Antibody Tracking Demonstrates Cell Type-Specific and Ligand-Independent Internalization of Guanylyl Cyclase A and Natriuretic Peptide Receptor C

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ABSTRACT

Atrial natriuretic peptide (ANP) binds guanylyl cyclase-A (GC-A) and natriuretic peptide receptor-C (NPR-C). Internalization of GC-A and NPR-C is poorly understood, in part, because previous studies used ^{125}I-ANP binding to track these receptors, which are expressed in the same cell. Here, we evaluated GC-A and NPR-C internalization using traditional and novel approaches. Although HeLa cells endogenously express GC-A, ^{125}I-ANP binding and cross-linking studies only detected NPR-C, raising the possibility that past studies ascribed NPR-C-mediated processes to GC-A. To specifically measure internalization of a single receptor, we developed an ^{125}I-IgG-binding assay that tracks extracellular FLAG-tagged versions of GC-A and NPR-C independently of each other and ligand for the first time. FLAG-GC-A bound ANP identically with wild-type GC-A and was internalized slowly (0.5%/min), whereas FLAG-NPR-C was internalized rapidly (2.5%/min) in HeLa cells. In 293 cells, ^{125}I-ANP and ^{125}I-IgG uptake curves were superimposable because these cells only express a single ANP receptor. Basal internalization of both receptors was 8-fold higher in 293 compared with HeLa cells and ANP did not increase internalization of FLAG-GC-A. For FLAG-NPR-C, neither ANP, BNP, nor CNP increased its internalization in either cell line. Prolonged ANP exposure concomitantly reduced surface and total GC-A levels, consistent with rapid exchange of extracellular and intracellular receptor pools. We conclude that ligand binding does not stimulate natriuretic peptide receptor internalization and that cellular environment determines the rate of this process. We further deduce that NPR-C is internalized faster than GC-A and that increased internalization is not required for GC-A down-regulation.

Introduction

Atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) are endogenous cardiac hormones that regulate blood pressure, extracellular volume, and cardiac load (Potter et al., 2009). ANP and BNP bind two distinct, single membrane-spanning, cell surface receptors: guanylyl cyclase-A (GC-A) and natriuretic peptide receptor-C (NPR-C). GC-A mediates the signaling functions of ANP and BNP by catalyzing the synthesis of cGMP in response to peptide binding (Potter, 2011). NPR-C controls natriuretic peptide concentrations via receptor-mediated endocytosis and lysosomal degradation (Nussenzveig et al., 1990). The extracellular domains of NPR-C and GC-A are similar; but unlike

GC-A, NPR-C has a short intracellular domain with no known enzymatic activity. Mice lacking GC-A are hypertensive with large hearts, whereas mice lacking NPR-C are hypotensive with dilute urine, consistent with a signaling role for GC-A and a clearance role for NPR-C (Lopez et al., 1995; Oliver et al., 1997; Jaubert et al., 1999; Matsukawa et al., 1999).

^{125}I-ANP binding studies have led to conflicting conclusions regarding natriuretic peptide processing and receptor trafficking due to uncertainty regarding which receptor, GC-A or NPR-C, binds the peptide and changing affinities of GC-A for ANP (Abe et al., 1995; Vieira et al., 2001). Some reports indicate that GC-A internalizes ANP and is rapidly degraded in response to ANP binding (Rathinavelu and Isom, 1991; Pandey, 2001). Other reports indicate that GC-A does not internalize ANP and is not degraded in response to ANP binding (Koh et al., 1992; Vieira et al., 2001). We found that GC-A is down-regulated in "regular" 293 cells but is down-regulated at much slower rates in 293T cells (Potter and

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ABBREVIATIONS: ANP, atrial natriuretic peptide; GC-A, natriuretic peptide receptor-A; BNP, B-type natriuretic peptide; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FBS, fetal bovine serum; NPR-C, natriuretic peptide clearance receptor; tTA, tetracycline transactivator; DMEM, Dulbecco's modified Eagle's medium; GFP, green fluorescent protein; PAGE, polyacrylamide gel electrophoresis.

Hunter, 1999; Fan et al., 2005; Flora and Potter, 2010). We have reported that GC-A is down-regulated when endogenously expressed in primary cells, in transfected Chinese hamster cells and in tissues from mice with congestive heart failure (Bryan et al., 2007; Dickey et al., 2007; Flora and Potter, 2010). Our current model is that GC-A is down-regulated under biological conditions, in which ANP is elevated for extended periods of time. The mechanistic details of GC-A internalization, however, are unknown.

Ligand-dependent increases in receptor internalization have been suggested to account for the down-regulation of GC-A, but this issue is controversial because of the lack of specificity of the assays used to measure this process. Likewise, the effect of ANP binding on the internalization rate of NPR-C is disputed. Two groups reported that ANP stimulates NPR-C down-regulation, whereas another group reported that NPR-C is constitutively internalized (Nussenzveig et al., 1990; Rathinavelu and Isom, 1991; Pandey, 1992).

For the first time, we investigated the effect of ANP binding on the internalization rates of GC-A and NPR-C in HeLa and 293 cells using a newly developed ^{125}I-IgG binding assay that tracks FLAG-tagged versions of each receptor independently of the other receptor or the presence of ligand. We found that FLAG-NPR-C is rapidly internalized regardless of the presence of ligand or cellular environment. Surprisingly, the initial internalization rate of FLAG-GC-A was not increased by ANP in HeLa cells and was internalized by an 8-fold faster, ANP-independent process in 293 cells. Importantly, despite the differences in internalization, GC-A was down-regulated at similar rates in both cell lines, indicating that accelerated internalization is not required for GC-A degradation.

Materials and Methods

Materials. Anti-mouse ^{125}I-IgG (goat), ^{125}I-ANP (rat), and ^{125}I-transferrin (human) were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). $[\alpha$-^{32}P]GTP was from PerkinElmer Life and Analytical Sciences. Unlabeled ANP, cycloheximide, FLAG peptide, and the anti-FLAG M2 antibody were from Sigma-Aldrich (St. Louis, MO).

Cell Culture. HeLa and stably transformed tetracycline transactivator (tTA) HeLa cells were cultured as described previously (Sever et al., 2000). Regular HeLa cells were acquired from Dr. Do-Hyung Kim (University of Minnesota, Minneapolis, MN) and propagated in DMEM plus 10% fetal bovine serum (FBS). tTA-HeLa cells were from Dr. Sean Conner (University of Minnesota) and grown in the presence of 200 μg/ml G418. 293PMA-FLAG-GCA cells were prepared by stably expressing pCMV1-FLAG-GC-A (Flora and Potter, 2010) in 293 cells as described previously (Potter and Hunter, 1999; Fan et al., 2005).

Plasmids and Transfections. pCMV1-FLAG-NPR-C was made by adding HindIII and EcoRI restriction sites to the N- and C-terminal ends, respectively, of the human NPR-C cDNA. The N-terminal restriction site was added immediately after the signal peptide sequence of NPR-C, and the cDNA was amplified by PCR, digested with HindIII and EcoRI, and subcloned in-frame into the pCMV1-FLAG construct (Sigma-Aldrich) digested with the same restriction enzymes. HeLa or tTA HeLa cells were transfected with GFP, FLAG-GC-A, or FLAG-NPR-C using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions 24 to 48 h before analysis. The 293 cells were transfected with FLAG-NPR-C using the standard calcium phosphate transfection protocol (Potthast et al., 2004).

Intracellular Accumulation Assays. Cells were removed from plates with PBS containing 5 mM EDTA. Suspended cells were cooled to 4°C and washed in 1 ml of DMEM containing 10% FBS. Cells were incubated with the anti-FLAG M2 antibody (1:10,000; Sigma-Aldrich) for 30 min at 4°C, washed in DMEM containing 0.5% bovine serum albumin (BSA), and incubated with anti-mouse ^{125}I-IgG for 30 min. The cells were washed twice with DMEM containing 0.5% BSA and resuspended in DMEM containing 10% FBS. Fifty microliters of cells was dispensed into tubes and incubated in a 37°C water bath for the indicated times. All tubes except for those designated "total counts" were transferred to 4°C and stripped with 0.2 M acetic acid and 0.5 M NaCl for 5 min at 4°C to remove surface-bound radioactivity. Cells were pelleted, the supernatant was removed, and the amount of radioactivity in the pellets was determined. Nonspecific counts, obtained from mock-transfected cells, were subtracted from the counts generated from each time point, and the resulting values were graphed as a percentage of the "total counts." Initial rates of intracellular accumulation were determined by linear regression, and a paired t test was used to determine statistical significance.

For the ^{125}I-ANP internalization assay, suspended cells were incubated with ^{125}I-ANP for 1 h at 4°C and then washed with DMEM containing 0.5% BSA before resuspension in DMEM containing 10% FBS. The assay was initiated by elevating the temperature of the cells to 37°C. After increasing periods of time, the cells were acid washed at 4°C to separate surface from internalized ^{125}I-ANP, and intracellular radioactivity was plotted as a function of time at 37°C.

For the ^{125}I-transferrin internalization assay, cells were resuspended in 1 ml of a phosphate-buffered saline solution containing 1 mM MgCl$_2$, 1 mM CaCl$_2$, 5 mM glucose, and 0.2% BSA. Suspended cells were incubated with ^{125}I-transferrin for 30 min at 4°C. Cells were then pelleted, washed in DMEM/BSA, and resuspended in DMEM containing 10% FBS. Fifty-microliter aliquots were incubated in a 37°C water bath for the times indicated. All tubes except for those designated "total counts" were washed with 0.2 M acetic acid and 0.5 M NaCl for 5 min at 4°C. The percentage of internalized counts was then determined as described above.

Whole-Cell cGMP Elevation Assays. The assays were performed as described previously (Dickey et al., 2009). In brief, cells were plated on poly(D-lysine)-coated 48-well plates and then incubated 4 to 12 h in serum-free media upon reaching 75 to 90% confluence. For the assay, the medium was aspirated and replaced with 0.15 ml of DMEM containing 1 mM 1-methyl-3-isobutylxanthine and 25 mM HEPES, pH 7.4, for 10 min at 37°C. After pretreatment, the medium was aspirated and cells were treated with DMEM containing 1 mM 1-methyl-3-isobutylxanthine and 25 mM HEPES, pH 7.4, with or without natriuretic peptide for 3 min. Treatment medium was then aspirated and the reaction was stopped with 0.2 ml of ice-cold 80% ethanol. An aliquot of the resulting supernatant was dried in a centrifugal vacuum concentrator and analyzed for cGMP content by radioimmunoassay.

Whole-Cell ANP Binding. Whole-cell ^{125}I-ANP binding assays were performed as described previously (Dickey et al., 2009). In brief, cells were added to 24-well plates precoated with poly(D-lysine). When 75 to 90% confluent, the cells were washed with DMEM and then incubated with DMEM containing 0.2% BSA at 37°C for 1 to 2 h. Medium was aspirated and 0.2 ml of binding medium containing 75 pM ^{125}I-ANP and 1% BSA, alone or with increasing concentrations of unlabeled ligand was added to the cells. The plates were incubated at 4°C for 1 h before the binding medium was aspirated, and the cells were washed twice with 0.5 ml of ice-cold PBS. The cells were solubilized in 0.5 ml of 1 N NaOH, transferred to glass tubes and bound radioactivity was measured in a Beckman 5500 γ counter (Beckman Coulter, Fullerton, CA).

Cross-Linking of ^{125}I-ANP-HeLa. Cells from three plates (10 cm) were resuspended in 3 ml of Hanks' balanced salt solution containing 10 mM HEPES, pH 7.4, and divided into three tubes. ^{125}I-ANP (0.28 nM) in the absence or presence of 1 μM unlabeled
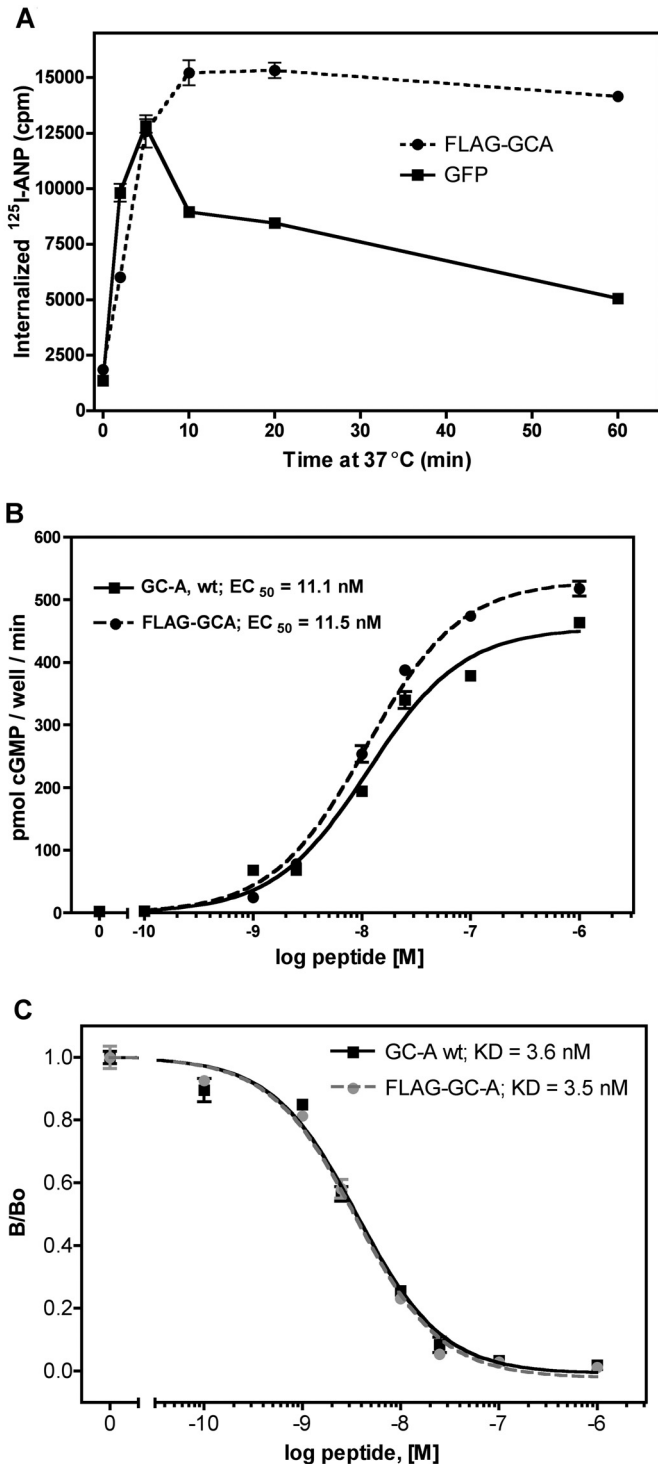
Fig. 1. A, ^{125}I-ANP uptake in tTA-HeLa cells transiently transfected with GFP or FLAG-GC-A. Cells were labeled with subsaturating concentrations of ^{125}I-ANP at 4°C. Aliquots of labeled cells were incubated at 37°C for the times indicated before acid washing and counting. Values represent average ± the range of the determinations, where n = 2. The graph is representative of multiple experiments. B, FLAG-GC-A and wild-type GC-A bind and are activated by ANP similarly. The 293 cells were transiently transfected with wild-type GC-A or FLAG-GC-A and incubated with increasing concentrations of ANP for 1 min. Cellular cGMP concentrations were measured and plotted as a function of peptide concentration. The data points represent the mean ± S.E.M. assayed in triplicate. C, FLAG-GC-A has similar affinity for ANP as wild-type GC-A. Transiently transfected 293 cells were incubated for 1 h at 4°C with ^{125}I-ANP in the presence or absence

ANP or CNP was added for 2 h at 4°C. A 0.25-ml portion of a 2.17 mM solution of freshly prepared disuccinimidyl suberate in Hanks' balanced salt solution was added to the cells at room temperature for 1 h for a final concentration of 0.5 mM to cross-link the bound radioligand. Cells were washed to remove unbound ligand and resuspended in 0.1 ml of 2× reducing SDS sample buffer. An aliquot was removed and lysed by passing through a 21-gauge needle 6 to 10 times. The cell extract was then fractionated by SDS-PAGE. The gel was dried, and cross-linked proteins were visualized by autoradiography.

ANP-Dependent Down-Regulation. Cells on 10-cm plates were incubated with DMEM in the presence or absence of 200 nM ANP and 10 μg/ml cycloheximide for the indicated times. The cells were washed at 4°C with phosphate-buffered saline before preparation of crude membranes as described previously (Dickey et al., 2009). For surface receptor measurements, cells were removed from the plate with PBS containing 5 mM EDTA and labeled with anti-FLAG/^{125}I-IgG as described for the internalization assay. Fifty microliters of labeled cells were then aliquoted into tubes and pelleted before counting in a γ counter.

Guanylyl Cyclase Assays. Guanylyl cyclase assays were performed on crude membranes at 37°C for 5 min using [^{32}P]GTP as described previously (Dickey et al., 2009). Reactions were started by the addition of 0.080 μl of the above reagents to 0.05 to 0.20 μg of crude membrane protein suspended in 0.020 μl of phosphatase inhibitor buffer (Bryan and Potter, 2002).

Quantification and Statistical Analysis. GraphPad Prism software was used for graphing and statistical analysis of the data (GraphPad Software Inc., San Diego, CA). The specific statistical tests performed are indicated in the text and figure legends.

Results

Different ^{125}I-ANP Uptake Profiles for Mock and GC-A-Transfected HeLa Cells. Because previous photoaffinity ^{125}I-ANP labeling studies indicated that HeLa cells express GC-A but not NPR-C (Watt and Yip, 1989), we investigated GC-A internalization in HeLa cells by measuring intracellular ^{125}I-ANP accumulation (Fig. 1). Intracellular radioactivity accumulated rapidly in control cells transfected with green fluorescent protein (GFP) and was maximal by 5 min. After 10 min, intracellular radioactivity had diminished, and by 1 h, intracellular radioactivity was less than half of maximum. In contrast, cellular uptake of ^{125}I-ANP was slower in cells transfected with FLAG-GC-A and did not decline with time. The slow internalization rate of FLAG-GC-A was not explained by differences in the ability of ANP to bind FLAG-GC-A verses wild type GC-A because similar EC_{50} and K_d values were obtained for each receptor (Fig. 1, B and C).

NPR-C, Not GC-A, Is the Major ANP Receptor in HeLa Cells. Because the ^{125}I-ANP uptake curve in the GFP-transfected cells was similar to that reported for NPR-C (Nussenzveig et al., 1990) and different from that observed in cells transfected with GC-A (Fig. 1A), we asked whether HeLa cells also express NPR-C. ^{125}I-ANP was chemically cross-linked to two independent HeLa cell lines. Reducing SDS-PAGE-fractionated membranes from cross-linked cells and labeled receptors were visualized by autoradiography (Fig. 2). In both cell lines, the major ^{125}I-ANP binding protein migrated at the molecular weight of NPR-C (~60 kDa) in the absence of unlabeled natriuretic peptides. In the presence of saturating concentrations of unlabeled ANP, no specific bind-

of increasing concentrations of unlabeled ligand. Specifically bound ^{125}I-ANP was plotted as a function of competing peptide concentration. The data points represent the mean ± S.E.M. assayed in triplicate.
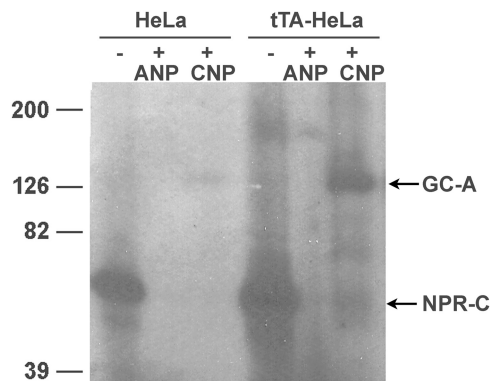
ing was observed, consistent with ANP blocking binding to both NPR-C and GC-A. The addition of excess unlabeled CNP reduced ^{125}I-ANP binding to NPR-C and increased binding to GC-A. These data indicate that HeLa cells express much higher concentrations of NPR-C than GC-A and that ^{125}I-ANP preferentially binds NPR-C, not GC-A. Thus, the vast majority of the internalization observed in the GFP-transfected HeLa cells in Fig. 1A was due to internalization of NPR-C, not GC-A.

Development of ^{125}I-IgG-Based Intracellular Receptor Accumulation Assay. Because most cells, like HeLa cells, express higher concentrations of NPR-C than GC-A, acid-resistant ^{125}I-ANP uptake primarily measures NPR-C internalization. Therefore, we developed a new assay that measures the uptake of a single class of receptors in the presence or absence of ligand. HeLa cells were transfected with plasmids expressing extracellular, amino-terminal FLAG-tagged versions of GC-A or NPR-C. Transfected cells were successively incubated with mouse anti-FLAG M2 and ^{125}I-conjugated anti-mouse IgG antibodies at 4°C to radioactively label surface receptors. Elevating the temperature of the cells to 37°C initiated the internalization assay and acid washing at 4°C separated internalized receptors from surface receptors.

With this technique, ^{125}I-IgG binding was dependent on expression of FLAG-GC-A as total counts were more than 10-fold higher in cells transfected with FLAG-GC-A compared with cells transfected with GFP (Fig. 3A). Binding was specific to the extracellular FLAG epitope because acid stripping removed the vast majority of bound counts and inclusion of the FLAG peptide in the medium reduced total counts from 10,525 ± 1576 to 715 ± 39 cpm (data not shown). Various primary and secondary antibody concentrations were tested to optimize the assay. The final conditions chosen (labeled as 1× in Fig. 3A) gave the highest signal-to-noise ratio but used the least amount of ^{125}I-IgG. These conditions do not saturate all secondary binding sites because this would have been cost-prohibitive.

To rule out the possibility that receptor overexpression artificially reduced the rate or magnitude of FLAG-GC-A internalization, 293 cells were transfected with increasing amounts of FLAG-GC-A plasmid, and receptor uptake rates were measured. Western blot analysis indicated that FLAG-
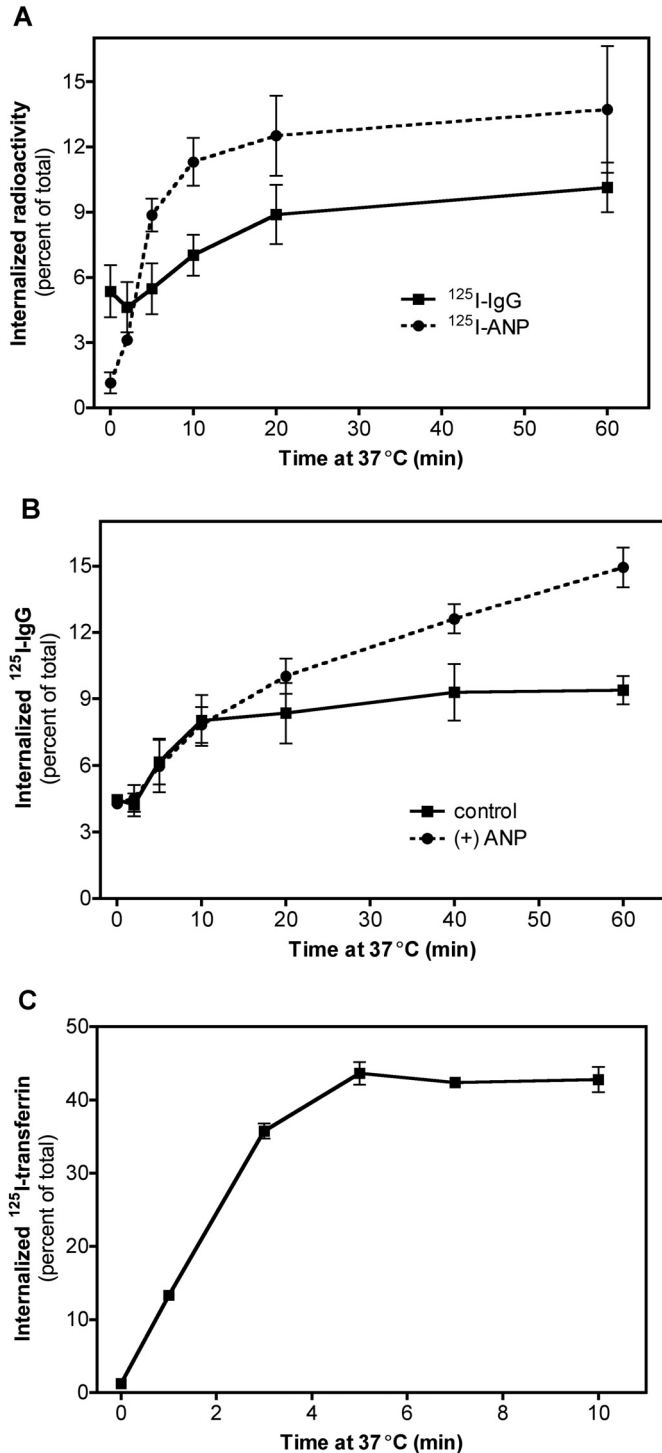
GC-A expression increased in proportion to the level of the transfected DNA. However, neither the rate nor the magnitude of FLAG-GC-A internalization was reduced in cells expressing higher levels of receptor (Fig. 3B). Thus, at all conditions tested, the same ratio of internalized-to-surface receptors was observed, which indicates that the ^{125}I-IgG uptake assay accurately measured the fate of the average cell surface receptor.

FLAG-GC-A Is Slowly Internalized in HeLa Cells. ^{125}I-ANP uptake was much faster than ^{125}I-IgG uptake in FLAG-GC-A transfected tTA-HeLa cells, which is consistent with NPR-C, not GC-A, mediating the majority of the ^{125}I-ANP internalization in these cells (Fig. 4A). ^{125}I-IgG uptake indicated that basal FLAG-GC-A internalization was linear for 10 to 20 min with approximately 4% of the surface recep-
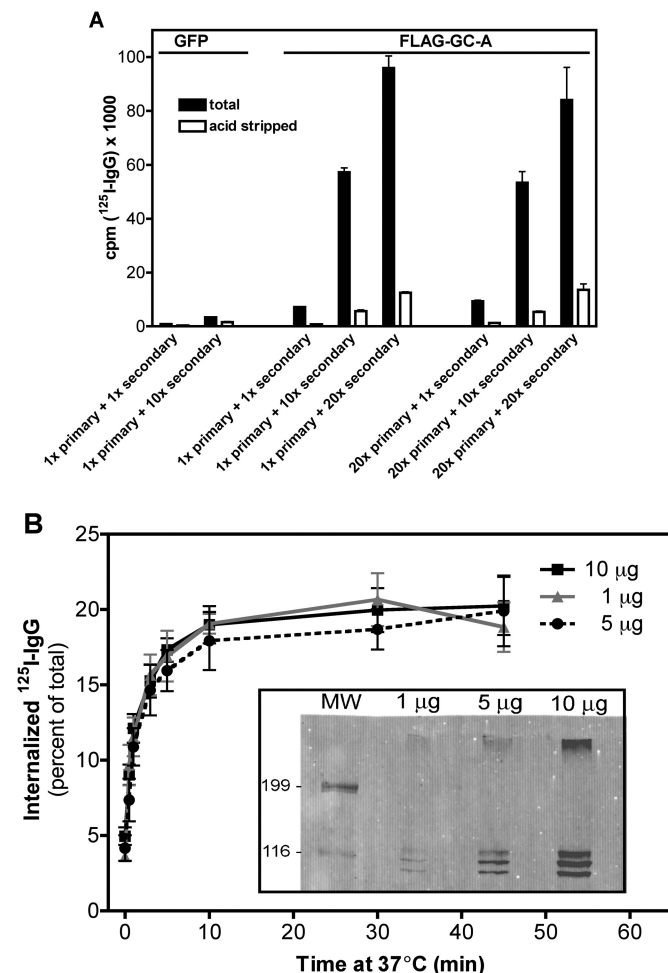
Fig. 3. The ^{125}I-IgG uptake assay specifically measures FLAG-GC-A internalization. A, tTA-Hela cells were transiently transfected with GFP or FLAG-GCA. The cells were dispensed into tubes and incubated with 0.05 (1× primary) or 0.1 μl (20× primary) of anti-FLAG-M2 antibody. Excess antibody was removed before addition of 5 (1×), 50 (10× secondary), or 100 μl of ^{125}I-IgG. Cellular radioactivity was measured directly or after acid-stripping to remove surface ^{125}I-sIgG. Values represent the range of determinations, where $n = 2$. The graph is representative of more than three experiments. B, 293 cells were transiently transfected with 10 (1×), 1, or 5 μg of FLAG-GC-A plasmid DNA. Internalization assays were performed 48 h later. An equal number of cells from each transfection were separated by SDS-PAGE, blotted to an Immobilon membrane, and GC-A expression was detected by Western blot using an anti-GC-A antibody (inset). Values represent average ± S.E.M., where $n = 6$.

Fig. 2. HeLa cells endogenously express high and low levels of NPR-C and GC-A, respectively. HeLa and tTA-HeLa cells were incubated with ^{125}I-ANP in the absence or presence of 1 μM ANP or CNP for 2 h at 4°C before cross-linking ^{125}I-ANP to the receptors with disuccinimidyl suberate. Membrane fractions were separated by SDS-PAGE, and ^{125}I-ANP-receptor complexes were visualized by autoradiography.

A

B

C

Fig. 4. GC-A is slowly internalized in HeLa cells. A, tTA-HeLa cells transiently transfected with FLAG-GC-A were labeled with ^{125}I-ANP or anti-FLAG-M2 antibody followed by anti-mouse ^{125}I-IgG at 4°C. Values represent average ± S.E.M. where n = 4 (B) ANP increases GC-A uptake at longer but not shorter periods of time. Cells transfected with FLAG-GC-A were labeled with anti-FLAG-M2 antibody and anti-mouse ^{125}I-IgG before incubation at 37°C in the absence or presence of 1 μM ANP for the periods of time shown. Samples were then acid-washed and counted. Values represent average ± S.E.M., where n = 6. C, ^{125}I-transferrin is rapidly internalized in tTA-HeLa cells. Aliquots of the tTA-HeLa cells transfected with FLAG-GC-A were labeled with ^{125}I-transferrin at 4°C. Internalized ^{125}I-transferrin was determined after the indicated periods of time at 37°C. Values represent the average ± S.E.M., where n = 8.

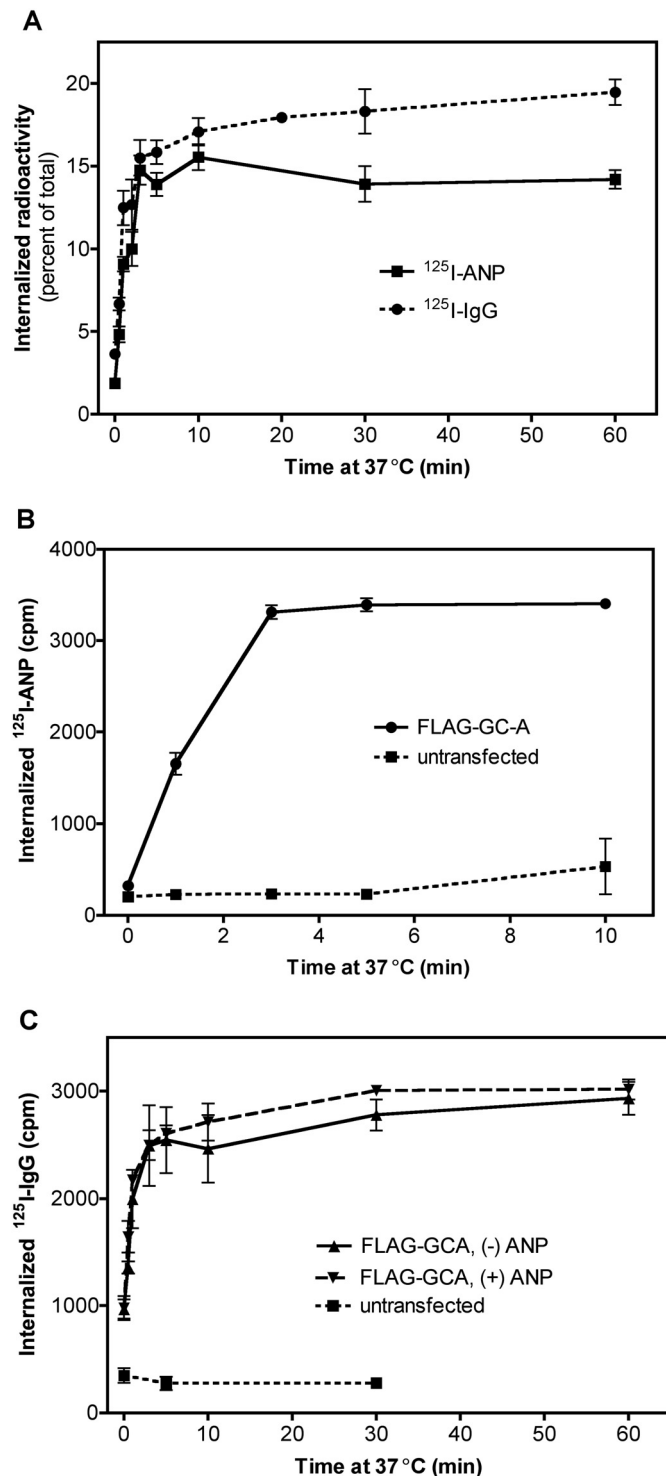tors being internalized during this period of time (Fig. 4B). Multiple experiments determined that the initial internalization rate was slow (0.2%/min ± 0.006, n = 4). Maximum receptor accumulation was achieved between 10 and 20 min. Inclusion of 1 μM ANP in the assay had no effect on initial internalization rates but increased accumulation after 1 h by 1.6-fold. To verify that the basal clathrin-dependent internalization pathway in these cells was functional, ^{125}I-transferrin uptake was measured and found to be robust, rapid, and saturable (Fig. 4C).

GC-A Internalization Is Rapid and ANP-Independent in 293 Cells. To measure internalization in cells where GC-A is the only measurable ^{125}I-ANP binding protein, FLAG-GC-A was stably expressed in 293 cells that do not endogenously express detectable levels of GC-A or NPR-C (Potter and Hunter, 1999). In contrast to the HeLa cells, the uptake curves for ^{125}I-ANP and ^{125}I-IgG were virtually indistinguishable in 293 cells (Fig. 5A). Internalization was clearly mediated by GC-A because cells lacking FLAG-GC-A failed to accumulate ^{125}I-ANP or ^{125}I-IgG (Fig. 5, B and C, untransfected). Thus, ^{125}I-IgG uptake faithfully mirrors ^{125}I-ANP internalization in these cells. Surprisingly, basal FLAG-GC-A internalization was 8-fold higher and more robust in the 293 cells compared with the HeLa cells. Eighteen percent of the total cell surface receptor population was internalized by 3 min at a rate of 4.9%/min ± 0.27. As in the HeLa cells, ANP did not increase the initial rate of FLAG-GC-A internalization in the 293 cells and, unlike the HeLa cells, had no effect on accumulation after 1 h (Fig. 5C).

NPR-C Internalization Is Rapid and Ligand-Independent in HeLa and 293 Cells. NPR-C internalization was also examined using the antibody-based assay. ^{125}I-IgG was internalized at an initial rate of 2.4%/min ± 0.01 in tTA-HeLa cells transfected with FLAG-NPR-C (Fig. 6A), which is similar to the rate of internalization of ^{125}I-ANP in GFP-transfected cells (Fig. 1A). In 293 cells transiently transfected with FLAG-NPR-C, receptor and initial ligand uptake were coincident with time. After 1 min, the rate of ^{125}I-ANP and ^{125}I-IgG accumulation were 18.8%/min ± 0.09 and 19.7%/min ± 0.11, respectively, and the percentage of surface receptor internalized as measured by ^{125}I-IgG was increased to 25 to 40% depending on the assay (Fig. 6B). Neither ANP, BNP, nor CNP increased NPR-C internalization as measured by ^{125}I-IgG uptake (Fig. 6C). Thus, the internalization of GC-A and NPR-C is ligand-independent.
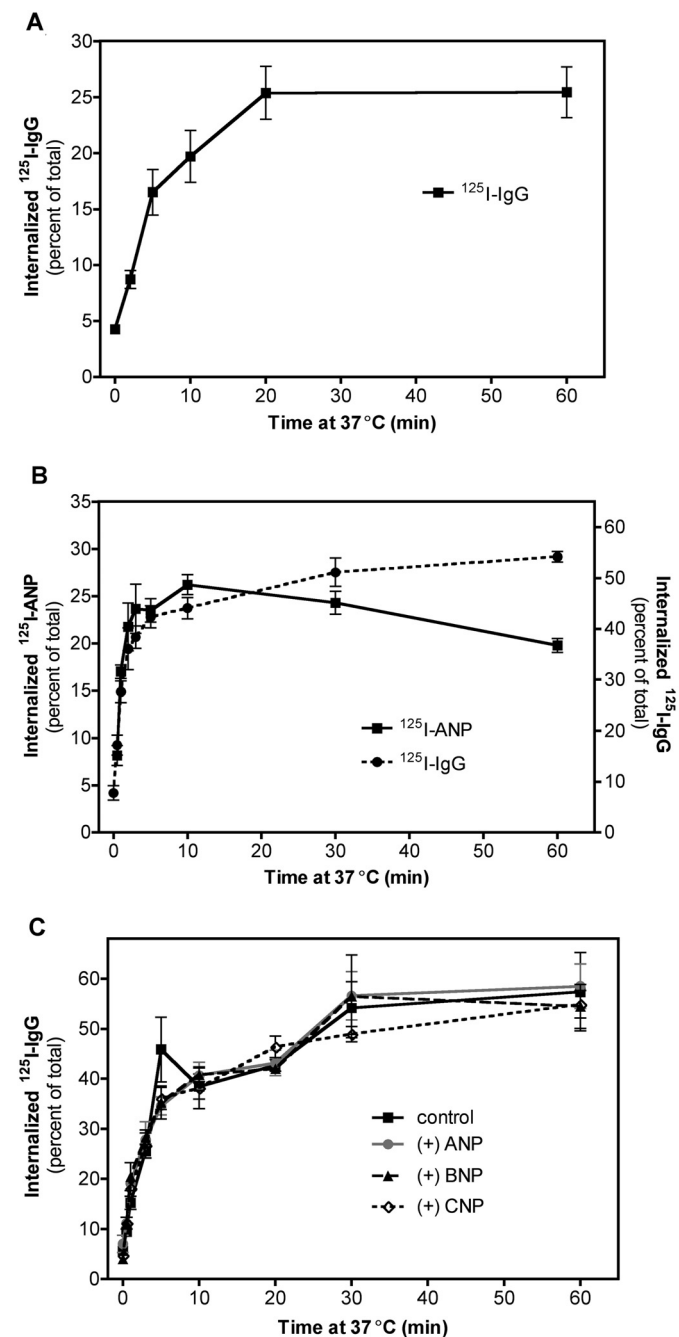
GC-A Down-Regulation Does Not Require Increased Internalization. We reported recently that GC-A is down-regulated in HeLa cells (Flora and Potter, 2010), but those experiments measured total receptor levels in cell lysates. To specifically measure surface levels of receptor, HeLa cells were transiently transfected with FLAG-GC-A and then incubated in the presence or absence of ANP for 8 h before labeling with anti-FLAG/^{125}I-IgG to measure surface receptor concentrations. Total counts (representing surface labeled receptors) were reduced by 53% in cells exposed to ANP (data not shown), which is consistent with previous studies showing that 8-h ANP exposure reduced total cellular GC-A concentrations by nearly 60% in HeLa cells (Flora and Potter, 2010).

We also examined the down-regulation of FLAG-GCA in stably expressing 293 cells. 293PMA-FLAG-GCA cells were incubated at 37°C with ANP for 0, 2, 4, and 8 h to stimulate

GC-A down-regulation (Fig. 7). In one experiment, crude membranes were prepared and assayed for detergent-dependent guanylyl cyclase activity to measure the effect of prior ANP exposure on total cellular receptor concentrations. In a separate experiment, ^{125}I-IgG binding was used to specifi-

Fig. 5. GC-A is rapidly internalized in 293 PMA cells. A, 293 cells stably expressing FLAG-GC-A were incubated at 4°C with either ^{125}I-ANP or anti-FLAG antibody followed by anti-mouse ^{125}I-IgG. Cells were incubated at 37°C for the indicated times before acid-washing and counting. Values represent average ± S.E.M., where $n = 14$. B, untransfected 293 cells or 293 cells stably expressing FLAG-GC-A were labeled at 4°C with ^{125}I-ANP. Internalized radioactivity as a function of time at 37°C is shown. Values represent average ± the range of two determinations. C, untransfected or 293 cells stably expressing FLAG-GC-A were labeled at 4°C with anti-FLAG antibody followed by ^{125}I-IgG secondary antibody. The cells were incubated at 37°C in the presence or absence of 1 μM ANP for the indicated periods of time. Values represent the average ± the range determinations, where $n = 2$.

Fig. 6. NPR-C is rapidly and constitutively internalized in HeLa and 293 cells. A, tTA-HeLa cells were transiently transfected with FLAG-NPR-C. Cells were then labeled with anti-FLAG antibody followed by anti-mouse ^{125}I-IgG at 4°C. Cells were incubated at 37°C for the indicated times before acid washing. Values represent the average ± S.E.M., where $n = 14$. B, 293 cells were transfected with FLAG-NPR-C and labeled with either ^{125}I-ANP or ^{125}I-IgG at 4°C. Aliquots were incubated at 37°C for the times shown and before acid washing. Values represent the average ± S.E.M., where $n = 6$. C, 293 cells transiently transfected with FLAG-NPR-C were labeled with anti-FLAG antibody and ^{125}I-IgG secondary antibody at 4°C. Aliquots were incubated at 37°C in the presence or absence of 1 μM ANP, BNP, or CNP for the times indicated, where $n = 4$.

cally measure receptors at the cell surface. Prior ANP exposure significantly reduced both activities after 4 and 8 h of exposure, and the reductions in surface and total receptors were temporally correlated. These data indicate that GC-A down-regulation does not require increased receptor internalization, because ANP clearly stimulates down-regulation but not internalization in 293 cells. Furthermore, it indicates that either the vast majority of receptors are at the cell surface or that intracellular and extracellular pools of receptor rapidly exchange.

Discussion

In this report, an antibody-based trafficking assay was developed that allowed the independent evaluation of GC-A and NPR-C internalization for the first time. The validity of the ^{125}I-IgG assay was established by demonstrating identical uptake rates for ^{125}I-IgG and ^{125}I-ANP in cells expressing a single ANP binding receptor. All previous uptake studies followed receptors with ^{125}I-ANP, which clearly measures NRP-C, not GC-A, trafficking in HeLa cells. A major advantage of the ^{125}I-IgG assay is that it measures a single receptor class without complicating contributions from related receptors. Another advantage is that it measures internalization rates of unbound receptors (basal rates), which was not possible using the ^{125}I-ANP binding approach. Although we demonstrated that ^{125}I-ANP binding accurately measured GC-A and NPR-C uptake in transfected 293 cells, ^{125}I-ANP binding is unlikely to accurately measure GC-A uptake in biological systems because most, if not all, cells express higher levels of NPR-C than GC-A. Leitman et al. (1986) studied eight cell lines and found that cells expressing GC-A also express higher concentrations of NPR-C. In contrast, Watt and Yip (1989) reported that HeLa cells only express GC-A. However, our ^{125}I-ANP chemical cross-linking studies indicated that NPR-C, not GC-A, is the major ANP receptor in the two HeLa cell lines that we examined. We suggest that past and future ^{125}I-ANP uptake studies should be carefully interpreted so that NPR-C uptake is not erroneously ascribed to GC-A.

Another clear conclusion from these studies is that cellular
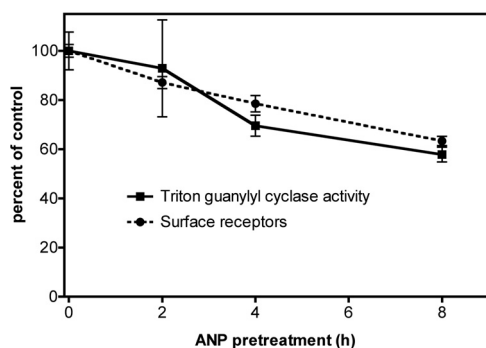
Fig. 7. Concomitant down-regulation of extracellular and intracellular FLAG-GC-A in 293 cells. The 293 cells stably expressing FLAG-GC-A were incubated with 10 μg/ml cycloheximide in the absence or presence of 200 nM ANP for the period of times indicated. In one experiment, crude membranes were prepared and then assayed for guanylyl cyclase activity in the presence of 1% Triton X-100 and Mn^{2+}-GTP. In a second experiment, cells were incubated with ANP as described above and then labeled with anti-FLAG antibody followed by ^{125}I-IgG at 4°C. Total ^{125}I-IgG radioactivity and guanylyl cyclase activities were normalized to activities obtained from cells not incubated with ANP (control) and plotted as a function of time of ANP exposure.

environment is a major determinant of receptor uptake. In HeLa cells, GC-A internalization was very slow, whereas in 293 cells, internalization was eight times faster for both GC-A and NPR-C. The rapid internalization of ^{125}I-ANP and ^{125}I-transferrin indicated that the meager FLAG-GC-A uptake in the HeLa cells was not due to a global internalization defect caused by transfection but was due to a difference in the GC-A internalization machinery. Likewise, the similar internalization rates of GC-A and NPR-C with either ^{125}I-ANP or the FLAG-based assay in the 293 cells suggest that the slow FLAG-GC-A internalization rate was not an artifact of the FLAG-tagged receptor and is representative of GC-A uptake in these cells. In addition, similar uptake rates in cells expressing various levels of receptors suggest that FLAG-GC-A uptake is representative of the internalization of endogenous GC-A.

Another conclusion that can be drawn from these studies is that FLAG-NPR-C internalization was several times faster than GC-A internalization regardless of cell line, which is consistent with unique trafficking pathways mediating uptake of each receptor. Future work will focus on the identification of these pathways. Surprisingly, ANP did not increase the internalization of either receptor in either cell line, although it did increase total GC-A uptake at later times in the HeLa cells. The fact that ANP increased uptake at later but not earlier time points is consistent with inhibition of recycling, not increased internalization and is inconsistent with ANP binding increasing the ability of GC-A or NPR-C to attach to cellular transport systems as occurs for other receptors.

As described in the introduction, many previous investigators have studied the trafficking of GC-A and/or NPR-C. However, an important question pertaining to all previous studies is which receptor is being measured. Our data suggest that previous internalization studies conducted on cells expressing both GC-A and NPR-C most likely measured NPR-C uptake. Nonetheless, regarding the debate over whether GC-A internalizes ^{125}I-ANP, it clearly does in the two cell lines that we tested. We do not know why our data differ from a previous study showing that intracellular ^{125}I-ANP radioactivity decreased rapidly in 293 cells transfected with GC-A (Pandey et al., 2002), but perhaps it is a function of the unique trafficking properties of the individual cell lines.

Down-regulation proceeds by three sequential processes: receptor internalization, endosomal sorting, and receptor proteolysis (Katzmann et al., 2002). Down-regulation of canonical G-protein coupled or tyrosine kinase receptors is associated with receptor internalization rates that are increased several fold in response to ligand binding (Vieira et al., 1996). However, because GC-A or NPR-C initial internalization rates were unchanged in the presence of ligand, we can conclude that increased uptake rates do not contribute to GC-A down-regulation in 293 cells. Furthermore, the similar loss of total and surface receptors indicates the intracellular and extracellular pools of receptor are closely linked and rapidly exchange.

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