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CLATHRIN-DEPENDENT INTERNALIZATION, SIGNALING, AND METABOLIC PROCESSING OF GUANYLYL CYCLASE/NATRIURETIC PEPTIDE RECEPTOR-A

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Abstract

Cardiac hormones, atrial and brain natriuretic peptides (ANP and BNP), have pivotal roles in renal hemodynamics, neuroendocrine signaling, blood pressure regulation, and cardiovascular homeostasis. Binding of ANP and BNP to the guanylyl cyclase/natriuretic peptide receptor-A (GC-A/NPRA) induces rapid internalization and trafficking of the receptor via endolysosomal compartments, with concurrent generation of cGMP. However, the mechanisms of the endocytotic processes of NPRA are not well understood. The present study, using ¹²⁵I-ANP binding assay and confocal microscopy, examined the function of dynamin in the internalization of NPRA in stably transfected human embryonic kidney-293 (HEK-293) cells. Treatment of recombinant HEK-293 cells with ANP time-dependently accelerated internalization of receptor from the cell surface to the cell interior. However, the internalization of ligand-receptor complexes of NPRA was drastically decreased by the specific inhibitors of clathrin and dynamin-dependent receptor internalization; almost 85% by monodansylcadaverine (MDC), 80% by chlorpromazine (CPZ) and 90% by mutant dynamin, which are specific blockers of endocytic vesicle formation. Visualizing internalization of NPRA and enhanced GFP (eGFP)-tagged NPRA (eGFP-NPRA) in HEK-293 cells by confocal microscopy, demonstrated the formation of endocytic vesicles after 5 min of ANP treatment; this effect was blocked by the inhibitors of clathrin and by mutant dynamin construct. Our results, suggest that NPRA undergoes internalization via clathrin-mediated endocytosis as part of its normal itinerary, including trafficking, signaling, and metabolic degradation.

Keywords

Guanylyl cyclase/natriuretic peptide receptor-A; recombinant HEK-293 cells; endocytosis; clathrin-coated pit; dynamin

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Disclosure Statement

None

INTRODUCTION

There are three types of natriuretic peptides (NPs). These are atrial (ANP), brain (BNP), and C-type (CNP); they principally mediate natriuretic, diuretic, vasorelaxant, and antimitogenic responses largely directed to the reduction of blood pressure and maintenance of fluid-volume homeostasis [1–7]. ANP and BNP are primarily synthesized in the granules of the heart and circulate in the plasma, while CNP, which is highly conserved among species, is basically present in endothelial cells. Natriuretic peptide receptors have been classified, namely natriuretic peptide receptor-A (NPRA), -B (NPRB), -C (NPRC), and ANF-RGC. NPRA and NPRB show similar homology and both also contain an extracellular ligand-binding domain, a single transmembrane region, an intracellular protein kinase-like homology domain (protein KHD), and a guanylyl cyclase (GC) catalytic domain [8–11]. ANP and BNP activate NPRA, which produces second-messenger cGMP in response to hormone binding [12, 13]. CNP activates NPRB, which also produces cGMP. However, all three natriuretic peptides indiscriminately bind to NPRC, which lacks GC activity [5, 14–16].

NPRA is considered to be the biologically active receptor of ANP and BNP, since most of the physiological effects of these peptide hormones are triggered by the generation of second-messenger cGMP [5, 17–22]. The biological function of NPRA is exhibited primarily through ANP-dependent GC catalytic activity and cGMP accumulation, which are regulated by several factors, including growth factors, physiological milieu, and the ligand itself [21, 23–29]. As we and others have found, gene targeting (gene disruption and duplication) of *Npr1* (coding for NPRA) in mouse models demonstrates the hallmark significance of NPRA signaling in lowering the arterial pressure and protecting against pathophysiological renal and cardiac conditions [30–34]. However, the use of ¹²⁵I-ANP binding of both NPRA and NPRB has demonstrated that the bound ligand-receptor complexes of NPRA are rapidly internalized within cells and largely degraded in the lysosomes [21, 27, 35, 36].

After ANP binding, NPRA dimerizes and the GC catalytic domain becomes activated [37]. ANP binding to the receptor also activates rapid internalization and trafficking of NPRA into intracellular compartments [13, 15, 21, 35, 36, 38, 39]. ANP is subsequently delivered, probably through endosomes, to lysosomal compartments, where it is largely degraded; however, populations of ligand-receptor complexes escape the lysosomal compartment, allowing these receptors to recycle back to the plasma membrane [12, 13, 27].

Ligand-mediated receptor endocytosis is considered to be a vital mechanism for physiological responses. Clathrin-mediated endocytosis is well established as a mechanism for several membrane-bound receptors, including insulin, glucagon, epidermal growth factor (EGF), and platelet-derived growth factor (PDGF), which are also downregulated by ligand-mediated endocytosis [40–42]. Although considerable progress has been made in structure-function studies of the receptor, the issue of the internalization of NPRA, an important member of the GC-coupled membrane receptor family, has been controversial. This controversy arose in response to reports that of the three natriuretic peptide receptors only NPRC is internalized with bound ligand [43–45]. In our recent study using confocal

immunofluorescence microscopy, we clearly visualized the internalization and subcellular trafficking of eGFP-tagged NPRA (eGFP-NPRA) in HEK-293 cells and murine mesangial cells [12, 13, 46]. NPRB, in response to CNP binding, has also been shown to be internalized and recycled back to the cell surface in cultured hippocampus neurons and C6 glioma cells [47]. Thus, the prevailing consensus is that in response to ligand binding both NPRA and NPRB are internalized into the cell interior. However, the exact mechanisms of endocytosis of NPRA and NPRB are not yet clearly understood.

The molecular mechanisms through which NPRA is internalized need to be investigated in a greater detail to determine the intracellular destiny of NPRA. To determine the role of clathrin-coated pits in the NPRA internalization process, we examined the effect of dynamin GTPase with blockers of clathrin-coated vesicle formation such as monodansylcadaverine (MDC), chlorpromazine (CPZ), and mutant dynamin, using recombinant HEK-293 cells expressing eGFP-NPRA fusion protein.

MATERIALS AND METHODS

Materials

ANP (rat-28) was purchased from Peninsula Laboratories (Belmont, CA). HEK-293 cells were obtained from the American Type Culture Collection (Manassas, VA). LT1 transfection reagent was obtained from Panvera (Madison, WI). ^{125}I -ANP was purchased from Amersham Biosciences. The mammalian expression vector p^{cDNA6} was obtained from Life Technologies (Grand Island, NY). Vectashield mounting medium was obtained from Vector Laboratories (Burlingame, CA). A cGMP complete-ELISA kit was obtained from Enzo Life Sciences. Tissue culture supplies were purchased from Life Technologies. CPZ, MDC, paraformaldehyde, and all other reagent grade chemicals were obtained from Sigma (St. Louis, MO).

Construction and stable transfection of eGFP-NPRA chimeric vector

The fusion of the C-terminus of murine NPRA cDNA [10] to the N-terminus of enhanced green fluorescent protein (eGFP) was done by subcloning it into p^{eGFP-NT} vector (Clontech, San Diego, CA) for its expression as an eGFP-NPRA fusion protein, as previously reported [13]. HEK-293 cells were grown in 6-well culture plates containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS). Initially, subconfluent cells were transfected with eGFP-NPRA cDNAs using LipofectamineTM reagents. To establish the stably expressing receptor cell lines, 400 µg/ml of geneticin was added to the culture medium after transfection as previously described [13, 21]. Antibiotic-resistant clones were isolated and established for receptor expression, using ANP as a ligand [21].

Plasmid construction of wild-type and mutant dynamin

The cDNA encoding wild-type and mutant K44A/dynamin, which was a gift from Dr. Sandra Schmid (Scripps, La Jolla, CA), was excised from pCB vectors and subcloned into pcDNA6/V5-His vector. The expression vector pcDNA6/V5-His, designed to function under the control of cytomegalovirus immediate early promoter, contains the simian virus-40

region of replication to increase transient expression of encoded protein in transfected cells. The plasmid of interest that had the insert in the correct orientation was identified by DNA sequencing.

Cell culture with stable and transient transfection

HEK-293 cells were grown in DMEM supplemented with 10% FBS at 37°C in an atmosphere of 5% CO₂ and 95% O₂. Cells were transfected with murine NPRA cDNA using LT-1 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 after transfection. Antibiotic-resistant clones were isolated and established for receptor expression by ¹²⁵I-ANP binding and cGMP assays as previously described [21]. For transient transfection, recombinant HEK-293 cells were harvested by trypsin and transfected with plasmids containing either full-length or mutant dynamin cDNAs. Transfection was done by electroporation using a gene pulser (BioRad, Richmond, CA) with 5 µg of plasmid DNA at 220 V with a capacitance setting of 960 µF and cells were then seeded into 60-mm² culture dishes. The medium was changed after 24 h; functional studies were done 48 h after DNA transfection.

Monodansylcadaverine and chlorpromazine treatment

Confluent HEK-293 cells in 6-cm² culture dishes were washed with assay medium (DMEM containing 0.1% bovine serum albumin; BSA) and independently pretreated in either the presence or absence of 100 µg/ml of MDC and 10 µg/ml of CPZ at 37° for 1 h. The CPZ and MDC concentrations and times of incubation were chosen so that cell viability was not affected.

Confocal microscopy

For all confocal fluorescence studies, cells were seeded on cover glasses and grown for 2 days, and incubated with 100 nM ANP for different lengths of time. After incubation, they were washed in PBS, fixed in 4% paraformaldehyde for 30 min, and washed with PBS three times, each time for 5 min. Cover glasses were allowed to dry and mounted on glass slides with Vectashield mounting medium, then sealed with Fixogum rubber cement. Cells were examined and images acquired using a TCS SP2 confocal laser scanning microscope (Leica Microsystems, Heidelberg, Germany). In all experiments, images of cells (1,024 × 1,024 pixels) were visualized using the same microscope settings (scans with wavelengths set as green 488–510), using a 63X Apo-oil immersion objective (NA 1.4) and 60-µm aperture, using LEICA Scan TCS-SP2 software (Leica Microsystems). The pinhole was adjusted to keep the same size of z-optical sections (1-µm z-axis) for green channels. In all experiments, images of cells were acquired as single midcellular optical sections and averaged over 8 scans per frame.

¹²⁵I-ANP binding assay

Confluent HEK-293 cells in 6-cm² culture dishes were washed with assay medium then labeled with ¹²⁵I-ANP in the presence or absence of a 10-fold excess molar concentrations of unlabeled ANP, essentially as previously described [21, 35]. After the completion of ¹²⁵I-

ANP binding at 4 °C, unbound ligand was removed from the culture dishes by four washes (2 ml each) with ice-cold DMEM assay medium. To quantitate the cell-surface-associated ^{125}I -ANP radioactivity, the acid wash method was used as we have previously reported [27, 35]. Each culture dish received 1 ml of ice-cold acetate buffer (pH 3.4) and cells were kept at 4 °C for 2 min. The acid eluates were removed and collected from each dish, after which all dishes were washed with additional 1 ml of ice-cold acid buffer. Both acid-wash solutions were combined to quantitate the acid-sensitive ^{125}I -ANP radioactivity on the cell surface. After which, cells were then dissolved in 0.5 N NaOH overnight and acid-resistant ^{125}I -ANP radioactivity in the intracellular compartments was determined. The acid-sensitive ^{125}I -ANP radioactivity was considered as an index of cell-surface-bound ANP; the acid-resistant radioactivity was used as a measurement of the internalized ligand-receptor complexes of ANP-NPRA.

Internalization and quantification of ligand-receptor complexes of ANP-NPRA

Incubation of culture dishes at 4°C for 60 min allowed ^{125}I -ANP to bind to HEK-293 cells expressing recombinant NPRA essentially as previously described [38]. Unbound ^{125}I -ANP was completely removed by washing the cells 4x (2ml each wash) with ice-cold DMEM assay medium as described above. The total cell-associated ^{125}I -ANP radioactivity was quantitated by dissolving cells in 0.5 N NaOH and counting the radioactivity in the cell lysate as previously reported [13, 21]. The initial zero-time control value of ^{125}I -ANP binding was represented by 100%. To begin the internalization of ligand-receptor complexes, cells were quickly warmed to 37 °C. At various times (1, 2.5, 5, 10, 15, 30, and 60 min), culture dishes were removed from 37°C and placed on ice at 4°C and medium was collected. The cell-surface-associated ^{125}I -ANP radioactivity was removed by washing the cells with ice-cold acetate buffer (pH 3.4) at 4°C. After acid wash, the amount of internalized ^{125}I -ANP radioactivity was quantified by dissolving cells in 0.5 N NaOH overnight. The treatment of cells with CPZ and MDC was maintained throughout the entire ^{125}I -ANP binding and internalization period. MDC or CPZ did not alter the capacity of ^{125}I -ANP to bind to intact HEK-293 cells at 4°C. Both the intact and degraded ligands into the culture medium were quantified by the precipitation of culture media with 10% trichloroacetic acid (TCA) containing 200 µg/ml BSA as carrier. The ^{125}I -ANP in the TCA precipitate was considered as the intact ligand, while in the supernatant it was counted as the degraded products, essentially as previously reported [13, 21, 35].

Quantification of endocytic vesicles

In the quantification of endocytic vesicle analysis, 50 cells or more were scored (control, MDC-treated, CPZ-treated, and dynamin wild-type and mutant transfected) per condition. The number of fluorescent vesicles per cell after different time of incubation was quantified and represented as the fold ratio with respect to untreated cells. Graph prism software was used to generate the bar graphs and statistical analyses of data. The results are presented as the means $\pm$ S.E.M. of 5–8 independent experiments.

cGMP assay

Cells were treated with 100 nM ANP for 5, 10, and 30 min in the presence of 0.2 mM IBMX and independently pretreated in either the presence or absence of 100 µg/ml of MDC and 10

µg/ml of CPZ at 37° for 1 h. Cells were also treated with 100 nM ANP for 5, 10, and 30 min after transfection with dynamin GTPase blocker (dynamin mutant). After treatment with ANP for different time points, cells were washed three times with PBS and scraped in 0.1 M HCl. Cell suspension was subjected to five cycles of freeze and thaw and then centrifuged at 10 000 *g* for 15 min at 4°C. The supernatant was utilized for cGMP assay by direct cGMP complete-EIA kit (Enzo Life Sciences) according to the manufacturer's procedure as previously reported [13].

Statistical analysis

Statistical analyses were done using Graph Pad Prism software (San Diego, CA). The results are presented as mean ± standard error (SE) of the average responses in multiple experiments done with different cell preparations. Results were normalized relative to untreated controls. Statistical significance was assessed using ANOVA followed by Dunnett's multiple comparisons test. The probability value of $p < 0.05$ was considered significant.

RESULTS

Effect of endocytotic inhibitors and dynamin GTPase blocker on the internalization of NPRA in recombinant HEK-293 cells

Bound ¹²⁵I-ANP-NPRA complexes were measured to examine the kinetics of internalization, trafficking, and sequestration of NPRA in stably expressing HEK-293 cells after treatment with the endocytic inhibitors (MDC and CPZ) and transfection with dynamin GTPase blocker (dynamin mutant). The radioligand ¹²⁵I-ANP was bound to cell-surface receptors during incubation at 4 °C, then allowed to internalize after the cells were warmed to 37 °C. The time course of ¹²⁵I-ANP internalization was determined for MDC and CPZ in HEK-293 cells expressing recombinant NPRA. The results show that ¹²⁵I-ANP bound to control cells as well as MDC- and CPZ-treated cells (Fig. 1A). In control cells, ligand-receptor complexes of ANP-NPRA were internalized to maximum levels in 30 min at 37°C. Results are expressed as percentages of the total cell-associated radioactivity internalized at each time point. The rate of internalization of ligand-receptor complexes was clearly decreased by almost 70%–80% in recombinant HEK-293 cells treated with MDC and CPZ. However, bound ¹²⁵I-ANP-NPRA was rapidly internalized in control recombinant HEK-293 cells and cells transfected with wild-type dynamin (Fig. 1B). In contrast, HEK-293 cells transfected with mutant dynamin did not show internalization of ANP-NPRA ligand-receptor complexes.

Effect of MDC and CPZ on the kinetics of internalization and sequestration of ligand-receptor complexes of NPRA in HEK-293 cells

After binding of ¹²⁵I-ANP to NPRA, the ligand-receptor complexes were internalized and both intact and degraded ligands were released into the culture medium. The dynamin inhibitors MDC (100 µg/ml) and CPZ (10 µg/ml) significantly inhibited endocytosis and intracellular degradation of internalized ¹²⁵I-ANP. For these experiments, cells were pretreated with MDC and CPZ at 37°C and cooled to 4°C; cell-surface receptors were labeled with ¹²⁵I-ANP for 1 h. After removal of the unbound ligand, cells were rapidly

warmed to 37°C in fresh medium. At the specified time intervals, the levels of radioactivity associated with cell surfaces, internalized into the cell, and released into the culture medium were quantified by the acid-wash procedure, which dissociated cell-surface-bound ^{125}I -ANP. After 30 min of incubation at 37°C, control HEK-293 cells expressing wild-type NPRA rapidly internalized 80%–90% of ligand-receptor complexes. However, after the same incubation period, MDC-treated HEK-293 cells expressing NPRA internalized only 10%–20% of ligand-receptor complexes (Fig. 2A). The amount of internalized receptor decreased rapidly after 10 min of incubation at 37°C. However, internalized ^{125}I -ANP radioactivity was reduced almost 80% to 85% in cells pretreated with both MDCs as compared with untreated control cells (Fig. 2B). As a result, most of the ^{125}I -ANP remained on cell surfaces in MDC-treated cultures.

The release of ^{125}I -ANP into the culture medium of HEK-293 cells expressing wild-type NPRA increased progressively, reaching 70%–80% with equilibrium after 30 min incubation at 37°C. However, only 10%–20% of ^{125}I -ANP was released into the culture medium in cells pretreated with MDC (Fig. 2C). Similarly, another dynamin inhibitor, CPZ, also time-dependently inhibited the internalization of ^{125}I -ANP-bound NPRA almost 85%–90% within 15 min of incubation at 37°C (Fig. 3A). Subsequently, as compared with wild-type receptor, the intracellular accumulation, recycling, and degradation of ligand-receptor complexes were all greatly diminished (Fig. 3 A–C).

Expression of mutant dynamin blocks the internalization of NPRA in recombinant HEK-293 cells

Using the acid-wash procedure, we determined the cell-surface-associated, internalized, and released ^{125}I -ANP radioactivity in recombinant HEK-293 cells expressing either wild-type or mutant dynamin. During the internalization period, culture dishes were removed from 37°C and placed at 4°C, medium was collected after increasing incubation periods. Cells were washed once with Hank's balanced salt solution (HBSS), then treated with acetate buffer (pH 3.4) at 4°C to release cell-surface-associated ^{125}I -ANP at the different time periods.

HEK-293 cells expressing wild-type dynamin internalized almost 75%–80% of receptors within 15 min of incubation at 37°C. However, HEK-293 cells expressing dynamin mutant internalized only 10%–15% of receptors within the same period (Fig 4A). In recombinant HEK-293 cells, the population of internalized ligand-receptor complexes expressing mutant dynamin was drastically lower after a 2.5-min incubation at 37°C as compared with HEK-293 cells expressing wild-type dynamin (Fig 4B). Controls as well as cells expressing wild-type dynamin accumulated intracellular ^{125}I -ANP radioactivity at much higher levels than did HEK-293 cells expressing mutant dynamin. In recombinant HEK-293 cells expressing wild-type dynamin, approximately 80%–90% of the ^{125}I -ANP bound to NPRA was in intracellular compartments and was resistant to removal by acid wash. In contrast, only about 10%–15% of ^{125}I -ANP-bound ligand-receptor complexes were localized in intracellular compartments in recombinant HEK-293 cells expressing mutant dynamin (Fig. 4B). We observed that a large proportion of ligand-receptor complexes were not internalized

and remained on the plasma membrane in recombinant HEK-293 cells expressing mutant dynamin.

The release of ^{125}I -ANP into the culture medium of control cells and recombinant HEK-293 cells expressing wild-type dynamin increased progressively, reaching 70%–80% with equilibrium after 30 min of incubation at 37 °C. However, only 10%–15% of ^{125}I -ANP was released into the culture medium of recombinant HEK-293 cells expressing mutant dynamin (Fig. 4C). Mutant dynamin blocked the internalization and degradation of bound ANP-NPRA complexes under physiological and steady-state conditions.

Analysis of degraded and intact ligand released into the culture media of MDC- and CPZ-treated cells and cells expressing wild-type and mutant dynamin

Intact and degraded ligand was quantitated by measuring the solubility of ^{125}I -ANP products in 10% TCA solution. The TCA precipitates, which contained intact ligand, and supernatants, which contained degraded ligand products, were separated by centrifugation of TCA-soluble extracts. Rapid release of ^{125}I -ANP radioactivity in control culture medium accounted for 75%–85% of the total radioactivity. After 30 min of incubation at 37°C, ^{125}I -ANP radioactivity released into the culture medium of control cells consisted of almost 70–80% degraded products and 15%–18% intact ligand. In MDC-pretreated cells after the same incubation period and at the same temperature, the release of ^{125}I -ANP accounted 10%–15% of degraded and 2%–4% of intact ligand (Fig. 5A). Similarly, in CPZ-pretreated cells, the release of ^{125}I -ANP accounted for 8%–10 % of degraded products and 3%–5% of intact ligand (Fig. 5B). Higher amounts of both intact and degraded ligand products were found in the culture medium of cells expressing wild-type dynamin than in the culture medium of cells expressing mutant dynamin. The total amount of released ^{125}I -ANP in the culture medium of cells expressing wildtype dynamin after 30 min of incubation at 37 °C was composed of approximately 85% degraded ligand products and 10%–15% intact ligand (Fig. 5C). On the other hand, the total amount of ^{125}I -ANP released into the culture medium of cells expressing the dynamin mutant was composed of 5%–10% degraded ligand products and only 2–5% intact ligand in the same experimental conditions (Fig 5D). However, control cells and those expressing wild-type dynamin had significant differences in the internalization of NPRA. The degraded and intact ligand released into the culture medium was quantitatively determined based on the total initial ^{125}I -ANP bound to the receptor on the cell surface.

Visualization of receptor internalization of eGFP-tagged NPRA by confocal microscopy

The dynamin-dependent internalization of eGFP-tagged NPRA was examined by confocal microscopy. To visualize eGFP-tagged receptor, cells were treated with 100 nM ANP for 5, 10, and 30 min (Fig. 6 A and B). The fixed control cells had the appearance of endocytic vesicles after 5 min of ANP treatment; these vesicles gradually decreased over 30 min, which is a characteristic of internalized membrane receptors within the cytoplasm (Fig. 6 A and B, control panels). In contrast, endocytic vesicles were absent or minimal in MDC- and CPZ-treated cells as compared to those in control cells (Fig. 6 A and B, MDC and CPZ panels).

The effect of wild-type and mutant dynamin on the internalization of eGFP-tagged NPRA was analyzed by confocal microscopy. To visualize eGFP-tagged receptors, cells were treated with 100 nM ANP for 5, 10, and 30 min. The fixed cells showed endocytic vesicles after 5 min of ANP treatment; these gradually decreased over 30 min (Fig. 7A and B, control panels). We also observed endocytic vesicles in wild-type dynamin-expressing cells after treatment with ANP for 5 min (Fig. 7A and B, dynamin wild-type panels). In contrast to controls cells expressing wild-type dynamin, endocytic vesicles were absent from cells expressing mutant dynamin (Fig. 7A and B, mutant dynamin panels).

Effect of endocytotic inhibitors and dynamin GTPase blocker to the intracellular accumulation of cGMP in recombinant HEK-293 cells

Intracellular accumulation of cGMP was quantified at different time points in intact cells treated with 100 nM ANP at 5, 10, and 30 min and independently pretreated with the endocytic inhibitors (MDC and CPZ) and transfection with dynamin GTPase blocker (dynamin mutant) (Fig. 8A). Increased intracellular accumulation of cGMP was observed in ANP-treated control cells as compared with pretreated cells with the endocytic inhibitors (MDC and CPZ). A significant reduction in the intracellular accumulation of cGMP was noted at 5 min (72%), 10 min (68%), and 30 min (62%) (Fig. 8A). Similarly, significant reduction of intracellular accumulation of cGMP also was observed by 70–80% in cells transfected with dynamin GTPase blocker (dynamin mutant) as compared with control cells (untransfected with dynamin mutant) or transfected with dynamin wild-type construct (Fig. 8B).

DISCUSSION

The results of this study provide new information regarding the mechanisms of NPRA endocytosis involving the classical clathrin-coated-pit-mediated pathway. The present findings demonstrated that ANP-mediated NPRA internalization in HEK-293 cells occurs via a clathrin-dependent pathway, which involves an initial clustering of receptor-ligand cargo in clathrin-coated pits with the invaginating plasma membrane, which forms clathrin-coated vesicles. We adopted two approaches, quantitative measurement of the internalization of ¹²⁵I-ANP-NPRA complex and the use of confocal microscopy to visualize the internalization of eGFP-NPRA. We used the recombinant HEK-293 cells expressing NPRA and/or eGFP-NPRA to study the endocytosis of ¹²⁵I-ANP-NPRA and visualized the receptor internalization in the presence of inhibitors of dynamin-dependent selective endocytic routes. Inhibition of ligand-induced endocytosis of the receptor is the most reasonable mode of action of CPZ and MDC, which blocked endocytosis of NPRA by 80%–90%. Confocal microscopy of eGFP-NPRA in HEK-293 cells clearly showed the formation of endocytic vesicles, which is a characteristic of internalized receptor within the cytoplasm. We determined the effect of inhibitors of clathrin-mediated endocytosis, such as CPZ, MDC or mutant dynamin, which are known to block the internalization of the membrane receptors, and also affect the assembly of clathrin components and the formation of clathrin-coated pits [48–52]. It is very explicable that the classical clathrin-mediated endocytic pathway is a major route for the internalization of NPRA, which is important towards the understanding

the role of natriuretic peptides and their receptors in hypertension and cardiovascular regulation.

The current findings demonstrate that the blockers of clathrin-coated vesicle formation (CPZ, MDC, and mutant dynamin) significantly blocked the internalization of ^{125}I -ANP-NPRA complexes compared with untreated control cells. Furthermore, confocal microscopic examination showed that after ANP treatments the inhibitors of dynamin prevented the formation of endocytic vesicles in a time-dependent manner. However, it is important to note that the internalization of NPRA was not affected by wild-type dynamin, demonstrating its critical role in the endocytic internalization of membrane receptors. Due to intrinsic mechanisms of the receptor-sorting machinery at the plasma membrane, clathrin-coated structures play pivotal roles in the internalization of ligand-receptor complexes. To inhibit the internalization and intracellular trafficking of the membrane receptors, the dynamin inhibitors, MDC and CPZ have been effectively utilized. Our results showed that MDC effectively blocked the internalization of ANP-NPRA complexes in the recombinant HEK-293 cells by almost 90%. MDC functions as a strong competitive inhibitor of transglutaminase that acts as a cross-linking agent by creating an isopeptide bond between a glutamin and lysine residue of two protein molecules [50, 53]. Interestingly, the inhibition of transglutaminase by MDC, has also been shown to block the internalization of Semiliki Forest virus, vesicular stomatitis virus, and other clathrin-coated vesicles-dependent endocytic processes [50, 54]. Previous studies have indicated that MDC was able to block the internalization and trafficking of ligand-receptor complexes of various hormones and cytokine, including insulin, EGF, and interleukin-8 [55–57]. Chlorpromazine, a cationic amphiphilic drug has been shown to inhibit the assembly of clathrin adapter protein 2 (AP2) on clathrin-coated pits and to block the coated vesicle-mediated internalization of membrane proteins [58–60]. In the present studies, CPZ inhibited the endocytosis of ANP-NPRA complexes by 80%–85%. This observation is consistent with the previous findings that CPZ was able to inhibit the endocytosis of proteoglycans and LDC receptors [60, 61]. CPZ also causes the loss of coated pits from the plasma membrane and the appearance of clathrin coats on endosomal membranes. Previously, it has been suggested that CPZ blocks the endocytotic entry of Japanese encephalitis virus into the cell interior [62]. It has been demonstrated that both MDC and CPZ efficiently inhibit the endocytosis of transferrin receptor protein [63]. Furthermore, CPZ, MDC, and dominant-interfering mutant proteins (dynamin-mutant) have also been shown to play inhibitory roles for endocytosis in Wnt signaling [64].

The present study, using various inhibitors of dynamin-dependent endocytosis, demonstrates that NPRA internalization occurs via clathrin-mediated pathways. Earlier studies from our laboratory and the reports from others have shown that endogenous NPRAs in PC-12 and MA-10 cells, as well as recombinant NPRAs in COS-7 cells, undergo rapid internalization in a ligand-dependent manner [21, 35, 36, 38]. Some controversy existed because it was earlier reported by the default that among the natriuretic peptide receptors (NPRA, NPRB, and NPPC), only NPPC (so called clearance receptor) is internalized with bound ligand [43, 44]. Studies in our laboratory have firmly established that ANP-NPRA complexes are rapidly internalized, sequestered into the intracellular compartments, and the degraded products released into the culture medium [12, 13, 46, 65]. We have demonstrated that the

μ 1B subunit of adaptor protein-1 (AP-1, μ 1B) directly binds to a phenylalanine-based FQOI motif in the cytoplasmic tail of NPRA. However, subcellular trafficking indicated that immunofluorescence colocalization of mutated receptors (FQOI/AAAA) with μ 1B marker was decreased by 50% for the subunit of AP-1 [12]. The present study demonstrated the involvement of clathrin-coated pits that participate in clathrin-mediated endocytosis of NPRA.

To address the mechanistic aspects of internalization of NPRA via clathrin-coated pit, we examined the role of dynamin in the endocytosis, sequestration, and metabolic degradation of NPRA. Early studies have indicated that the clathrin-mediated endocytosis plays a central role in the internalization of various growth factors and G-protein-coupled receptors [66–68]. The coated-vesicle formation is largely facilitated by the dynamin GTPase, which is recruited to the clathrin-coated pits during the assembly of clathrin-coated vesicles of membrane receptors [66, 69–73]. It has been shown that the expression of a dominant-negative mutant of dynamin (K44A/dynamin) blocks the genesis of coated vesicles during the internalization of various ligand-receptor complexes [40, 67, 68, 74]. Furthermore, the previous studies have also demonstrated that dynamin plays a critical role in the localization of the clathrin-coated pits and clathrin-mediated endocytosis through its interaction with the AP2 [75, 76]. After recruitment to coated pits, dynamin forms a ring structure and pinches off the coated vesicles into the cell-interior [77, 78]. Dynamin is also known as pinchase and through its intrinsic GTPase activity, it generates the clathrin-coated vesicles during clathrin-mediated endocytosis of membrane receptors [59, 79]. The findings of the present study indicate that after ligand-binding, ANP-NPRA complexes are endocytosed through the clathrin-coated pits and traffic into the intracellular compartments (Fig. 9). The ligand-receptor complexes into the endocytic vesicles-endosomes are directed to either degradative pathways of lysosomes or via recycling pathways of endosomes return to the plasma membrane. However, it needs to be determined whether during the endocytic itinerary of NPRA, the gradual loss of its catalytic activity corresponds to the trafficking process of the receptor molecules. However, the GTPase-defective dominant mutant of dynamin interferes with the endogenous wild-type dynamin and prevents the formation of endocytic coated-vesicles and internalization of membrane receptors [80–84]. In the present studies, using the dominant-negative mutant of dynamin, our results demonstrate that the mutant dynamin inhibits the internalization and degradation of bound ANP-NPRA complexes. The quantitation and visualization of receptor internalization in the presence of dynamin inhibitors strongly supports the dynamin-dependent internalization and trafficking of NPRA into the cell-interior.

The present results suggest that ANP/NPRA continuously produces intracellular cGMP at various time points after treatment with ANP during the internalization and trafficking process of this receptor protein. However, endocytic inhibitors (MDC and CPZ) and dynamin GTPase blocker (dynamin mutant) significantly decreased the intracellular accumulation of cGMP. Moreover, cGMP regulates several processes, including cardiovascular homeostasis, antimitogenic and vascular reactivity, sensory transduction, anti-inflammation responses, neuronal plasticity and learning process [85]. cGMP interacts with three distinct kinds of intracellular effector protein molecules that include cGMP-dependent protein kinases, cGMP-activated phosphodiesterases, and cGMP-regulated ion

channels [86]. It is possible that cGMP binding proteins transduce the cGMP signal to alter cellular function through different mechanisms, comprising an inhibitory effect and/or by stimulation of protein phosphorylation [87, 88]. Our previous studies have shown that cGMP production is not activated exclusively at the cell surface, but also occurs even when receptors have been internalized and continue trafficking in the subcellular compartments [12]. However, earlier reports have suggested that G-protein-coupled receptor (GPCRs) continue to signal by generating cAMP throughout the internalization processes with their ligands [89]. Signaling from inside the cell is persistent, which appears to trigger specific downstream effects. In the present study, we found that intracellular accumulation of cGMP was significantly decreased after treatment with the endocytic inhibitors (MDC and CPZ) and transfection with dynamin GTPase blocker (dynamin mutant). Diminution in the intracellular accumulation of cGMP suggests that endocytic inhibitors (MDC and CPZ) and dynamin GTPase blocker (dynamin mutant) seems to have a concerted regulatory role in NPRA trafficking and signaling process.

In summary, the present study provides compelling evidence of the specificity of ANP-NPRA-induced endocytosis through clathrin-coated vesicle formation and the role of the dynamin GTPase in this process. Further detailed investigation of endocytic proteins is necessary to elucidate the precise functions of these proteins in NPRA internalization and trafficking in intracellular compartments. Our study is an early step toward mechanistic understanding of the ANP-NPRA trafficking itinerary, in which it undergoes internalization via clathrin-mediated endocytosis and paves the way for pharmacological and pathophysiological conditions such as hypertension and cardiovascular disease states.

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Figure 1. Ligand-dependent internalization of NPRA in stably expressing recombinant HEK-293 cells after treatments with MDC and CPZ or mutant dynamin expression:

A) HEK 293 cells expressing wild-type NPRA were treated with MDC and CPZ at 37 °C for 1h and then preincubated for 1 h at 4 °C with labelled ^{125}I -ANP. Cells were subsequently warmed at 37 °C for the indicated time periods to permit internalization of ligand receptor complexes into the cell interior. Internalization of ^{125}I -ANP was quantified at each time point as described in the Materials and methods section. The results represent the means $\pm$ S.E.M. for three independent determinations. B) Recombinant HEK-293 cells expressing wild-type or mutant dynamin preincubated for 1 h at 4 °C with labelled ^{125}I -ANP. Cells

were subsequently warmed at 37 °C for the indicated time periods to permit internalization of ligand receptor complexes into the cell interior. Internalization of ^{125}I -ANP was quantified at each time point as described in the Materials and methods section. The results represent the means $\pm$ SEM from 6–8 independent determinations.

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Figure 2. Quantitative analyses of cell-surface-associated, internalized, and released ^{125}I ANP radioactivity in HEK-293 cells expressing wild-type NPRA treated with MDC: Confluent HEK-293 cells in 6 cm² dishes expressing wild-type NPRA were treated with MDC and allowed to bind ^{125}I -ANP at 4 °C for 1 h. Cells were washed four times, each with 2 ml of assay medium, to remove the unbound ligand and then re-incubated in fresh medium at 37 °C. At the indicated time points, dishes were transferred at 4 °C and the media were collected. The cell-surface-associated (acid-sensitive) radioactivity was eluted with ice-cold acidic buffer (pH 3.4) and cells were dissolved in 0.5 NaOH to measure the internalized (acid-resistant) radioactivity. A) Cell-surface-associated, B) internalized, and C) released

^{125}I -ANP radioactivity was determined in the acid eluate, cell extract and culture medium, respectively, as described in the Materials and methods section. Results represent the means $\pm$ SEM from 4–6 independent experiments with triplicate dishes in each replicate.

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Figure 3. Effect of CPZ on distribution of cell-surface-associated, internalized and released ^{125}I -ANP radioactivity in HEK-293 cells expressing wild-type NPRA:

Confluent HEK-293 cells expressing wild-type NPRA receptors were seeded in 6 cm² dishes, treated with CPZ, and then allowed to bind ^{125}I -ANP at 4 °C for 1 h. Cells were washed four times, each with 2 ml of assay medium to remove the unbound ligand and then re-incubated in fresh medium at 37 °C. At the indicated time points, dishes were transferred at 4 °C and the media were collected. The cell-surface-associated (acid-sensitive) radioactivity was eluted with ice cold acidic buffer (pH 3.4) and cells were dissolved in 0.5 NaOH to measure the internalized (acid-resistant) radioactivity. A) Cell-surface-associated,

B) internalized, and C) released ^{125}I -ANP radioactivity was determined in the acid eluate, cell extract, and culture medium, respectively, as described in the Materials and methods section. Results represent the means $\pm$ SEM from 5–6 independent experiments with triplicate dishes in each replicate.

Figure 4. Quantitative analyses of cell-surface-associated, internalized, and released ^{125}I -ANP radioactivity in recombinant HEK-293 cells expressing wild-type or mutant dynamin: Confluent recombinant HEK-293 cells in 6 cm² dishes expressing wild-type or mutant dynamin were allowed to bind ^{125}I -ANP at 4 °C for 1 h. Cells were washed four times, each with 2 ml of assay medium, to remove the unbound ligand and then re-incubated in fresh medium at 37 °C. At the indicated time points, dishes were transferred at 4 °C and the media were collected. The cell-surface-associated (acid-sensitive) radioactivity was eluted with ice cold acidic buffer (pH 3.4) and cells were dissolved in 0.5 N NaOH to measure the internalized (acid-resistant) radioactivity. A) Cell-surface-associated, B) internalized, and C)

released ^{125}I -ANP radioactivity was determined in the acid eluate, cell extract and culture medium respectively as described in the Materials and methods section. Results represent the means $\pm$ SEM. for 3–4 independent experiments with triplicate dishes in each replicate.

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Figure 5. Effect of MCD, CPZ, and mutant dynamin on the release of degraded and intact ^{125}I -ANP into the culture medium of HEK-293 cells expressing wild-type NPRA:

A) HEK-293 cells expressing wild-type NPRA treated with MDC were allowed to bind ^{125}I -ANP at 4 °C for 1 h. Then, the cells were washed four times with ice-cold assay medium to remove the unbound ligand and then re-incubated in 2 ml of fresh assay medium at 37 °C. At the indicated time points, dishes were transferred at 4 °C and the media were collected. The intact and degraded ligand products were quantified as described in the Materials and methods section. B) HEK-293 cells expressing wild-type NPRA receptors treated with CPZ were allowed to bind ^{125}I -ANP at 4 °C for 1 h. Then, the cells were washed four times with ice-cold assay medium to remove the unbound ligand and then re-incubated in 2 ml of fresh assay medium at 37 °C. At the indicated time points, dishes were transferred at 4 °C and the media were collected. The intact and degraded ligand products were quantified. Confluent recombinant HEK-293 cells in 6 cm² dishes expressing C) wild-type or D) mutant dynamin cDNA were allowed to bind ^{125}I -ANP at 4 °C for 1 h. Cells were washed four times with ice-cold assay medium to remove the unbound ligand and then re-incubated in 2 ml of fresh assay medium at 37 °C. At the indicated time points, dishes were transferred at 4 °C and the

media were collected. The intact and degraded ligand products were quantified as described. The results represent the means $\pm$ SEM from 5–6 independent determinations.

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Figure 6. ANP-induced internalization of eGFP-NPRA in stably expressing recombinant HEK-293 cells treated with MDC and CPZ:

A) HEK-293 cells stably expressing eGFP-NPRA fusion protein were pretreated with MDC and CPZ at 37°C for 1h. Series of single confocal plane images were taken from the cells fixed in 4.0 % formaldehyde to visualize the internalization of eGFP-NPRA after stimulation by 100 nM ANP. In untreated cells, receptors were localized on the plasma membrane. Endocytic vesicles (magnified images of areas indicated with red squares) were observed after 5, 10, and 30 min treatment, which is a characteristic of internalized receptor within the cytoplasm. B) The number of fluorescent vesicles per cell after different time of

incubation was quantified and represented as the fold ratio with respect to untreated cells. These images of mid-focal planes are the representation from 5–8 independent experiments. Scale bar = 50 μm .

Figure 7. Ligand-induced internalization of eGFP-NPRA in recombinant HEK-293 cells expressing wild-type or mutant dynamin:

HEK-293 cells stably expressing eGFP-NPRA fusion protein were utilized. Series of single confocal plane images were taken from recombinant HEK-293 cells expressing wild-type or mutant dynamin. Cells were fixed in 4.0 % formaldehyde to visualize the internalization of eGFP-NPRA after stimulation by 100 nM ANP for different time course. In untreated cells, receptors were localized in the plasma membrane. Endocytic vesicles (magnified images of areas indicated with red squares) were observed after 5, 10, and 30 min treatment, which is a characteristic of the internalized receptors within the cytoplasm. B) The number of

fluorescent vesicles per cell after different time of incubation was quantified and represented as the fold ratio with respect to untreated cells as described under Materials and Methods section. These images of mid-focal planes are the representative from 6–8 independent experiments. Scale bar = 50 μm .

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Figure 8. Effect of endocytotic inhibitors and dynamin GTPase blocker to the intracellular accumulation of cGMP in recombinant HEK-293 cells:

A) HEK-293 cells stably expressing eGFP-NPRA fusion protein were pretreated with MDC and CPZ at 37°C for 1h. Stimulation of intracellular accumulation of cGMP in cells treated with 100 nM ANP for 5, 10, and 30 min at 37°C in the presence of IBMX. Intracellular accumulation of cGMP was quantified at different time points in treated and untreated cells. B) Confluent recombinant HEK-293 cells in 6 cm² dishes expressing wild-type or mutant dynamin were treated with 100 nM ANP at 5, 10, and 30 min in the presence of IBMX and

cGMP was quantified by ELISA. Bars represent the mean $\pm$ S.E.M of 5–6 independent experiments. *** $P < 0.001$ relative to untreated control cells.

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Figure 9. Schematic representation of clathrin-dependent endocytosis of NPRA in HEK-293 cells:

The scheme describes the sequential events of internalization, trafficking, recycling, and degradation of ligand-receptor complexes in the intracellular compartments. After binding of ANP to NPRA, ligand-receptor complexes enter the cell via clathrin-coated pits with the help of dynamin GTPase and the ligand-bound receptor complex is endocytosed and traffics intracellularly through the endosomes, lysosomes, and a population of receptor recycles back to the plasma membrane through the recycling endosomes. On the other hand, with the expression of mutant dynamin and the blockers of dynamin GTPase (monodansylcadaverine,

chlorpromazine) inhibiting the clathrin-coated vesicle formation, the internalization of NPRA was significantly prevented.

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