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

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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 compartments appears to indicate this fact.

Atrial natriuretic peptide (ANP)1 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 ~250 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 α-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 retroendocytotic 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.
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 ($B_{max}$) 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 $^{125}$I-ANP and Generation of cGMP in Intact 293 Cells Stably Expressing NPRA—Binding of $^{125}$I-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 $^{125}$I-ANP. However, the specific $^{125}$I-ANP binding was not discernible in nontransfected 293 cells. The binding of $^{125}$I-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 $2.5 \times 10^{-10}$ M and a density ($B_{max}$) 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 $^{125}$I-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 $^{125}$I-ANP (≥95%) was acid-sensitive, regardless of the incubation time periods (Fig. 3A). At 37 °C, the bound $^{125}$I-ANP was largely acid-sensitive at the initial incubation time points, however, $^{125}$I-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.

![Fig. 1. Specific competition and saturation binding of $^{125}$I-ANP in 293 cells stably expressing recombinant NPRA.](image1)

![Fig. 2. Concentration- and time-dependent formation of intracellular cGMP in response to ANP treatment in 293 cells stably expressing recombinant NPRA.](image2)
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-associated, 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 I

| Treatment          | 125I-ANP radioactivity | % control |
|--------------------|------------------------|-----------|
|                    | Cell surface 125I-ANP | Internized 125I-ANP | Released 125I-ANP |
| None               | 45 ± 4                 | 5 ± 1      | 62 ± 6                 |
| Amonium chloride   | 24 ± 2                 | 60 ± 1     | 20 ± 2                 |
| Chloroquine        | 20 ± 3                 | 58 ± 7     | 24 ± 2                 |
| Dinitrophenol      | 22 ± 3                 | 62 ± 8     | 18 ± 2                 |
| Monensin           | 18 ± 2                 | 68 ± 5     | 24 ± 3                 |
| Nigercin           | 25 ± 3                 | 56 ± 6     | 17 ± 2                 |

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.

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.

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—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
plasma membrane, and this could account for a significant
preexisting intracellular pool (Table II). Trypsin inactivates
receptor binding in trypsin-treated solubilized cell prepara-
tions indicated that most of the receptors were present on the
intracellular compartments until it reaches a steady-state
level after 30 min ($t_{1/2}$). The distribution of $^{125}$I-ANP
radioactivity to the cell surface, intracellular compartments,
and into the culture medium revealed a dynamic equilibrium
between the rates of $^{125}$I-ANP uptake, subcellular sequestra-
tion, degradation, and extrusion from the cell interior to the
extracellular space. The majority of the internalized $^{125}$I-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 $^{125}$I-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 $^{125}$I-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 $^{125}$I-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 $^{125}$I-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 $^{125}$I-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-guanylyl 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 of trypsin-treated cells was also exposed to cycloheximide. Internalization and down-regulation of NPRA. The cells were then further incubated at 37 °C in fresh medium containing 10% serum, and 125I-ANP was determined in the absence or presence of cycloheximide (20 µg/ml) as described under "Experimental Procedures." The solid bar represents the binding of 125I-ANP in control cells, which were never exposed to trypsin. Each data point represents the mean ± S.E. of three separate experiments. ● without cycloheximide; △ with cycloheximide.

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 return 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 cycloheximide 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 envision that the receptor-mediated endocytosis of ANP-NPRA complexes may involve a number of sequential sorting steps through which ligand-receptor complexes could be eventually 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 endocytosed receptor back to the plasma membrane occurs simultaneously with the process leading to the degradation of the majority of ligand-receptor complexes into lysosomes.

The present findings indicate that internalized ANP bifurcates 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 retroendocytotic pathway that accelerates the release of intact ANP. This approach should provide a direct assessment of ligand-bound receptor trafficking for various ligand-receptor complexes in different cell types. It is conceivable that some remarkable differences do exist with regard to the internalization, 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 transfected Chinese hamster ovary cells (59), and thyrotropin hormone receptors in GH3 cells (60). However, the degradation of asialoglycoprotein and its receptor complexes is not observed.

### Table II

| Treatment                      | % Specific 125I-ANP | % Relative binding |
|--------------------------------|--------------------|--------------------|
| Untreated control cells        | 18.75 ± 1.89       | 100                |
| Trypsin-treated cells          | 1.25 ± 0.14        | 6.60               |
| Triton X-100 solubilized cells | 26.89 ± 2.92       | 100                |
| Trypsin-treated solubilized cells | 5.85 ± 0.36   | 21.75              |

The confluent 293 cells in 60-mm² culture dishes were washed with binding assay medium (Dulbecco’s modified Eagle’s medium containing 0.1% bovine serum albumin). Cells were allowed to bind 125I-ANP in the presence of 0.025% trypsin and incubating them in fresh medium at 37 °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 return 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 cycloheximide 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

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See also Table II.
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 inhibits 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.

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