligomers, seemingly inconsistent with the ligand independence of oligomerization observed in the co-immunoprecipitation assays. A similar shift in concentration response was observed when EGF receptor (which dimerizes in a ligand-dependent fashion) was co-expressed with a truncated EGF receptor (57). Another explanation for the shift in concentration response could be that overexpressed ΔKC might bind a substantial percentage of the ANP in the medium at low, but not high, ANP concentrations, thus reducing the amount of ANP available to the wild-type receptor. This explanation would seem to rule out by the observation (Fig. 9B) that co-culturing cells expressing ΔKC with cells expressing NPR-A did not result in a shift in the ANP concentration response. Curiously, however, it did result in a small but reproducible reduction in the maximal response. Since the co-cultured cells compete for growth in the same plate, this decrease in maximal response could be an artifact of more rapid growth by ΔKC-transfected cells than by the control vector-transfected cells. It is difficult to imagine other mechanisms by which ΔKC on one cell may reduce signaling by NPR-A on another cell at high ANP concentrations.

The observation that ΔKC inhibits signaling by NPR-A could be important not only in elucidating the biochemical mechanisms of signaling but in exploring the normal physiological roles of the natriuretic peptides in cardiovascular homeostasis, which are poorly understood (24). If this dominant negative mutant can be used to block the function of natriuretic peptide receptors in transgenic animals (7), we could test directly the importance of natriuretic peptides in specific physiological responses in vivo.

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