Down-Regulation Does Not Mediate Natriuretic Peptide-Dependent Desensitization of Natriuretic Peptide Receptor (NPR)-A or NPR-B: Guanylyl Cyclase-Linked Natriuretic Peptide Receptors Do Not Internalize

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Received May 6, 2004; accepted September 20, 2004

ABSTRACT

Natriuretic peptide receptor A (NPR-A/GC-A) and B (NPR-B/GC-B) are members of the transmembrane guanylyl cyclase family that mediate the effects of natriuretic peptides via the second messenger, cGMP. Despite numerous reports of these receptors being down-regulated in response to various pathological conditions, no studies have actually measured desensitization and receptor internalization in the same cell line. Furthermore, the ligand-dependent trafficking properties of NPR-A remain controversial, whereas nothing is known about the trafficking of NPR-B. In this report, we tested whether down-regulation explains the ligand-dependent desensitization of NPR-A and NPR-B and characterized their trafficking properties using a combination of hormone-binding and antibody-based assays. Quantitative partition analysis indicated that ^{125}I-atrial natriuretic peptide (ANP) was rapidly released into the medium after 293T cells stably expressing NPR-A were warmed from 4° to 37°C. High-performance liquid chromatography fractionation of medium supplemented with the protease inhibitor phosphoramidon indicated that the ^{125}I-ANP was mostly intact. In contrast, ^{125}I-ANP purified from medium bathing cells expressing NPR-C, a receptor known to internalize natriuretic peptides, was degraded. Cleavable biotinylation and noncleavable biotinylation assays indicated that neither NPR-A nor NPR-B was internalized or degraded in response to natriuretic peptide binding. In contrast, agonist-dependent internalization of a G protein-coupled receptor was clearly apparent in the same cell line. Finally, we show that NPR-A and NPR-B are desensitized in cells in which they are not internalized. We suggest that mechanisms other than receptor down-regulation account for the desensitization of NPR-A and NPR-B that occurs in response to various physiological and pathological stimuli.

Atrial natriuretic peptide (ANP), brain natriuretic peptide, and C-type natriuretic peptide (CNP) comprise the mammalian natriuretic peptide family (Levin et al., 1998). ANP and brain natriuretic peptide are endocrine cardiac hormones that decrease blood pressure through the stimulation of renal sodium and water excretion, vasorelaxation, and antagonization of the renin-angiotensin-aldosterone system. CNP signals in a paracrine fashion to stimulate vasorelaxation and long bone growth. The known effects of natriuretic peptides are mediated through the two cell-surface guanylyl cyclase

receptors (Schulz and Waldman, 1999; Potter and Hunter, 2001; Silberbach and Roberts, 2001; Misono, 2002; Tremblay et al., 2002). NPR-A, also known as GC-A, is selectively activated by ANP and brain natriuretic peptide, whereas NPR-B, also known as GC-B, is activated by CNP. Both receptors consist of an extracellular ligand-binding domain, a single membrane-spanning region, and intracellular kinase homology, dimerization, and carboxyl-terminal guanylyl cyclase domains. In addition, all three natriuretic peptides bind a third protein called the natriuretic peptide clearance receptor (NPR-C), which lacks enzymatic activity and functions to remove natriuretic peptides from the circulation via receptor-mediated, but ligand-independent, internalization and degradation (Nussenzveig et al., 1990).

Activation of the guanylyl cyclase-linked natriuretic peptide receptors is incompletely understood. Both receptors are

ABBREVIATIONS: ANP, atrial natriuretic peptide; CNP, C-type natriuretic peptide; NPR-A, natriuretic peptide receptor-A; NPR-B, natriuretic peptide receptor-B; BES, *N,N*-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid; HA, hemagglutinin; HPLC, high-performance liquid chromatography; NHS, *N*-hydroxysuccinimide; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; TBST, Tris-buffered saline containing 0.05% Tween; NHS, *N*-hydroxysuccinimide.

homo-oligomers in the absence and presence of their respective ligands, indicating that receptor activation does not simply result from ligand-dependent dimerization (Chinkers and Wilson, 1992). However, ANP binding does cause a conformational change in NPR-A that brings the extracellular juxtamembrane regions of each monomer closer together (Huo et al., 1999; Labrecque et al., 1999, 2001). The stoichiometry of the ligand-receptor complex is 1:2 (Ogawa et al., 2004). In addition to natriuretic peptides, ATP is also required to maximally activate these receptors in broken cell preparations (Kurose et al., 1987). Because receptors lacking the kinase homology domain are constitutively active and are not further stimulated by ATP (Chinkers and Garbers, 1989), it has been suggested that ATP binding to the kinase homology domain relieves the basal repression of the guanylyl cyclase domain.

With respect to the mechanism by which an activated receptor is turned off, a process commonly referred to as desensitization, a number of hypotheses have been put forth. Initial in vitro data suggested that direct phosphorylation of NPR-A by protein kinase C mediated its desensitization (Duda and Sharma, 1990), but subsequent studies conducted in live cells indicated that desensitization in response to prolonged natriuretic peptide exposure (homologous desensitization) or activators of protein kinase C results in a net loss of phosphate from NPR-A and NPR-B (Potter and Garbers, 1992, 1994; Foster and Garbers, 1998; Potter, 1998; Joubert et al., 2001). Consistent with this latter scenario, mutations that mimic phosphorylated or dephosphorylated forms of NPR-A and NPR-B yield receptors that are hormonally responsive and unresponsive, respectively (Potter and Hunter, 1998a,b, 1999).

Another well-characterized deactivation mechanism used by cell-surface signaling receptors is ligand-mediated internalization and degradation, a process often referred to as down-regulation (Katzmann et al., 2002). Although ligand-dependent internalization and degradation of NPR-A has been intensely studied by several groups for many years, a consensus understanding of the importance of this process in the regulation of natriuretic peptide receptors has not emerged. Early studies conducted on PC-12 pheochromocytoma cells suggested that both NPR-A and NPR-C internalize ANP and that both receptors are recycled back to the cell surface (Rathinavelu and Isom, 1991). Pandey and colleagues, using Leydig, Cos, and 293 cell lines, have reported that ANP binding to NPR-A stimulates its internalization, which results in the majority of the receptors being degraded with a smaller portion being recycled to the plasma membrane (Pandey et al., 1986, 2000; Pandey, 1993, 2001). In contrast, Maack and coworkers reported that NPR-A in primary kidney or stably expressing Chinese hamster ovary cells is a constitutively membrane-resident protein that neither undergoes endocytosis nor mediates lysosomal hydrolysis of ANP (Koh et al., 1992; Vieira et al., 2001). Likewise, Jewett and colleagues (1993) found that 293 cells expressing NPR-A bound less ANP over time but concluded that the reduced binding was caused by a reduction in the affinity of NPR-A for ANP and not by decreased amounts of NPR-A at the cell surface.

Because the role of this fundamental mechanism in the regulation of NPR-A and NPR-B remains ambiguous and because receptor down-regulation has been implicated in the desensitization of natriuretic peptide receptors that accompanies various pathologies, such as congestive heart failure (Tsutamoto et al., 1993; Kim et al., 1999) and diabetes (Sechi et al., 1995), we initiated experiments designed to resolve these important issues. For the first time, we measured both the desensitization and internalization of NPR-A and NPR-B in the same cell system. Also for the first time, we measured internalization with techniques that directly track the location of each receptor. We found that in contrast to the vast majority of cell-surface signaling proteins, ligand binding does not trigger the internalization of guanylyl cyclase-linked natriuretic peptide receptors.

Materials and Methods

Materials. The μ-opioid receptor cDNA and etorphine were generous gifts from Dr. Ping-Yee Law (University of Minnesota, Minneapolis, MN). The 293T cell line was a kind gift from Dr. Nikunj Somia (University of Minnesota). The cDNA for human NPR-C was kindly provided by Dr. Thomas Maack (Cornell Medical School, Ithaca, NY).

The following materials and substances were purchased commercially: ^{125} I-ANP (Amersham Biosciences Inc., Piscataway, NJ), rat ANP$_{1-28}$ and rat CNP (Peninsula Laboratories, Belmont, CA, or Sigma-Aldrich, St. Louis, MO), horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody (PerkinElmer Life and Analytical Sciences, Boston, MA), anti-HA antibody (Covance Inc., Princeton, NJ), ECF and ECL Western Blot Detection System (Amersham Biosciences), and sulfo-NHS-biotin and sulfo-NHS-S-S-biotin (Pierce, Rockford, IL).

Cell Culture and Stable Cell Lines. The majority of the studies presented in this report were conducted on stably transfected 293T-NPR-A, 293T-NPR-B, and 293T-NPR-C cell lines. Unlike some varieties of 293 cells that endogenously express NPR-A, the parental cell that was used to make this line does not endogenously express detectable amounts of any known natriuretic peptide receptor. We made the stable cells lines by transfecting the pCMV-NPR-A, pcDNA3.1(+)-NPR-B, and pcDNA 3.1(+)-NPR-C constructs into the same parental cell line. In brief, cells were grown to 40 to 50% confluence in 10-cm dishes, then the cells were transfected with 5 μg of the constructs mentioned above and 0.5 μg of pcDNA3.1-hygro plasmid (Invitrogen, Carlsbad, CA) to convey hygromycin resistance using the BES-buffered calcium phosphate coprecipitation method. Forty-eight hours later, the cells were switched to medium containing 100 μg/ml hygromycin to select for cells that had incorporated the resistance plasmid into their genome. After 10 to 14 days, individual colonies were isolated and tested for expression with hormone-binding assay and/or Western blot analysis. Control experiments with the μ-opioid receptor were conducted on 293T cells that had been transiently transfected with the μ-opioid cDNA using the transfection method described above.

Saturation Binding Assay. Cultured 293T-NPR-A or 293T-NPR-C cells grown to confluence in 24-well dishes (precoated with 50 μg/ml poly-L-lysine), were washed twice with 1 ml of serum-free Dulbecco's modified Eagle's medium (DMEM) and incubated with 0.2 ml of binding buffer (DMEM, 0.2% bovine serum albumin, HEPES, pH 7.8, and 1% penicillin/streptomycin) for 1 h. Then, cells were incubated for 2 h at 4°C with an additional 50 μl of binding buffer containing various concentration (0.22–18 nM) of ^{125}I-ANP. After incubation, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and then dissolved in 1 N NaOH. The cell extract-associated radioactivity was determined in a γ counter (5500; Beckman Coulter, Fullerton, CA). Specific binding was determined as the difference between total binding and nonspecific binding (binding of ^{125}I-ANP in the presence of 2000-fold unlabeled ANP).

Internalization Experiments. Cultured 293T-NPR-A and 293T-NPR-C cells grown to confluence in 24-well dishes were washed once with 1 ml of ice-cold serum-free DMEM medium and then

incubated for 1 h with 0.2 ml of binding solution with or without 200 μM chloroquine at 37°C. At this point, 50 μl of binding solution containing 200 nM ^{125}I-ANP was added for a final concentration of 40 nM ^{125}I-ANP. After incubating for 2 h at 4°C, the cells were washed once with ice-cold PBS to remove unbound ligand. Then, cells were incubated at 37°C with fresh binding solution for various periods of time. Unlabeled ANP (80 μM) was added to prevent rebinding of the radioactive form of ANP. At the end of the incubation, medium was collected, and the cells were treated with a hypertonic acid solution (0.2 M acetic acid and 0.5 M NaCl) at 4°C for 5 min to remove surface-bound ANP. The cells were rinsed one time to remove residual ^{125}I-ANP. At first, experiments with two and three rinses removed more radioactivity and reduced background binding, but they also washed a significant number of the cells off the plates. Therefore, only one wash was used in the experiments presented here. After the acid wash and rinse were collected, the cells were dissolved in 0.5 N NaOH for 30 min at 37°C. The medium, acid wash, and NaOH cell extracts were then counted and represent released, surface-bound, and internalized radioactivity, respectively. In some experiments, ANP from the medium was fractionated by reverse-phase HPLC by injecting 0.25 ml into a 4.6 × 250-mm reverse-phase C18 column. The solvent systems were 0.1% (v/v) trifluoroacetic acid in water (A) and 0.1% (v/v) trifluoroacetic acid in 100% acetonitrile (B). After running for 5 min under 85% A and 15% B, the separation was achieved with a linear 0 to 60% B gradient over 30 min at a flow rate 1 ml/min. Radioactivity in each fraction was measured in a gamma counter.

Change of Surface Receptor Amount Measured by a Noncleavable Biotin Assay. The cleavable and noncleavable biotinylation assays were performed as described by Vickery and von Zastrow (1999). Stably transfected cells were incubated with the appropriate agonist at 37°C for the indicated periods of time. Next, the cells were placed on ice, washed twice with ice-cold PBS, and the surface proteins were biotinylated with 30 mg/ml sulfo-NHS-biotin for 30 min at 4°C. The cells were rinsed twice with ice-cold PBS to quench the biotinylation reaction. The cells were then solubilized in detergent extraction buffer (0.5% v/v Triton X-100, 10 mM Tris-Cl, pH 7.5, 120 mM NaCl, 25 mM KCl, 1 μg/ml pepstatin, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Immunoprecipitation and SDS-PAGE were performed as described previously (Bryan and Potter, 2002). In brief, the extract was cleared by centrifugation at 10,000g for 10 min at 2°C. A 500-μl sample of the supernatant from each treatment was incubated with 1 μl of polyclonal antiserum from rabbit 6325 (NPR-A) or 6327 (NPR-B) overnight at 4°C as described previously (Abbey and Potter, 2002). A 50-μl sample of a 50% slurry of protein A-agarose was added to the extract and incubated for an additional hour. The protein A immunocomplex was pelleted by low-speed centrifugation and washed three times with 1 ml of extraction buffer. Receptors were released from the agarose beads by boiling for 3 min in the presence of 50 μl of reducing SDS sample buffer, fractionated by SDS-PAGE on an 8% resolving gel, and electroblotted to an Immobilon-P membrane. The membrane was incubated in blocking buffer for 30 min and then rinsed with Tris-buffered saline containing 0.05% Tween (TBST) for 30 min and incubated with avidin-biotinylated enzyme complex–alkaline phosphatase reagent for an additional 30 min. The membranes were then washed three times with TBST for 10 min each and then incubated with ECF solution for 1 min, dried, and visualized by autofluorography or on a PhosphorImager.

Measurement of Internalized Receptor Pools with a Cleavable Biotin Assay. Stably transfected cells were surface-biotinylated with 30 mg/ml sulfo-NHS-*S-S*-biotin for 30 min at 4°C and quenched with two washes of ice-cold PBS. Some samples were incubated on ice to measure total surface-biotinylated receptor. The remaining samples were incubated with media in the presence or absence of the appropriate agonist (100 nM ANP, 100 nM CNP, or 1 μM etorphine) for various times at 37°C and then immediately chilled on ice to stop internalization and washed twice with ice-cold

PBS. To estimate the amount of residual biotinylated receptor remaining in the endocytic pathway, monolayers were treated for 15 min at 4°C with glutathione strip solution (50 mM glutathione, 75 mM NaCl, 75 mM NaOH, and 10% fetal bovine serum) to cleave biotin groups from receptors accessible at the cell surface. Cells were then washed three times with ice-cold PBS to remove the residual glutathione, and then the receptors were extracted, immunoprecipitated, and detected as described above. Because the μ-opioid receptor contained an HA tag, we precipitated it with 50 μl of immobilized streptavidin and performed the Western blot by incubating the membrane with the monoclonal antibody HA.11 (1:1000) for 40 min at room temperature, washing with TBST four times for 5 min each, and incubating with anti-mouse horseradish peroxidase (1:10,000) for 40 min at room temperature. The NPR-A, NPR-B, and μ-opioid antibody complexes were detected by chemiluminescence using the ECL Western Blot Detection System (Amersham Biosciences).

Results

Characterization of the 293T-NPR-A and 293T-NPR-C Cell Lines. We began our investigation into the down-regulation and trafficking characteristics of natriuretic peptide receptors by performing saturation-binding experiments on 293T cells that had been stably transfected with the rat cDNA for NPR-A (293T-NPR-A) or the human cDNA for NPR-C (293T-NPR-C). This particular line of 293 cells does not contain detectable levels of any natriuretic peptide receptor (data not shown). Therefore, it represents a complete null background for their expression. By using these cells, we could be sure that we were measuring binding to a single family member, not multiple members, which has proved problematic in previous studies (Rathinavelu and Isom, 1991; Koh et al., 1992). Because it is generally accepted that internalization of surface receptors does not occur at 4°C, we performed our initial binding experiments at this temperature. We found that the 293T-NPR-A cells expressed approximately $9.6 × 10^5$ receptors/cell and that the affinity of the binding reaction was relatively strong, having a K_d value of 417 pM (Fig. 1, left column). The 293T-NPR-C cells expressed $2.5 × 10^6$ receptors/cell and had a K_d value of 675 pM for ANP (Fig. 1, right column). The dissociation constants for both NPR-A and NPR-C are similar to those that have been reported previously in untransfected cells.

Partitioning of ^{125}I-ANP in 293T-NPR-A and 293T-NPR-C Cells. Fig. 2 depicts data on the fate of ^{125}I-ANP initially bound to NPR-A (left column) or NPR-C (right column) at 4°C in 293T cells. The top shows the amount of radioactivity found in the media at different time periods after the incubation temperature of the cells was increased from 4° to 37°C. This corresponds to the radioactivity released from the cells under physiological conditions. The middle depicts the amount of radioactivity remaining on the surface of the cells. It was obtained by incubating the cells for 5 min with a solution of acetic acid and salt and is referred to as the acid wash. The bottom of the figure represents the amount of radioactivity located inside the cells, which was obtained by solubilizing the cells in 1 N NaOH.

In the 293T-NPRA cells (left column), specifically bound radioactivity disappeared rapidly from the plasma membrane when cells were warmed to 37°C. The time required for half of the total specifically bound ^{125}I-ANP to appear in the media was less than 5 min, which is similar to the value obtained in cultured glomerular and renomedullary intersti-

tial cells (Koh et al., 1992). This rapid appearance of radio-activity in the medium is a unique characteristic of NPR-A and suggests that the receptor is behaving normally in the 293T cell environment. After 10 min, the partitioning of radioactivity reached a plateau, with approximately 80% residing in the medium and less than 20% remaining on the cell surface. In contrast, the amount of intracellular radioactivity was low and did not show a time-dependent increase. The lysosomotrophic agent chloroquine (squares), which increases the pH of lysosomes and inhibits the degradation of receptor-ligand complexes targeted to this organelle, had no effect on the distribution of radioactivity in the NPR-A–expressing cells.

As a positive control for the internalization of ^{125}I-ANP, we used 293T-NPR-C cells because NPR-C has been shown by many investigators to constitutively internalize and degrade ANP (Nussenzveig et al., 1990; Pandey, 1992; Cohen et al., 1996). After the prebound 293T-NPR-C cells were warmed from 4° to 37°C, specifically bound radioactivity was released at a slower rate than was released from the 293T-NPR-A cells having a half-time of approximately 10 min (Fig. 2, right column). Radioactivity in the medium bathing the 293T-NPR-C cells plateaued between 30 and 60 min. Similarly to 293T-NPR-A cells, the majority of the remaining radioactivity was found associated with the cell surface (acid wash). However, in contrast to the NPR-A–expressing cells, chloroquine treatment coordinately increased the amount of intracellular radioactivity and decreased the amount of radioactivity found in the medium bathing the 293T-NPR-C cells, which is consistent with ^{125}I-ANP being degraded through a lysosomal pathway (Fig.2, right column, top and bottom). Intracellular accumulation of radioactivity in the presence of chloroquine was increased to slightly more than 40% at 10 min, which is similar to that observed in bovine vascular smooth muscle cells that endogenously express NPR-C (Nus-

senzveig et al., 1990). At the 0 time points for both the 293T-NPR-A and 293T-NPR-C cells, we found approximately 20% of the radioactivity remained in NaOH wash. One possible reason for this observation is that the acid wash is less effective at removing membrane-bound ^{125}I-ANP at the reduced temperature. In addition, because the cells tended to come off the plate, they were only washed once to remove the nonspecifically bound ANP. This also contributed to slightly higher backgrounds. Nevertheless, these results indicate that the 293T cells contain the necessary intracellular machinery required for the internalization of ANP but are not consistent with a scenario in which NPR-A mediates this process.

Phosphoramidon Inhibits ANP Degradation in 293T-NPR-A but Not 293-NPR-C Cells. We next investigated whether the radioactivity in the medium bathing the 293T-NPR-A and 293T-NPR-C cells was associated with intact or degraded ANP. The idea was that if the appearance of radioactivity is caused by a rapid receptor-mediated internalization process, then it should be associated with a markedly degraded, not a mostly intact, form of ANP. Medium from cells that had been incubated for 1 h with ^{125}I-ANP at 4°C and then warmed to 37°C for 30 min was fractionated by HPLC. A media sample (250 μl) was loaded onto a reverse-phase C18 column, and the bound peptides were eluted with a linear 0 to 60% gradient of acetonitrile. Fractions (1 ml) were collected, and the radioactivity in each sample was determined (Fig. 3). Preliminary experiments indicated that freshly synthesized intact ^{125}I-ANP eluted at fraction 17 under these conditions (data not shown). When the radioactivity contained in the medium bathing the 293T-NPR-A or the 293T-NPR-C cells was purified, it eluted in fractions 9 and 10, suggesting that the ^{125}I-ANP was degraded in cells expressing both receptors. Because ANP is degraded by neutral endopeptidases expressed on the extracellular face of the
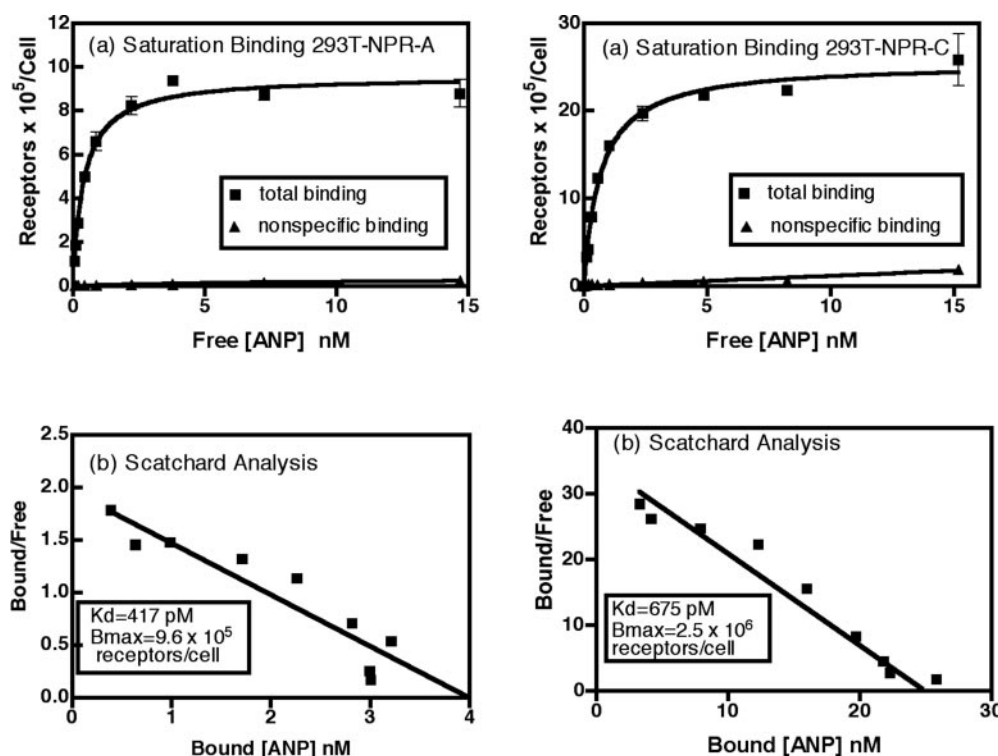

Fig. 1. Saturation binding analysis of ^{125}I-ANP in 293T cells stably expressing recombinant NPR-A (left column) or NPR-C (right column). Cells were incubated with the indicated concentration of ^{125}I-ANP at 4°C for 2 h in the absence (■) or presence (▲) of 2000-fold excess of unlabeled ANP. Data are plotted as saturation binding (top), and the Scatchard transformation is shown at the bottom. The results shown were taken from duplicate dishes. The vertical bar within each symbol represents the range of two values. The results are representative of three separate experiments.

plasma membrane, we repeated these experiments in the presence of phosphoramidon, a potent inhibitor of neutral endopeptidases (Stephenson and Kenny, 1987; Abe et al., 1995). We found that in the presence of phosphoramidon, the vast majority of the radioactivity isolated from 293T-NPR-A cells now eluted in fractions 15 and 16. In contrast, the elution profile of the medium from the 293T-NPRC cells was unaffected by the presence of phosphoramidon. Although we cannot rule out the possibility that multiple species are contained in each peak, we can conclude that unique processes in
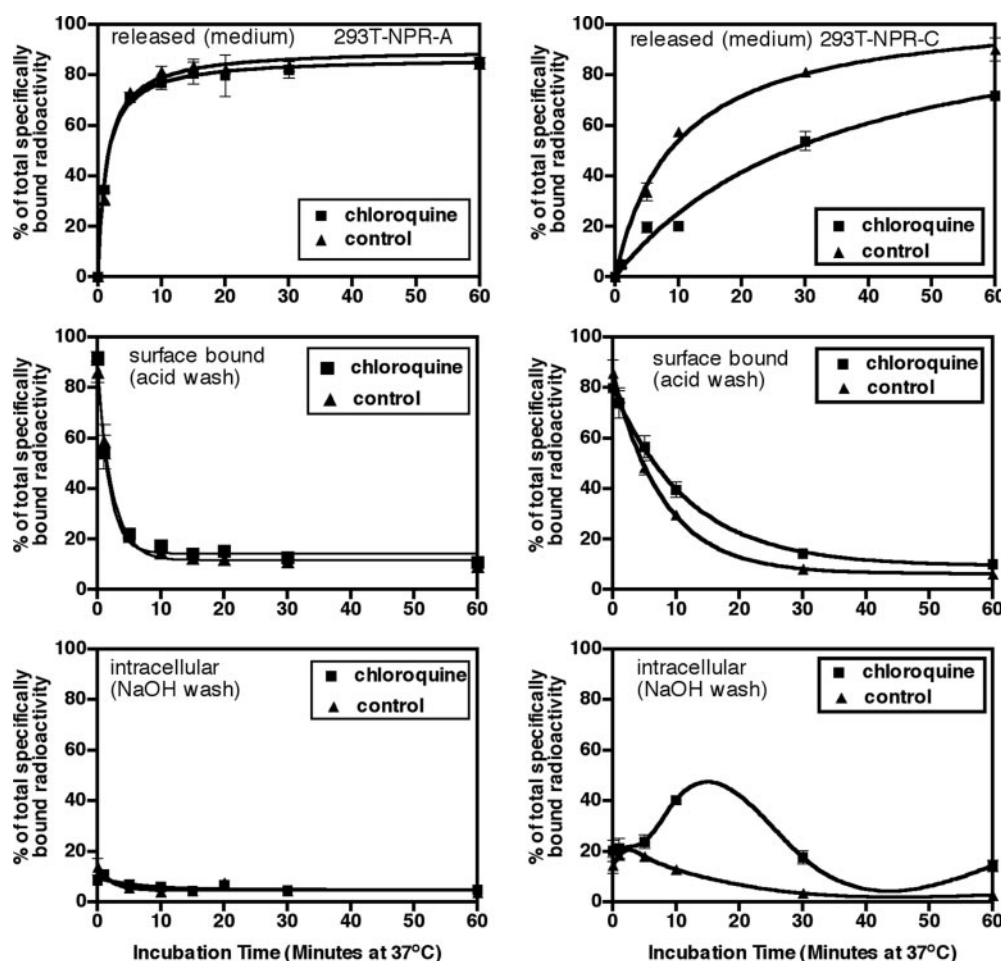
Fig. 2. Quantitative cellular partition analysis of ^{125}I-ANP in 293T-NPR-A or 293T-NPR-C cells. 293T-NPR-A (left column) and 293T-NPR-C (right column) cells were pretreated in the presence (■) or absence (▲) of 200 μM chloroquine at 37°C for 1 h. Cells were then incubated with ^{125}I-ANP at 4°C for 2 h, washed to remove the unbound ligand, and warmed to 37°C. At the indicated time periods, released (top), cell-surface–associated (middle), and intracellular (bottom) ^{125}I-ANP radioactivity was determined in the medium, acid wash, and NaOH extract, respectively. Each data point represents the average of duplicate wells. This experiment was repeated at least three times with similar results.
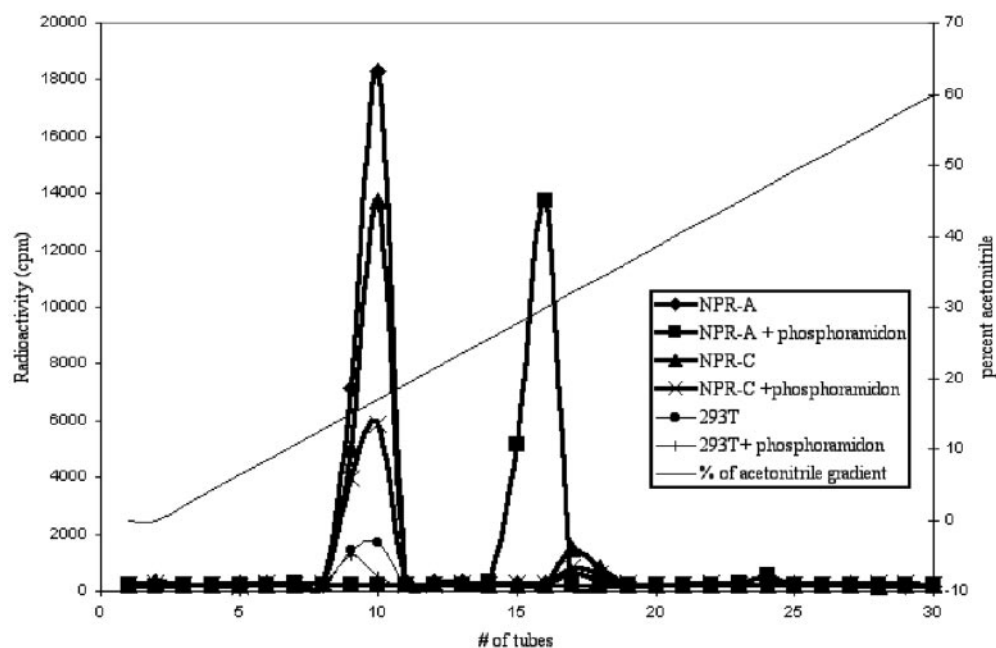
Fig. 3. HPLC fractionation of radioactivity in medium from 293T cells expressing NPR-A or NPR-C. Medium (250 μl) obtained from the cells shown in the figure legend was purified by HPLC on a C18 column. The separation was achieved with linear 0 to 60% acetonitrile gradient from 2 to 30 min.

the two cell lines degrade ANP. In the NPR-A–expressing cells, ANP is degraded by an extracellular phosphoramidon- but not by a chloroquine-sensitive protease, whereas a path- way sensitive to chloroquine but not to phosphoramidon de- grades ANP in the NPR-C–expressing cells.

Lack of Evidence for Down-Regulation or Recycling of Biotinylated NPR-A or NPR-B. To determine whether the rapid disappearance of surface-bound ^{125}I-ANP is corre- lated with the degradation of NPR-A as would be expected if NPR-A undergoes ANP-dependent endocytosis and degrada- tion, we exposed 293T-NPR-A cells to ANP for varying peri- ods of time and then performed Western blot analysis on extracts from these cells to determine whether ANP exposure stimulated NPR-A degradation. We observed no differences in the amount of NPR-A obtained from cells treated with or without ANP (data not shown). Because this crude technique does not distinguish the cell-surface pool of NPR-A from the intracellular pool, we performed a noncleavable biotin assay in which surface NPR-A, but not NPR-A inside the cell, is covalently conjugated to biotin. In this experiment, cells were incubated with or without ANP for the indicated periods of time at 37°C to trigger possible internalization. After ligand exposure, the surface proteins on the cells were biotinylated. The top of Fig. 4 shows that biotinylated protein bands mi- grating at the appropriate molecular mass of NPR-A (130 kDa) were specifically recovered in immunoprecipitates pre- pared from cells expressing NPR-A (lanes 2–9) but not from untransfected cells (lane 1). After incubating the cells with ANP for 10, 60, or 840 min, there was no discernable differ- ence in the amount of surface NPR-A detected by this method. When we reversed this process and precipitated with streptavidin-agarose and blotted with antibodies

against NPR-A, we observed similar results (data not shown). We also performed the same experiment on 293T cells stably expressing NPR-B and found no significant change in the amount of cell-surface NPR-B after incubation with 1 μM CNP for 10, 60, or 840 min (Fig. 4, bottom). These results indicate that receptor occupation does not result in detectable degradation of NPR-A or NPR-B in 293T cells.

Although the noncleavable biotin assay indicated that NPR-A and NPR-B were not being degraded in a natriuretic peptide-dependent manner, this assay cannot rule out the possibility that a receptor is being internalized and then recycled to the cell surface without being degraded. To ad- dress this issue, cell-surface proteins were labeled with a biotin moiety that contains a disulfide bond between the biotin and the conjugation group. Hence, if cells are exposed to a membrane-impermeable reducing agent, such as gluta- thione, the disulfide bond will be cleaved, and the biotin group will be dissociated from the receptor. On the other hand, if ligand binding stimulates the internalization of the receptor, then the internalized pool of receptors will be pro- tected from the membrane-impermeable reducing solution, and the amount of biotinylated receptor will increase in re- sponse to ligand binding.

To test whether NPR-A is internalized in response to ANP exposure, 293T-NPR-A cells were labeled with a disulfide- conjugated biotin moiety at 4°C and then incubated with fresh medium with or without natriuretic peptide at 37°C (Fig. 5, top). After the indicated time periods, one plate of cells was treated with a stripping buffer containing glutathi- one to cleave the biotin from cell-surface proteins. A dupli- cate plate was incubated without stripping buffer and repre- sents the total amount of biotinylated receptor. As in the
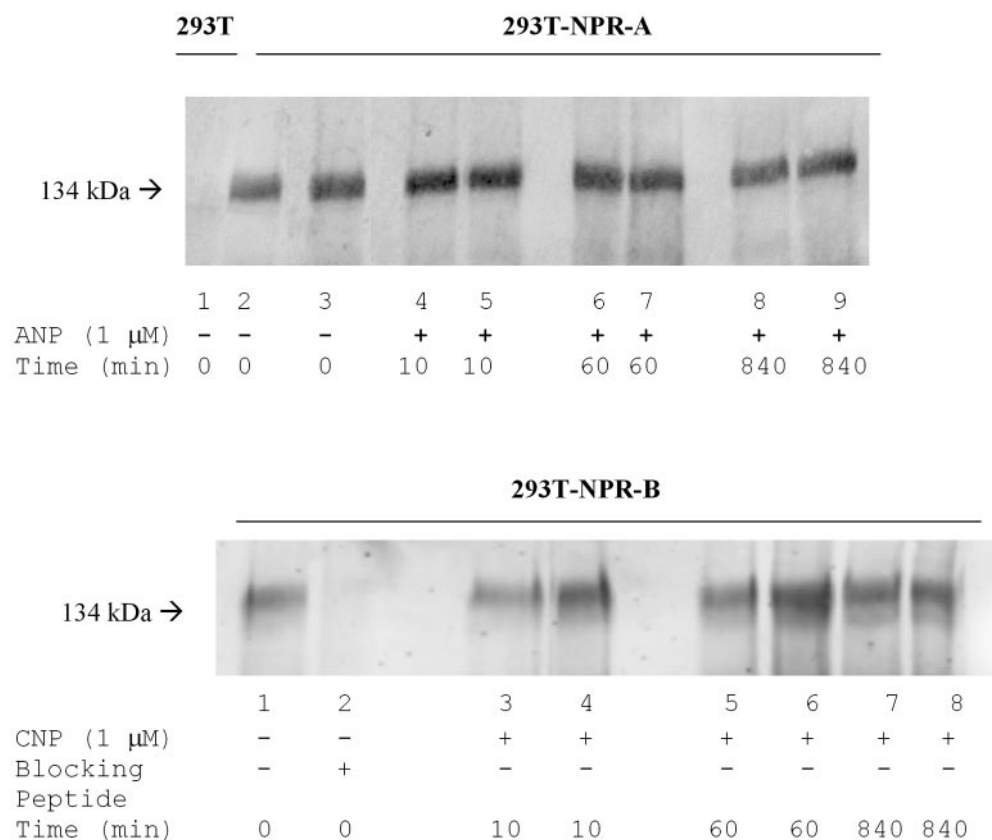
Fig. 4. Lack of evidence for ligand- dependent degradation of NPR-A or NPR-B from a noncleavable biotin as- say. Top, 293T (lane 1) or 293T-NPR-A cells (lanes 2–9) were incubated with or without ANP as shown. Cell-sur- face proteins were biotinylated, then cell extracts were prepared, and NPR-A was immunoprecipitated. Im- munocomplexes were fractionated by SDS-PAGE, transferred to an Immo- bilon membrane, probed with alkaline phosphatase-conjugated avidin, and visualized by enhanced chemifluores- cence. Bottom, 293T-NPR-B cells were incubated with or without CNP for the periods of time shown. Cell-surface proteins were biotinylated, then cell extracts were prepared, and NPR-B was immunoprecipitated in the pres- ence (lane 2) or absence (lanes 1 and 3–8) of a peptide that blocks antibody binding to NPR-B. Immunocomplexes were fractionated by SDS-PAGE, transferred to an Immobilon mem- brane, probed with alkaline phospha- tase-conjugated avidin, and visualized by enhanced chemifluorescence. These results are representative of three in- dependent experiments.

previous experiment, there is no detectable biotinylated natriuretic peptide receptor in the untransfected 293T cells (Fig. 5, lane 1, top and middle). When cells were briefly incubated with ligand at 4°C and then exposed to the stripping buffer, the vast majority of the biotin was removed, indicating that the cleavage reaction is highly effective (Fig. 5, lane 3, top). However, exposure of the cells to ANP at 37°C for 10 or 60 min did not result in significant increases in the amount of biotinylated NPR-A detected (Fig. 5, lanes 5 and 9, top). Similar data also were observed for NPR-B in response to CNP (Fig. 5, middle). These data indicate that natriuretic peptide binding does not stimulate the internalization of NPR-A or NPR-B. Hence, they do not support the hypothesis that guanylyl cyclase-linked receptors internalize natriuretic peptides or are down-regulated.

To rule out the possibility that the lack of internalization of NPR-A and NPR-B was caused by a generalized defect of the endocytotic machinery in these cells or by our inability to properly perform this experimental technique, we conducted the same experiment on 293T cells transiently transfected with the μ-opioid receptor, a receptor known to internalize upon ligand binding (El Kouhen et al., 2001). The bottom of Fig. 5 clearly demonstrates that either a 30- or 60-min exposure of these cells to the μ-opioid receptor agonist, etorphine, caused a substantial increase in the number of its cognate receptors inside the cell compared with cells that were not exposed to etorphine. Hence, 293T cells are capable of ligand-dependent internalization.

NPR-A and NPR-B Desensitize Normally in 293T Cells. Finally, because several reports have suggested that the diminished effects of natriuretic peptides observed during pathological conditions, such as congestive heart failure and diabetes, result from receptor down-regulation, we asked whether NPR-A and NPR-B undergo homologous (natriuretic peptide-dependent) desensitization in the stably expressing 293T cells. We found that membranes obtained from cells exposed to ANP or CNP for increasing periods of time contained diminished hormone-dependent guanylyl cyclase ac-
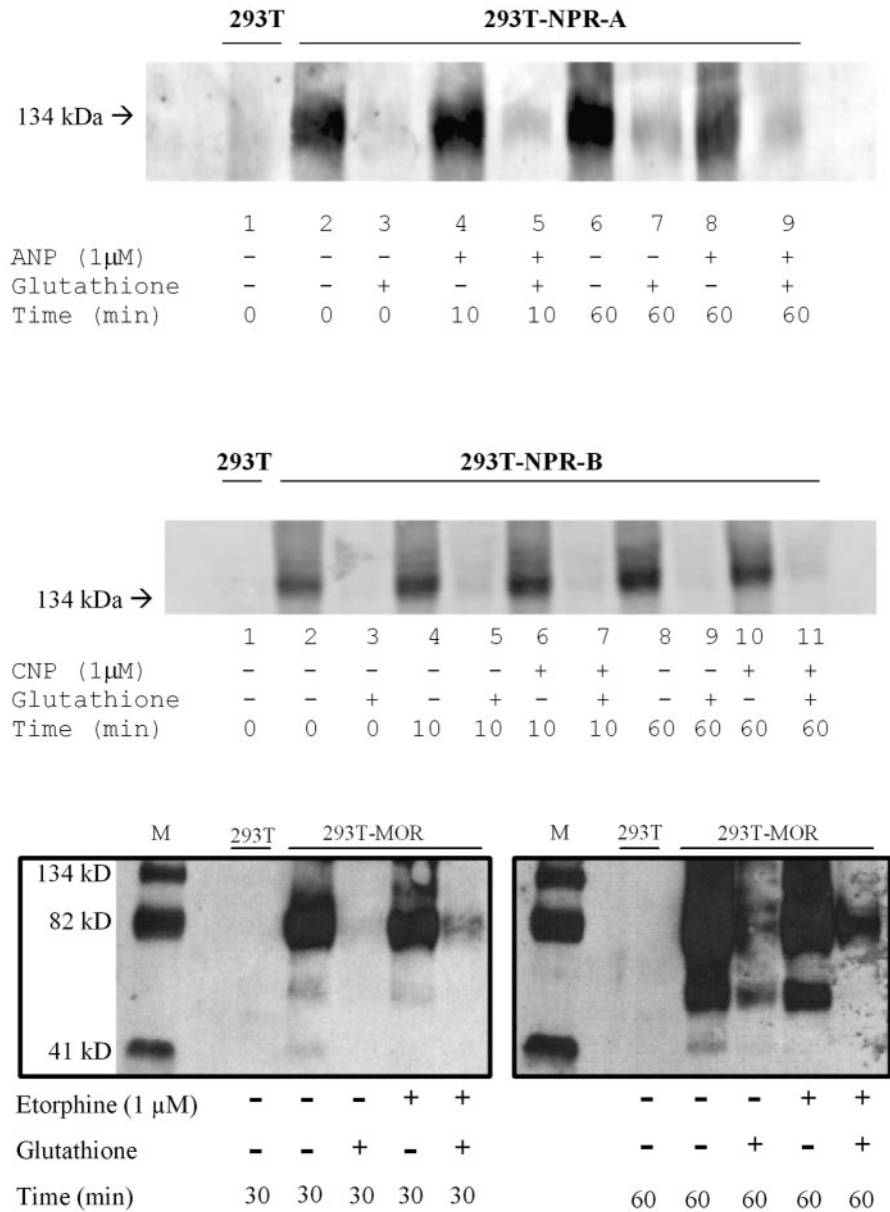
Fig. 5. Lack of evidence for ligand-dependent internalization of NPR-A or NPR-B from a cleavable biotin assay. Top, 293T (lane 1) or 293T-NPR-A cells (lanes 2–9) were biotinylated with a cleavable biotin analog, and then cells were incubated with or without ANP as shown. After the cleavage of cell-surface biotin with glutathione, cell extracts were prepared, and NPR-A was immunoprecipitated. Immunocomplexes were fractionated by SDS-PAGE, transferred to an Immobilon membrane, probed with alkaline phosphatase-conjugated avidin, and visualized by enhanced chemifluorescence. Middle, 293T (lane 1) or 293T-NPR-B cells (lanes 2–11) were biotinylated with a cleavable biotin analog, and then cells were incubated with or without CNP as shown. Surface biotin was cleaved, and NPR-B was purified and processed as described above for NPR-A. Bottom, 293T cells or 293T cells transiently transfected with the μ-opioid receptor (293T-MOR) were biotinylated with a cleavable biotin analog. After removal of the excessive biotin, the cells were treated with or without 1 μM etorphine for 30 or 60 min at 37°C. The biotinylated molecules on the cell surface were stripped in a glutathione-containing solution, and the cells were extracted with lysis buffer. Streptavidin immobilized on agarose was added to the clarified cell extracts to immunoprecipitate the biotin-conjugated proteins. Immunocomplexes were fractionated by SDS-PAGE, transferred to an Immobilon membrane, and detected by sequential incubation with mouse anti-HA.11 followed by a sheep anti-mouse secondary antibody conjugated to horseradish peroxidase. The immunocomplex was visualized by enhanced chemiluminescence. M, molecular weight markers.

tivity (i.e., NPR-A and NPR-B were desensitized) (Fig. 6). It is important to note that the reductions were not explained by receptor degradation because cyclase activity measured in the presence of nonionic detergent and manganese was only slightly reduced. These data are consistent with our previous findings indicating that neither NPR-A nor NPR-B is degraded in response to ligand binding. Thus, in cell lines in which NPR-A and NPR-B are not internalized, they desensitize normally. The desensitization most likely results from receptor dephosphorylation (Potter and Garbers, 1992; Potter, 1998).
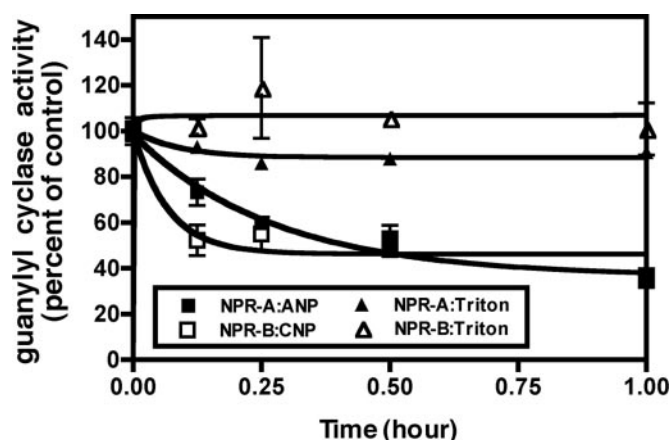
Discussion

The goal of these studies was to clarify our understanding of the postbinding properties of NPR-A and NPR-B, an issue that is decidedly controversial (Koh et al., 1992; Vieira et al., 2001; Pandey et al., 2002), and to determine whether downregulation is required for the homologous desensitization of these receptors. Hormone binding experiments demonstrated that ^{125}I-ANP bound to NPR-A at 4°C, dissociated quickly when cells were warmed to 37°C, and free radioactivity levels reached a steady state after approximately 10 min. NPR-A–mediated internalization and degradation of ^{125}I-ANP has been suggested to be the mechanism for the appearance of radioactivity in the medium. However, we disagree with this scenario for several reasons. First, the time course of appearance of radioactivity in the medium from 293T-NPR-A cells is faster than that from 293T-NPR-C cells. Second, unlike cells expressing NPR-C, we did not observe a time-dependent increase of intracellular radioactivity in NPR-A–expressing cells in the presence of the lysosome inhibitor chloroquine. Third, in the presence of a neutral endopeptidase inhibitor, the released ^{125}I-ANP from NPR-A–expressing cells was mostly intact, whereas that isolated from cells expressing NPR-C was degraded. Fourth, cell biotinylation assays failed to reveal significant internalization or degradation of surface NPR-A or NPR-B but readily detected ligand-dependent in-

Fig. 6. Natriuretic peptide-dependent desensitization of NPR-A and NPR-B is not explained by receptor down-regulation in 293T cells. Confluent, serum-starved 293T cells stably transfected with NPR-A or NPR-B cells were incubated with 100 nM ANP or CNP for the indicated periods of time, and then crude membranes were prepared and assayed for guanylyl cyclase activity in the presence of 1 mM ATP, 1 μM natriuretic peptide, and 5 mM MgCl$_2$ (squares) or 1% Triton X-100 and 5 mM MnCl$_2$ (circles) for 3 min. Values are the mean of individual determinations performed on membranes prepared from two separate plates, which were assayed in duplicate ($\pm$ range). The data are representative of two separate experiments.

ternalization of the μ-opioid receptor. Finally, confocal microscopy failed to detect a ligand-dependent change in the cellular location of NPR-A or NPR-B, whereas it easily detected hormone-dependent changes in the location of the platelet-derived growth factor receptor and the μ-opioid receptor expressed in the same cells (data not shown). From these data, we conclude that neither NPR-A nor NPR-B undergoes internalization or degradation in response to receptor occupation, nor do they mediate the internalization or degradation of natriuretic peptides.

Although the expression of NPR-A in the 293T cells was abnormally high ($\sim$1 $\times$ 10^6 receptors/cell), we believe that this expression system was well-suited for our studies for at least two reasons. First, because the 293T cells do not endogenously express any natriuretic binding proteins, they provide a complete null background for their expression. In other words, we can be sure that natriuretic peptide binding is a result of the specifically expressed natriuretic peptide receptor because in the mock-transfected cells, there is no specific binding. The fact that most cells simultaneously express NPR-A or NPR-B and NPR-C poses serious experimental difficulties and has complicated the interpretation of ANP binding data on many occasions (Rathinavelu and Isom, 1991; Koh et al., 1992). Second, by expressing receptors at high levels, we have significantly increased the signal-to-noise ratio in our assay, which has resulted in very clear and unambiguous data.

On the other hand, one could argue that the postbinding properties of NPR-A and NPR-B in the overexpressing 293T cells are artificial and may not represent the true trafficking characteristics of these receptors that would be observed if they were endogenously expressed in native tissue at normal levels. This is clearly not the situation, because the ANP binding and, more importantly, the dissociation kinetics observed in the 293T cells (Fig. 2) nicely parallel those observed in primary glomerular mesangial or renomedullary interstitial cells (Koh et al., 1992). Furthermore, this phenomenon has been observed in every cell line (both transfected and untransfected) tested. The real question examined here is not whether a physiologically relevant phenomenon is occurring in these 293T-NPR-A cells; it is obvious that the phenomenon is occurring because the rapid appearance of radioactivity in the medium bathing the 293T-NPR-A cells is striking (Fig. 2, top left). On the contrary, the question under investigation is whether NPR-A internalization is the mechanism for this phenomenon. Our data indicate that it is not.

Koh et al. (1992) first reported that ANP-NPR-A complexes were not processed intracellularly in renomedullary interstitial cells. They suggested that a rapid dissociation of receptor-ligand complexes occurs upon ANP binding to NPR-A at 37°C, and the intact ligand is released into the culture medium. In a subsequent study, they reported similar behavior for NPR-A in transfected Chinese hamster ovary cells, suggesting that the rapid off-rate of ANP from NPR-A is not a cell-type–specific phenomenon (Vieira et al., 2001). In contrast, the groups led by Isom or Pandey have reported that NPR-A undergoes rapid endocytosis in Leydig tumor (MA-10), PC-12, COS-7, and human embryonic kidney 293 cells (Pandey et al., 1986, 2000, 2002; Rathinavelu and Isom, 1991; Pandey, 1993). Although we have never directly investigated the internalization of NPR-A, we have observed that prolonged exposure of "regular" 293 cells stably expressing

NPR-A to ANP results in decreased receptor levels (Potter and Hunter, 1999). Because we do not see a similar down-regulation in the 293T stable cells lines in our current study, we cannot rule out the possibility that the ligand-dependent degradation of NPR-A is a cell-type–specific phenomenon. That various 293 cell lines are not uniform in their signaling properties was nicely demonstrated by Lefkowitz and colleagues (2002) within the pages of this journal. Hence, although the rapid release of ANP from cells seems universal, down-regulation may depend on the cell type.

If the rapid appearance of radioactivity in the media bathing 293T-NPR-A cells is not caused by receptor-mediated internalization and degradation of ANP, then what causes it? Koh et al., (1992) have suggested that it results from a temperature-dependent increase in the off-rate of ANP for NPR-A. Subsequent studies led them to conclude that intact cell architecture and a complete intracellular domain are required for the shift in binding (Vieira et al., 2001). The effect of temperature on the binding properties of the wild-type receptor is remarkable. Between 22° and 37°C, the off-rate increases approximately eight times, whereas between 4° and 22°C, it only increases 1.5 times. The intracellular region requirement was suggested because NPR-A deletion mutants missing either the kinase homology domain, the cyclase domain, or the whole intracellular domain had greatly reduced off-rates and failed to display marked temperature-dependent binding. It is interesting that Koh et al., (1992) failed to observe the effect of temperature on ANP binding in membrane preparations and concluded that proper cell architecture, a missing cytosolic factor, or both are required for the effect. In a separate report using bovine lung membranes, Abe and coworkers (1995) also observed a dramatic decrease in ANP binding to NPR-A, but not NPR-C, as a function of increased temperature. However, unlike Koh et al. (1992), they observed a clear temperature effect in membrane preparations; intact cell architecture was not required for the temperature-dependent binding in their assays.

Because cells incubated at lower temperatures have markedly reduced flux through their metabolic pathways, and therefore decreased ATP production, the effect of temperature on NPR-A binding may be related to intracellular ATP concentrations. De Lean (1986) initially observed that ATP inhibits ANP binding to NPR-A, whereas the loop diuretic amiloride increased ANP binding in a manner that is competitive with ATP. Thereafter, Kurose et al. (1987) demonstrated that ATP is required for maximal activation of NPR-A in membrane preparations, and Koh et al. (1992) found that amiloride markedly decreases the dissociation of ANP from intact cells. A similar phenomenon was observed by Jewett and colleagues (1993) when studying the time course of ^{125}I-ANP binding to NPR-A expressed in 293 cells. At early time points (15 min), they found that cells bound the highest amount of ANP, but soon thereafter (30 min), the amount of bound ANP was markedly reduced. It is interesting that mutant receptors lacking either the kinase homology or cyclase domains displayed only high-affinity binding. This was also observed by Vieira and colleagues (2001). Exactly what causes the shift from high-affinity to low-affinity binding is not known, but one theory that is consistent with the current data is that upon ANP binding, a conformational change occurs within the intracellular region of NPR-A that facilitates direct ATP binding to the kinase homology domain, which, through an unknown mechanism, decreases the affinity of the extracellular domain for ANP. This view is consistent with reports showing that ATP can modulate the ligand binding (Meloche et al., 1988) and cyclase activities (Wong et al., 1995) of highly purified preparations of NPR-A.

From our studies that directly track NPR-A and NPR-B, we conclude that guanylyl cyclase-linked natriuretic peptide receptors are neither internalized nor degraded in response to receptor occupation, nor do they play a significant role in the internalization of natriuretic peptides in 293T cells. Hence, our data do not support the hypothesis that down-regulation is responsible for natriuretic peptide receptor desensitization observed in response to various physiological or pathological stimuli (Gauquelin et al., 1991; Yasunari et al., 1992; Tsutamoto et al., 1993; Kim et al., 1999; Matsumoto et al., 1999). We believe that the inability of these receptors to internalize natriuretic peptides is reasonable because in most tissues, NPR-C is present in higher concentrations than NPR-A or NPR-B and is known to constitutively internalize natriuretic peptides. It thereby controls the local concentrations of natriuretic peptides that are able to bind the guanylyl cyclase-linked receptors. Finally, although the mechanism responsible for the rapid release of natriuretic peptides from NPR-A and NPR-B is currently undefined, to our knowledge, it is without precedent in the cell-signaling literature and therefore represents an exciting avenue for future investigation.

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