Ligand-regulated Internalization, Trafficking, and Down-regulation of Guanylyl Cyclase/Atrial Natriuretic Peptide Receptor-A in Human Embryonic Kidney 293 Cells*

We examined the kinetics of internalization, trafficking, and down-regulation of recombinant guanylyl cyclase/natriuretic peptide receptor-A (NPRA) utilizing stably transfected 293 cells expressing a very high density of receptors. After atrial natriuretic peptide (ANP) binding to NPRA, ligand-receptor complexes are internalized, processed intracellularly, and sequestered into subcellular compartments, which provided an approach to examining directly the dynamics of metabolic turnover of NPRA in intact cells. The translocation of ligand-receptor complexes from cell surface to intracellular compartments seems to be linked to ANP-dependent down-regulation of NPRA. Using tryptic proteolysis of cell surface receptors, it was found that ~40–50% of internalized ligand-receptor complexes recycled back to the plasma membrane with an apparent $t_{1/2} = 8\text{ min}$. The recycling of NPRA was blocked by the lysosomotropic agent chloroquine, the energy depletor dinitrophenol, and also by low temperature, suggesting that recycling of the receptor is an energy- and temperature-dependent process. Data suggest that ~70–80% of internalized $^{125}$I-ANP is processed through a lysosomal degradative pathway; however, 20–25% of internalized ligand is released intact into the cell exterior through an alternative mechanism involving an chloroquine-insensitive pathway. It is implied that internalization and processing of bound ANP-NPRA complexes may play an important role in mediating the biological action of hormone and the receptor protein. In retrospect, this could occur at the level of receptor regulation or through the initiation of ANP-mediated signals. It is envisioned that the endocytotic pathway of ligand-receptor complexes of ANP-NPRA would lead to termination and/or diminished responsiveness of ANP in target cells.

Atrial natriuretic peptide (ANP) is synthesized in cardiac atrial myocytes and regulates sodium excretion, water balance, steroidogenesis, and cell proliferation (1–3). Natriuretic peptides belong to a family that comprises at least three members, ANP, brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP), but each is derived from a separate gene (4). The biological actions of these peptide hormones are triggered by interactions with highly selective and specific receptors. Three subtypes of natriuretic peptide receptors have been identified and characterized by molecular cloning, namely natriuretic peptide receptor-A, -B, and -C, also designated as NPRA, NPRB, and NPRC, respectively (5, 6). Two of these receptors, NPRA and NPRB, contain guanylyl cyclase (GC) activity and produce the intracellular second messenger cGMP in response to ligand binding. The third receptor, NPRC, lacks the GC catalytic domain and has been termed the clearance receptor (7). The evidence suggest that both ANP and BNP selectively bind to NPRA, and CNP has been shown to activate primarily NPRB. However, all three natriuretic peptides (ANP, BNP, and CNP) indiscriminately bind to NPRC. NPRA is essentially thought to be the primary ANP signaling molecule because most of the physiological effects of the hormone can be triggered by cGMP or its cell-permeable analogs (8–10).

NPRA is a unique class of cell surface receptors that contains an extracellular ligand-binding domain, a single transmembrane-spanning region, an intracellular protein kinase-like homology domain (protein-KHD), and a GC catalytic domain (5). The GC catalytic region of NPRA has been assigned to ~250 amino acid residues that presumably constitute the catalytic active site of the receptor (11–13). Although the transmembrane GC receptors contain a single cyclase catalytic active site per polypeptide chain, they function as homodimers (14, 15). The protein-KHD is a region of ~280 amino acids that immediately follows the transmembrane-spanning domain of the receptor. It has been suggested that the dimerization region of the receptor is located between the protein-KHD and GC catalytic domain, predicted to form an amphipathic a-helix (16). The integrity of these regions of NPRA are conserved across the species. Previous studies as well as recent data have indicated that protein-KHD seems to be important for ANP-dependent activation of NPRA (17, 18). It has also been suggested that ANP binding to NPRA activates ATP binding to protein-KHD in the intracellular cytoplasmic space, which in turn activates the GC catalytic domain of the receptor (19–21). However, the exact mechanisms of activation and relay of signals from protein-KHD to GC catalytic active site of the receptor remains to be established. Previous studies have proposed that NPRA exists as a dimer and that one molecule of ANP binds to a receptor dimer, suggesting a receptor-to-ANP binding stoichiometry of 2:1 (22). In contrast, recent observations have indicated that an equimolar binding stoichiometry between the...
extracellular domain of NPRA and ANP for ligand-induced dimerization was 1:1 (23, 24). Nevertheless, the validity of these schemes remains to be firmly established, and the interactive role of receptor dimers with bound ligands have yet to be elucidated.

Despite the considerable progress on the structure-function studies, the issue of internalization of NPRA, an important member of the GC-coupled membrane receptor family, is controversial. There is currently a debate over whether ANP-NPRA complexes internalize at all or whether the cell utilizes some other mechanisms to release ANP from NPRA. Indeed, controversy exists because it has been reported earlier by default that among the three natriuretic peptide receptors only NPRC is internalized with bound ligand (25, 26). Hence, from a thematic standpoint, it is clearly evident that there is a current need to provide a consensus forum that establishes the cellular trafficking and processing of ANP-NPRA complexes in intact cells. The present study was undertaken to resolve this important issue and to elucidate unequivocally the ligand-regulated internalization and trafficking of NPRA in stably expressing human embryonic kidney (HEK 293) cells without interference from other natriuretic peptide receptor proteins. It is implied that after internalization, ligand-receptor complexes dissociate inside the cell and a population of the receptor recycles back to the plasma membrane. Subsequently, some of the dissociated ligand molecules escape the lysosomal degradative pathway, are released intact into culture media, and may reenter the cell by retroendothelial mechanisms. In the present report, utilizing pharmacological and physiological perturbants, we have studied the cellular regulation and processing of ligand-receptor complexes in intact HEK-293 cells stably expressing recombinant NPRA.

**EXPERIMENTAL PROCEDURES**

**Materials—**ANP (rat-28), angiotensin II, and endothelin-1 were purchased from Peninsula Laboratories, Inc (Belmont, CA). HEK-293 cells were received from American Type Culture Collection (Manassas, VA). L71 transfection reagent was obtained from Panvera Inc. (Madison, WI). 125I-ANP was purchased from Amersham Biosciences, Inc. The mammalian expression vector pGEMEX was obtained from Invitrogen. cGMP immunoassay kit was purchased from Assay Design (Ann Arbor, MI). Chloroquine, cycloheximide, diithothreitol, monensin, and nigericin were obtained from Sigma. Genetin, LipofectAMINE, and tissue culture supplies were purchased from Invitrogen. All other chemicals were reagent grade.

**Plasmid Construction—**Full-length murine NPRA cDNA (27) was excised from Bluescript vector by digestion with Not I and subcloned into a site of pDNA3 vector previously digested with Not I (28). The expression vector pDNA3 is designed to function under the control of cytomegalovirus immediate early promoter and contains the simian virus-40 region of replication to increase the transient expression of the plasmid. The plasmid of interest that had the insert in the correct orientation was identified by restriction mapping and DNA sequencing according to previously published methods (29).

**Cell Culture and Stable Transfection—**HEK-293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Cells were transfected with murine NPRA cDNA using L71 and LipofectAMINE reagents. For transient expression, cells were examined 48 h after transfection. To establish the stably expressing cell lines, 500 μg/ml Geneticin was added to the culture medium following trypsin (0.025%) treatment for 10 min at 4°C. Free ligand was removed from the dishes by four washes (2 ml each wash) with ice-cold assay medium. To determine the cell surface-associated radioactivity, the acid wash procedure was utilized as described previously (30). After binding was completed, each culture dish received 1 ml of ice-cold acetate buffer, pH 3.5, and cells were placed at 4 °C for 2 min. The acid eluates from the dishes were collected, and each dish received another 1 ml of ice-cold acid buffer to wash the cells. Both solutions were combined to determine the acid-sensitive radioactivity. Cells were then dissolved in 0.5 N NaOH, and acid-resistant radioactivity was determined. The acid-resistant radioactivity was accounted for in the acid-resistant index of cell-associated 125I-ANP, and the acid-resistant radioactivity was used as a measurement of the internalized ligand-receptor complexes.

**Internalization of Ligand-Receptor Complexes—**125I-ANP was allowed to bind to 293 cells expressing NPRA by incubation at 4 °C for 60 min. The unbound 125I-ANP was removed by washing cells with ice-cold assay medium. The cell-associated radioactivity was determined by dissolving cells in 0.5 N NaOH and counting the radioactivity in the cell lysate. This represented the initial zero time control value of 100%. To permit the internalization of ligand-receptor complexes, cells were warmed quickly to 37 °C. At the indicated times the culture dishes were removed from 37 °C and placed on ice and media were collected. The cell surface-associated radioactivity was removed by washing the cells with ice-cold acetate buffer (pH 3.5) at 4 °C. After acid wash, the internalized 125I-ANP radioactivity was determined by dissolving cells in 0.5 N NaOH. To determine the rate of lysosomal degradation of ligand-receptor complexes, cells were pretreated with chloroquine (200 μM) at 37 °C for 1 h. Cells were allowed to bind 125I-ANP at 4 °C for 60 min, washed with assay medium, and reincubated in fresh medium at 37 °C. The chloroquine treated through the binding and internalization period of the experiment. It should be noted that chloroquine did not alter the binding capacity of ligand to intact 293 cells at 4 °C. To assess the internalization of ligand-receptor complexes at the indicated time intervals, culture dishes were removed from 37 °C, the medium was collected, surface-associated radioactivity was removed by acetate buffer (pH 3.5), and cells were dissolved in 0.5 N NaOH. The radioactivity in acid eluate, cell lysate, and culture medium was considered as cell surface-associated, internalized, and released into medium, respectively. To determine whether the sequestration of ligand-receptor complexes was an energy-dependent process, cells were pretreated with dithothreitol. The quantitation of intact and degraded ligand released into the culture medium after internalization of ligand-receptor complexes was performed by precipitation of medium with 10% trichloroacetic acid containing 500 μg/ml bovine serum albumin as carrier. The recovered 125I-ANP in trichloroacetic acid precipitate were considered intact 125I-ANP molecules, and those in the supernatant were regarded as degraded products as described previously (28, 30).

**Recycling of Internalized NPRA in 293 Cells—**The recycling of NPRA in 293 cells was determined by trypsin-dependent loss of cell surface receptor radioactivity. The recycling of NPRA were washed with ice-cold binding assay medium and exposed to trypsin (0.025%) treatment for 10 min at 4 °C. At the end of the trypsin treatment, soybean trypsin inhibitor (200 μg/ml) was added, and cells were washed quickly three times with assay medium. Cells were reincubated in fresh medium at 37 °C in the absence or presence of cycloheximide (20 μg/ml), and 125I-ANP binding was determined as described above. The 10-min exposure of cells to trypsin treatment essentially abolished the cell surface-bound 125I-ANP, and it did not cause any significant cell detachment.

**Quantitative Measurement of Intracellular NPRA in Solubilized 293 Cells—**The cellular distribution of NPRA was analyzed by measuring both the total and intracellular receptors. Total cellular receptor content was quantitated by solubilizing 293 cells in a buffer containing 20 mM HEPES, pH 7.4, 1% Triton X-100, 15% glycerol, 0.15 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 2 mM N-ethylmaleimide, 1 mM EDTA, and 10 μg/ml each of leupeptin and aprotinin. The mixture was centrifuged for 5 min at 1000 × g to remove the insoluble material and then reconstituted at 100,000 × g for 60 min to obtain the clear supernatant. The 125I-ANP binding activity was assayed at 25°C for 60 min by adding 50 μl of solubilized supernatant to 400 μl of binding buffer containing 50 mM Tris-HCl, pH 7.4, 0.15 mM NaCl, 5 mM MgCl2, 0.1% bovine serum albumin, 0.5 mg/ml bacitracin, and 1 mM 125I-ANP with and without an excess of unlabeled ANP. The 125I-ANP bound to the solubilized receptors was precipitated by adding 0.25% bovine γ-globulin and 2.5 ml of 10% polyethylene glycol 8000 in 20 mM Tris-HCl, pH 7.4, 0.15 mM NaCl as described previously (28). The mixture was filtered under a vacuum through Whatman GF/B filters treated with 0.3% (w/v) polyethyleneimine. To quantitate only intracellular receptors, cells were first trypsinized to degrade almost all receptor binding activity and internalization period of the experiment. It should be noted that chloroquine did not alter the binding capacity of ligand to intact 293 cells at 4 °C. To assess the internalization of ligand-receptor complexes at the indicated time intervals, culture dishes were removed from 37 °C, the medium was collected, surface-associated radioactivity was removed by acetate buffer (pH 3.5), and cells were dissolved in 0.5 N NaOH. The radioactivity in acid eluate, cell lysate, and culture medium was considered as cell surface-associated, internalized, and released into medium, respectively. To determine whether the sequestration of ligand-receptor complexes was an energy-dependent process, cells were pretreated with dithothreitol. The quantitation of intact and degraded ligand released into the culture medium after internalization of ligand-receptor complexes was performed by precipitation of medium with 10% trichloroacetic acid containing 500 μg/ml bovine serum albumin as carrier. The recovered 125I-ANP in trichloroacetic acid precipitate were considered intact 125I-ANP molecules, and those in the supernatant were regarded as degraded products as described previously (28, 30).

**CAMP Assay—**293 cells stably expressing NPRA were treated with ANP at 37 °C in a dose- and time-dependent manner in the presence of...
0.2 mM 3-isobutyl-1-methylxanthine as described previously (29). To stop the reaction, the culture medium was aspirated, and cells were washed three times with phosphate-buffered saline and scraped in 0.5 N HCl. Cell suspension was subjected to five cycles of freeze and thaw and then centrifuged at 10,000 rpm for 15 min. In the supernatant, cGMP concentration was determined using an enzyme-linked immunosorbent assay kit (Assay Design) according to the manufacturer’s protocols.

Statistical Analysis—The dissociation constant \( K_d \) and the receptor density \( B_{\text{max}} \) were determined by Scatchard analysis. Data are presented as the means ± S.E. of triplicate determinations in at least three separate sets of experiments. Statistical significance was ascertained by the use of an unpaired, two-tailed \( t \) test.

RESULTS

Equilibrium Binding of \( ^{125}\text{I}-\text{ANP} \) and Generation of cGMP in Intact 293 Cells Stably Expressing NPRA—Binding of \( ^{125}\text{I}-\text{ANP} \) to 293 cells stably expressing recombinant NPRA was specific and was displaced by unlabeled hormone (Fig. 1A). The peptides unrelated to ANP, such as angiotensin II or endothelin-1, were unable to displace bound \( ^{125}\text{I}-\text{ANP} \). However, the specific \( ^{125}\text{I}-\text{ANP} \) binding was not discernible in nontransfected 293 cells. The binding of \( ^{125}\text{I}-\text{ANP} \) in recombinant 293 cells was rapid and saturable with increasing concentrations of radiolabeled ligand (Fig. 1B). Scatchard analysis of these binding data using a one-site model indicated a dissociation constant \( (K_d) \) value of \( 2.5 \times 10^{-10} \text{ M} \) and a density \( (B_{\text{max}}) \) of \( 2-3 \times 10^6 \) receptor sites/cell. ANP stimulated the intracellular accumulation of cGMP between 250- and 300-fold in a time- and concentration-dependent manner in 293 cells expressing recombinant NPRA as compared with untreated control cells (Fig. 2, A and B). To determine the time course of ligand binding, cells were incubated with \( ^{125}\text{I}-\text{ANP} \) at either 4 or 37 °C, and the amounts of cell surface-associated (acid-sensitive) and internalized (acid-resistant) radioactivity were determined as a function of time after the addition of hormone. The results showed that at 4 °C, most of the bound \( ^{125}\text{I}-\text{ANP} \) (≥95%) was acid-sensitive, regardless of the incubation time periods (Fig. 3A). At 37 °C, the bound \( ^{125}\text{I}-\text{ANP} \) was largely acid-sensitive at the initial incubation time points, however, \( ^{125}\text{I}-\text{ANP} \) radioactivity declined rapidly with increasing incubation time (Fig. 3B). The time course of the accumulation of acid-sensitive and acid-resistant radioactivity at 37 °C followed a precursor-product relationship, whereas acid-sensitive radioactivity became acid-resistant as a function of time.
Internalization and Sequestration of Ligand-Receptor Complexes of NPRA in 293 Cells—After binding of 125I-ANP to NPRA, the ligand-receptor complexes were internalized, and both the intact and degraded ligands were released into culture medium. The lysosomotropic agent chloroquine (200 μM) profoundly inhibited the intracellular degradation of internalized 125I-ANP. To perform these experiments, cells were pretreated with chloroquine at 37°C and cooled to 4°C, and cell surface receptors were labeled with 125I-ANP for 1 h. After the removal of the unbound ligand, cells were rapidly warmed to 37°C in fresh medium. At the indicated time intervals, the levels of radioactivity associated with the cell surface, internalized into the cell interior, and released into the culture medium were quantified utilizing the acid-wash procedure, which specifically dissociated cell surface-bound 125I-ANP (Fig. 4a). After a 20-min incubation at 37°C, 40–50% 125I-ANP radioactivity was cell surface-associated in control cells as compared with only 20% in chloroquine-treated groups. The intracellular (acid-resistant) 125I-ANP radioactivity increased rapidly to ~60% in 10-min in chloroquine-treated cells as compared with only 28% in control groups (Fig. 4b). However, after a 15-min incubation of the cells at 37°C, the effect of chloroquine was diminished, and acid-resistant radioactivity decreased to a level of 20% in 60 min and then remained at a plateau for almost 2 h. The release of radioactivity into the culture medium increased progressively, reaching equilibrium in 30–40 min (Fig. 4c). Initially, chloroquine inhibited the release of 125I-ANP in a time-dependent manner, but after a longer incubation time, the release of radiolabeled ligand increased steadily. Nevertheless, the treatment of cells with the lysosomotropic agents chloroquine, ammonium chloride, monensin, or nigericin, as well as the energy depleter dinitrophenol, significantly blocked the degradation of internalized 125I-ANP as compared with control cells (Table I).

The quantitative analysis of the intact and degraded ligand released into the culture medium was determined by meas-
Confluent 293 cells expressing recombinant NPRA were washed with assay medium and preincubated in 2 ml of fresh medium with indicated agents at 37 °C for 60 min. Cells were then washed and loaded with 125I-ANP for 60 min at 4 °C. After binding was completed, cells were washed four times with assay medium and placed at 37 °C. After a 20-min internalization and incubation period, dishes were removed from 37 °C and placed on ice, and medium was collected. Each dish was treated with 2 ml of acetate buffer (pH 3.5) for 2 min, acid eluate was collected, and cells were dissolved in 0.5 N NaOH as described under “Experimental Procedures.” The radioactivity in acid eluate, cell extract, and culture medium was counted to determine the cell surface-bound, internalized, and released radioactivity, respectively. Non-specific binding was determined by adding 100-fold excess concentrations of unlabeled ANP. The values shown are the mean ± S.E. of four independent determinations in triplicate dishes.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>125I-ANP radioactivity</th>
<th>% control</th>
<th>125I-ANP</th>
<th>Released 125I-ANP</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>45 ± 4</td>
<td>5 ± 1</td>
<td>62 ± 6</td>
<td></td>
</tr>
<tr>
<td>Amonium chloride</td>
<td>24 ± 2</td>
<td>60 ± 1</td>
<td>20 ± 2</td>
<td></td>
</tr>
<tr>
<td>Chloroquine</td>
<td>20 ± 3</td>
<td>58 ± 7</td>
<td>24 ± 2</td>
<td></td>
</tr>
<tr>
<td>Dinitrophenol</td>
<td>22 ± 3</td>
<td>62 ± 8</td>
<td>18 ± 2</td>
<td></td>
</tr>
<tr>
<td>Monensin</td>
<td>18 ± 2</td>
<td>68 ± 5</td>
<td>24 ± 3</td>
<td></td>
</tr>
<tr>
<td>Nigercin</td>
<td>25 ± 3</td>
<td>56 ± 6</td>
<td>17 ± 2</td>
<td></td>
</tr>
</tbody>
</table>

The radioactivity in acid eluate, cell extract, and culture medium was also analyzed by determining the trichloroacetic acid-soluble (Degraded 125I-ANP) and -precipitable (Intact 125I-ANP) products. The data represent the mean ± S.E. of three separate experiments.

FIG. 5. Composition of degraded and intact 125I-ANP in culture medium of chloroquine preloaded cells after 15- and 30-min incubation periods at 37 °C. Control and chloroquine-treated cells were incubated with 10 ng/ml 125I-ANP for 1 h at 4 °C. Unbound 125I-ANP was removed by washing the cells with assay medium, cells were collected, and the composition of degraded and intact 125I-ANP was analyzed by determining the trichloroacetic acid-soluble (Degraded 125I-ANP) and -precipitable (Intact 125I-ANP) products. The data represent the mean ± S.E. of three separate experiments.

- **Ligand-regulated Metabolic Processing of Internalized 125I-ANP/NPRA Complexes in 293 Cells**—Confluent 293 cells stably expressing recombinant NPRA were pretreated with chloroquine at 37 °C and then exposed to 125I-ANP at 4 °C for 60 min. Cells were washed to remove unbound ligand and then reincubated at 37 °C for different time periods. One set of culture dishes also received unlabeled ANP (10 nM). Extracellular unlabeled ANP inhibited the degradation of internalized ligand–receptor complexes as compared with untreated control cells (Fig. 6). In these experiments, at 37 °C, ~70–80% internalized 125I-ANP radioactivity was released into the culture medium. In chloroquine-treated cells, the extracellular unlabeled ANP affected the release of both the degraded and intact radiolabeled ligand (Fig. 6, A and B). Chloroquine inhibited the degradative processing of internalized 125I-ANP, thus causing an intracellular accumulation of intact 125I-ANP and a decrease in the release of degraded 125I-ANP products. We determined the effect of extracellular unlabeled ANP (10 nM) on the release of the intact and degraded 125I-ANP in control and chloroquine-treated cells. Extracellular unlabeled ANP effectively enhanced the release of both degraded and intact 125I-ANP under conditions in which the ANP degradative pathway was significantly impaired by chloroquine (Fig. 6, A and B). It was intriguing to find that chloroquine had only little or no effect on the release of intact 125I-ANP but dramatically inhibited the release of degraded 125I-ANP, indicating that endocytosed 125I-ANP can be processed through two separate and independent pathways. The data show that extracellular unlabeled ANP had no major effect on the release of degraded 125I-ANP in the presence of chloroquine. However, it effectively triggered the release of intracellular intact 125I-ANP in cells treated with chloroquine (Fig. 6, A and B).

**ANP-Dependent Down-Regulation of NPRA in 293 Cells**—The pretreatment of 293 cells with unlabeled ANP caused a substantial decrease in the 125I-ANP binding capacity of NPRA both in a time- and dose-dependent manner. Cells were treated with various concentrations of unlabeled ANP for the indicated time periods and then washed with acetate buffer (pH 3.5) to remove any bound ligand. The treatment of cells with 10 nM ANP markedly reduced the cell surface 125I-ANP binding by 55–65% in 60 min, and the micromolar concentrations of ANP produced almost a complete loss of cell surface ligand binding capacity of NPRA. The maximum effect of ANP on down-regulation of NPRA occurred within 60 min after treatment of cells with hormone.

**Recycling of Internalized NPRA in 293 Cells**—To examine whether the internalized NPRA is recycled back to the plasma membrane, recombinant 293 cells were incubated with 100 nM ANP at 37 °C for 2 h to deplete the cell surface receptors. After pretreatment with unlabeled ANP, cells were washed with acetate buffer (pH 3.5) to remove any bound hormone and then reincubated at 37 °C in fresh medium, after which a gradual return in the cell surface 125I-ANP binding was observed. After a 30-min incubation at 37 °C, cell surface binding returned to ~55–60% of the original levels (Fig. 7). A parallel set of culture dishes was also incubated in the presence of 20 μg/ml cyclohex-
Internalization and Down-regulation of NPRA

Discussion

These present studies were undertaken to address the kinetics of internalization, sequestration, recycling, and down-regulation of recombinant NPRA in intact 293 cells. The data establish that NPRA is a dynamic macromolecule that traverses through the subcellular compartments before it is degraded in the lysosomes. In 293 cells, the recombinant NPRA was expressed at a very high density with a B_max of 2–3 × 10^6 receptor sites/cell and a K_d value of 2.5 × 10^{-11} M. The expression of recombinant NPRA in 293 cells seems to be at a higher density compared with endogenous receptors present in Leydig tumor cells, as reported previously (30). The 125I-ANP binding assay was utilized to determine the post-binding kinetics and cellular itinerary of the labeled ligand-receptor complexes in intact cells. In the past, the interrelationship between the endocytic mechanisms, intracellular sequestration, and ANP-dependent down-regulation of NPRA has been controversial. It is conceivable that the homeostatic regulation of NPRA and cellular sensitivity of ANP would be dependent on a dynamic equilibrium and reutilization of the ligand-receptor complexes from the cell surface to the cell interior. Therefore, to understand the dynamics of the interrelationship between these processes, the events that occur after the binding of ligand to the receptor were investigated in 293 cells expressing a high density of recombinant NPRA. The results presented herein demonstrate that 125I-ANP binds to cell surface NPRA, enters through the subcellular compartments and is delivered to the intracellular compartments until it reaches a steady-state level after 30 min (t_1/2 = 15 min). The distribution of 125I-ANP radioactivity to the cell surface, intracellular compartments, and into the culture medium revealed a dynamic equilibrium between the rates of 125I-ANP uptake, subcellular sequestration, degradation, and extrusion from the cell interior to the extracellular space. The majority of the internalized 125I-ANP was degraded and rapidly released into the culture medium.

Fig. 6. Effect of unlabeled ligand on the release of internalized 125I-ANP in 293 cells. Confluent cells were preincubated in the absence or presence of 200 μM chloroquine at 37 °C for 1 h, and then cells were allowed to bind 125I-ANP at 4 °C for 1 h. After being washed with assay medium, one group of the chloroquine-treated cells was exposed to unlabeled extracellular ANP (10 nM). Both the treated and control cells were warmed to 37 °C, and the released 125I-ANP radioactivity levels were determined at the indicated times as described under “Experimental Procedures.” Panels A and B represent the degraded and intact 125I-ANP released into the culture medium, respectively, as a function of incubation time. The data represented are the mean of three independent experiments.

Discussion

These present studies were undertaken to address the kinetics of internalization, sequestration, recycling, and down-regulation of recombinant NPRA in intact 293 cells. The data establish that NPRA is a dynamic macromolecule that traverses through the subcellular compartments before it is degraded in the lysosomes. In 293 cells, the recombinant NPRA was expressed at a very high density with a B_max of 2–3 × 10^6 receptor sites/cell and a K_d value of 2.5 × 10^{-11} M. The expression of recombinant NPRA in 293 cells seems to be at a higher density compared with endogenous receptors present in Leydig tumor cells, as reported previously (30). The 125I-ANP binding assay was utilized to determine the post-binding kinetics and cellular itinerary of the labeled ligand-receptor complexes in intact cells. In the past, the interrelationship between the endocytic mechanisms, intracellular sequestration, and ANP-dependent down-regulation of NPRA has been controversial. It is conceivable that the homeostatic regulation of NPRA and cellular sensitivity of ANP would be dependent on a dynamic equilibrium and reutilization of the ligand-receptor complexes from the cell surface to the cell interior. Therefore, to understand the dynamics of the interrelationship between these processes, the events that occur after the binding of ligand to the receptor were investigated in 293 cells expressing a high density of recombinant NPRA. The results presented herein demonstrate that 125I-ANP binds to cell surface NPRA, enters through the subcellular compartments and is delivered to the intracellular compartments until it reaches a steady-state level after 30 min (t_1/2 = 15 min). The distribution of 125I-ANP radioactivity to the cell surface, intracellular compartments, and into the culture medium revealed a dynamic equilibrium between the rates of 125I-ANP uptake, subcellular sequestration, degradation, and extrusion from the cell interior to the extracellular space. The majority of the internalized 125I-ANP was degraded and rapidly released into the culture medium.
Exposed to 100 nM unlabeled ANP for 1 h. After being washed free of ANP with acid buffer (pH 3.5), cells were reincubated in fresh medium with and without cycloheximide. Specific 125I-ANP binding was determined at the indicated time intervals after the initial ANP exposure as described under “Experimental Procedures.” One group of cells was exposed in the presence of ANP throughout (dotted line) as a control. A second group of cells was washed with acidic buffer (pH 3.5) and cultured in fresh medium at 16 °C for 45 min, and the temperature was then shifted to 37 °C (shown by the arrow). The solid bar represents the binding of 125I-ANP in control cells (C), which were never exposed to ANP treatment.

Which consisted of ~70–75% degraded products and 20–25% intact ligand. The rates of internalization, degradation, and release of 125I-ANP were markedly decreased in the presence of metabolic inhibitors such as chloroquine and dinitrophenol and also at low temperature, which suggested that the metabolic processing of 125I-ANP in 293 cells is, in part, lysosomal. However, after longer incubation periods, the effect of chloroquine was only partially effective in blocking the release of both the degraded and intact ligands.

Previous studies from this laboratory as well as reports by others have suggested that endogenous NPRA undergoes rapid endocytosis in Leydig tumor (MA-10) cells (30, 32) and in PC-12 cells (33). On the other hand, studies by Maack and colleagues (25) suggested that ANP-NPRA complexes were not processed intracellularly in renomedullary interstitial cells. These authors suggest that a rapid dissociation of receptor-ligand complexes probably occurs upon ANP binding to NPRA at 37 °C, and the intact ligand is released into the culture medium. A trace amount of 125I-ANP may be released from the receptor by the neutral endopeptidase, as described in neuroblastoma cells (34). However, further studies have not been carried out to support those postulates. Our recent data in COS-7 cells transiently expressing NPRA have provided the evidence that its internalization seems to be controlled by sequences located within the carboxyl-terminal domain of this receptor protein (28). The phenomena of receptor-mediated internalization and metabolic processing of ANP through the non-guanosine cyclase-containing receptor, NPRC, have been investigated in a number of laboratories utilizing vascular smooth muscle cells, which contain a predominantly high density of endogenous NPRC (35–43). Early studies of the post-binding events of NPRC were greatly facilitated because of its predominant presence in vascular smooth muscle cells, which are among the important target cells for ANP. In contrast, studies on the post-binding events of NPRA were hampered because of the lack of suitable target cells that exclusively contained this receptor protein. The results from this present study with recombinant 293 cells, which predominantly express NPRA, firmly establish that ANP-NPRA complexes are rapidly internalized and sequestered into the intracellular compartments and the degraded products released into culture medium.

Further experiments revealed that a short-term exposure of recombinant 293 cells with increasing concentrations of unlabeled ANP at 37 °C resulted in an accelerated loss of cell surface receptors. The maximal effect of ANP on down-regulation of NPRA occurred between 40 and 60 min, suggesting the involvement of receptor-mediated endocytosis and subsequent metabolic degradation and redistribution of ligand-receptor complexes in the cell interior. The data suggested that ~30–35% of NPRA returned to the cell surface in ANP-treated cells. The treatment of cells with unlabeled ANP accelerated the release of intact ligand, indicating that ANP-dependent down-regulation of its receptor involves the internalization of ligand-receptor complexes, which dissociate intracellularly and probably escape the lysosomal degradative pathway. Nevertheless, an alternate mechanism also seems to exist for the release of intact ligand (Fig. 9). Dual pathways for the intracellular processing of ligand-receptor complexes have also been proposed previously for insulin and epidermal growth factor (EGF) receptors (44–47). In our metabolic processing studies of NPRA, ANP binding has been used as an index of NPRA activity. Degradation, on the other hand, is considered as the actual loss of receptor from the cell interior by proteolysis into amino acids. Temporally, inactivation may precede degradation, or both events may occur simultaneously. An invaluable experimental tool in the elucidation of NPRA inactivation would be the use of ANP-induced receptor down-regulation. Essentially, down-regulation may result in a loss of cellular NPRA by means of an accelerated rate constant for receptor internalization and presumably inactivation. Desensitization and/or inactivation of NPRA have been suggested by mechanisms involving ANP-dependent dephosphorylation of this receptor protein (48). However, the exact mechanisms of dephosphorylation-dependent inactivation of NPRA are not well understood. In contrast, it has also been suggested that phosphorylation of NPRA occurs (17, 18, 20, 48, 49) and is probably essential for its activation process (18, 50). It would be anticipated that inactivation of NPRA might occur intracellularly, and the inhibition
Interestingly, in cycloheximide-treated cells, binding of
125I-ANP binding was determined from the specific
125I-ANP binding parameters.

The cells were then further incubated at
37 °C in fresh medium containing 10%
sodium bicarbonate, and 125I-ANP was determined in
37
°
C in the absence or presence of cycloheximide
treated (0.025%) and in control groups as described under
Experimental Procedures. The solid bar
represents the binding of 125I-ANP in control
cells, which were never exposed to tryp-
sin. Each data point represents the
mean ± S.E. of three separate experi-
ments. ○, without cycloheximide; ■, with
cycloheximide.

Table II
Quantitative analysis of the total and intracellular pool of NPRA in
intact and solubilized 293 cells after treatment with and without
Trypsin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Specific 125I-ANP Binding</th>
<th>% Relative Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control cells</td>
<td>18.75 ± 1.89</td>
<td>100</td>
</tr>
<tr>
<td>Trypsin-treated cells</td>
<td>1.25 ± 0.14</td>
<td>6.60</td>
</tr>
<tr>
<td>Triton X-100 solubilized cells</td>
<td>26.89 ± 2.92</td>
<td>100</td>
</tr>
<tr>
<td>Trypsin-treated solubilized cells</td>
<td>5.85 ± 0.36</td>
<td>21.75</td>
</tr>
</tbody>
</table>

of internalization should prevent this process. However, the
molecular and biochemical nature of the inactivation process of
NPRA and the subcellular localization of the events have yet to
be determined.

To examine the recycling of recombinant NPRA, 293 cells
were treated with trypsin at 4 °C for 10 min, which abolished
cell surface receptors. However, after washing the cells free of
trypsin and incubating them in fresh medium at 37 °C, a re-
turn in 125I-ANP binding was observed. In parallel, one group
of trypsin-treated cells was also exposed to cycloheximide.
Interestingly, in cycloheximide-treated cells, binding of 125I-ANP
was 25–30% lower as compared with control cells without cy-
cloheximide treatment. These observations provided further
evidence that a return in 125I-ANP binding was due to the
recycling of NPRA. However, a complete return in ANP binding
did not occur, suggesting that a new protein synthesis may also
be required. Both the lysosomotropic agent chloroquine and the
metabolic inhibitor dinitrophenol, which deplete cellular ATP,
disrupted the internalization and recycling process of NPRA.
However, it has been reported that ATP is not required for
internalization of insulin receptors (51, 52), but it seems to be
essential for the internalization of EGF receptors (53). It is
envisioned that the receptor-mediated endocytosis of ANP-
NPRA complexes may involve a number of sequential sorting
steps through which ligand-receptor complexes could be eventu-
ally degraded, recycled back to the cell surface, or released
into the cell exterior. A number of these events may take place
sequentially, as shown in Fig. 9. The first step would be the
noncovalent binding of ligand to the cell surface receptor. The
receptors, through some intrinsic affinity or aggregation
induced by protein binding, must cluster into pits on the cell
membrane. The proposed itinerary would be consistent with
the current data indicating that ligand-receptor complexes
should be delivered to the lysosomes. Acidification of lysosomes
may induce the dissociation of ligand from the receptor, and a
population of receptor molecules may recycle back to the
plasma membrane. The data are consistent with the following
findings: (a) a lysosomotropic agent such as chloroquine is
unable to completely block the ligand-receptor degradation in
lysosomes; (b) the release of intact ANP seems to occur through
a lysosome-independent pathway; and (c) recycling of endocy-
tosed receptor back to the plasma membrane occurs simulta-
neously with the process leading to the degradation of the
majority of ligand-receptor complexes into lysosomes.

The present findings indicate that internalized ANP bifur-
cates into two major pathways: a degradative pathway, through
which the majority of internalized ANP (70–80%) of
all incoming ligand is processed in lysosomes, and a retroen-
docytic pathway that accelerates the release of intact ANP.
This approach should provide a direct assessment of ligand-
bound receptor trafficking for various ligand-receptor com-
plexes in different cell types. It is conceivable that some
remarkable differences do exist with regard to the internaliza-
tion, processing, and metabolic turnover among various types
of membrane receptors. Our results show that recombinant 293
cells begin to release degraded ANP within minutes after
internalization of ligand-receptor complexes at 37 °C. The
phenomenon of ANP-NPRA degradation is similar to that reported
for low density lipoprotein receptors in human fibroblasts (54,
55), insulin receptors in adipocytes (56–58) as well as in trans-
rected Chinese hamster ovary cells (59), and thyrotropin hor-
mones receptors in GH3 cells (60). However, the degradation of
asialoglycoprotein and its receptor complexes is not observed.
until about 30 min after endocytosis in hepatoma cells (61). Similarly, the degradation of EGF is not detectable for at least 20 min in hepatocytes (62). Although there is no apparent explanation to account for such differences, several possibilities may be considered. Because the internalization of receptor-bound ligand should not be the limiting factor, there may be multiple pathways leading to the eventual metabolic turnover of ligand-receptor complexes, perhaps utilizing different intermediate vesicles for the transfer of ligands to the site of degradation. If this was the case, then the route could be determined by either intrinsic properties of ligand-receptor complexes or the way various cells process the incoming ligands. It is also possible that there may be a single metabolic pathway composed of several distinct processing steps, which should be unique for a specific ligand-receptor complex.

The present results demonstrating that chloroquine effectively interrupts the degradative process without exerting a deleterious effect on the retroendocytic pathway is novel and intriguing. In agreement with our findings, several other types of ligand-receptor complexes recycle through the chloroquine-insensitive pathway, including EGF, insulin, and asialoglycoprotein receptors (46, 47, 56, 63, 64). This establishes the notion that after internalization, many types of ligand-receptor complexes can recycle through the chloroquine-insensitive pathway and finally be degraded via chloroquine-sensitive lysosomal pathway.

Acknowledgment—We thank Kamala Pandey for typing the manuscript.

REFERENCES


Fig. 9. Schematic representation of internalization, recycling, and intracellular degradation of 125I-ANP bound to NPR-A in 293 cells. The schematic diagram shown postulates the stoichiometric kinetics of internalization, subcellular sequestration, recycling, and ultimately metabolic turnover of ligand-receptor complexes from cell surface to cell interior and back to the plasma membrane. The scheme depicts that after synthesis: (i) the receptor is inserted in the plasma membrane; (ii) the ligand-receptor complex enters the cell via coated pits; and (iii) the complex is processed intracellularly through endosome-, lysosome-, and/or chloroquine-insensitive pathways. Sorting of bound ANP-NPR-A complexes into the intracellular compartments may occur by (i) lysosomal degradative metabolic pathway, (ii) endosomal dissociation metabolic pathway, and/or (iii) release through the chloroquine-insensitive pathway.