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Cellular Trafficking of Human α_{1a}-Adrenergic Receptors Is Continuous and Primarily Agonist-Independent[S]

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ABSTRACT

α_{1a}-Adrenergic receptors (α_{1a}ARs) are present intracellularly and at the cell surface in cultured and natural cell models, where they are subject to agonist-mediated desensitization and internalization. To explore α_{1a}AR trafficking, a hemagglutinin (HA)-tagged α_{1a}AR/enhanced green fluorescent protein (EGFP) fusion protein was expressed in rat-1 fibroblasts and tracked by EGFP fluorescence and antibody labeling of surface receptors. Confocal analysis of antibody-labeled surface receptors revealed unexpected constitutive internalization in the absence of agonist stimulation. In partial agreement, the inverse agonist prazosin also caused a modest 20 ± 2% increase in surface receptor levels, suggesting a partial block of constitutive internalization caused by decreased basal activation. However, prazosin was unable to prevent internalization of antibody-tagged surface receptors observed by confocal microscopy or cause obvious redistribution of intracellular receptor to the surface, suggesting that the α_{1a}AR is internalizing even in a basal-inactive state. In contrast to the α_{1a}AR, surface labeling of an HA-tagged α_{1b}-EGFP fusion protein did not result in any apparent constitutive internalization. Constitutive internalization of the α_{1a}AR seems to occur alongside reversible agonist-induced internalization, and both seem to involve clathrin-mediated endocytosis but not degradation in lysozymes. Surface receptor density must be maintained by recycling, because the protein synthesis inhibitor cycloheximide has no effect on total or surface receptor density in agonist-treated or untreated cells for 6 h. Constitutive agonist-independent trafficking of α_{1a}ARs may provide a novel mechanism by which an internal pool of α_{1a}ARs are maintained and recycled to allow continuous agonist-induced signaling.

α_{1a}-Adrenergic receptors (α_{1a}ARs) are 1 of 3 members of the α_1AR subfamily (α_{1a}, α_{1b}, and α_{1d}) of G protein-coupled receptors (GPCRs) that mediate physiological effects such as prostatic smooth muscle contraction and myocardial hypertrophy in response to sympathetic stimulation (Michelotti et al., 2000; Milligan, 2003). Activated α_1ARs interact predominantly with G_q to induce phospholipase-Cβ activity, hydrolysis of membrane phospholipids, and ultimately increased levels of intracellular calcium (Graham et al., 1996). Although α_1AR family members are highly homologous (e.g., 75% amino acid identity in transmembrane domains), recent data suggest that divergent regulatory mechanisms characterize expression, function, and subcellular distribution of different α_1AR subtypes. For example, long-term agonist stimulation (24–72 h) of all three α_1ARs natively expressed in rat neonatal cardiac myocytes leads to down-regulation of α_{1b} and α_{1d} mRNA transcription, whereas α_{1a}AR expression is enhanced (Rokosh et al., 1996; Autelitano and Woodcock, 1998).

A series of reports in heterologous expression systems using both stable and transient transfection has demonstrated that the α_{1a}AR (Hirasawa et al., 1997; Coge et al., 1999; Chalothorn et al., 2002; Sugawara et al., 2002) and α_{1d}AR (Daly et al., 1998; McCune et al., 2000; Chalothorn et al., 2002) are present both on the cell surface and as intracellular pools; in contrast, the α_{1b}AR is predominantly found on the cell surface (Fonseca et al., 1995; Hirasawa et al., 1997; Hrometz et al., 1999; McCune et al., 2000; Stevens et al., 2000; Chalothorn et al., 2002; Sugawara et al., 2002). Two studies also examined localization of α_1ARs in native human smooth muscle cells and found internal as well as membrane-

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ABBREVIATIONS: AR, adrenergic receptor; GPCR, G protein-coupled receptor; EGFP, enhanced green fluorescent protein; PKC, protein kinase C; HA, hemagglutinin; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; [^{125}I]HEAT, 2-[β-(4-hydroxy-3-[^{125}I]iodophenyl)-ethylaminomethyl]tetralone; PMA, 4β-phorbol-12-myristate-13-acetate; ABTS, 2,2′-azino-di-[3-ethylbenzthiazolinesulfonate]; PCR, polymerase chain reaction; NE, norepinephrine; PBS, phosphate-buffered saline.

bound populations of α_{1a}AR (Hrometz et al., 1999; Mackenzie et al., 2000). This is similar to the situation for the α_2ARs, where α_{2A}- and α_{2B}ARs are predominantly surface localized, whereas the α_{2C}AR is predominantly intracellular (von Zastrow et al., 1993; Wozniak and Limbird, 1996; Daunt et al., 1997) but contrasts with βARs, which are surface localized (Guillaume et al., 1994; Barak et al., 1997; McLean and Milligan, 2000).

The demonstrated intracellular localization of α_{1a}ARs in both recombinant and native systems raises questions regarding subcellular trafficking of α_{1a}ARs both basally and in the presence of agonist. Cellular trafficking is an important step in the regulation of many GPCRs, particularly in response to receptor stimulation by agonist (reviewed in Ferguson, 2001). After agonist exposure, GPCRs are usually taken into the cell, where they are sorted into various intracellular compartments and then targeted either to lysosomes for degradation or recycled back to the cell surface. The ultimate effect on receptor signaling and fate of sequestered receptors varies with receptor, duration of agonist exposure, and cellular environment. For example, rapid agonist-mediated subcellular redistribution of the α_{1b}AR is thought to involve internalization of the receptor through a PKC-dependent pathway as well as recycling of at least a portion of the internalized receptors back to the cell surface (Fonseca et al., 1995).

We recently demonstrated that α_{1a}ARs are acutely desensitized and phosphorylated and that a portion of α_{1a}AR surface receptors subsequently internalize (Price et al., 2002). To further characterize the regulation of α_{1a}AR trafficking, we have created a fusion construct consisting of an amino-terminal hemagglutinin (HA) and carboxyl-terminal EGFP-tagged α_{1a}AR and have stably expressed this protein in rat-1 fibroblast cells. This combination of tags allowed us not only to identify the receptor, but to compare surface (anti-HA antibodies) and total (EGFP fluorescence) receptor pools by cell sorting and to visually distinguish between particular subpopulations of receptors in the cell using confocal microscopy. Our data confirm the surface and intracellular localization of α_{1a}ARs and agonist-induced receptor internalization. We were surprised to find that the α_{1a}AR constitutively internalizes even in the absence of agonist. It is noteworthy that constitutive internalization continues even in the presence of the α_1AR-selective antagonist and inverse agonist prazosin, suggesting that internalization can occur without α_{1a}AR activation. Thus, although agonist stimulation of α_{1a}ARs may alter the balance of α_{1a}ARs at the cell surface, mechanisms underlying α_{1a}AR trafficking, cellular location, and degradation seem distinct from those regulating many other GPCRs, providing a possible mechanism for continued signaling even after prolonged agonist exposure.

Materials and Methods

Materials. DMEM and fetal bovine serum (FBS) were obtained from Invitrogen (Carlsbad, CA). [^{125}I]HEAT, [^{3}H]inositol, and [^{32}P]orthophosphate were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). Dowex AG1-X8 was obtained from Bio-Rad (Hercules, CA), PMA was obtained from Sigma-Aldrich (St. Louis, MO), and norepinephrine was obtained from Sigma/RBI (Natick, MA) or Sigma-Aldrich. Goat anti-rat IgG conjugated to either Oregon Green or Texas Red, Texas Red-conjugated transferrin, and Texas Red-conjugated dextran were obtained from Molecular Probes (Eugene, OR). Bisindolylmaleimide I was obtained from Calbiochem (San Diego, CA). ABTS, ABTS buffer, 3F10 monoclonal antibody, and 3F10/peroxidase conjugate were obtained from Roche Diagnostics (Indianapolis, IN).

Construction of HA-Tagged α_{1a}AR and α_{1a}EGFP. cDNA encoding the human α_{1a} was modified using PCR to include the sequence of the HA epitope at the amino terminus of the protein. The 5′ (sense) 59-mer oligonucleotide (5′-AAAAGAATTCATG***TACCCATACGACGTCCCAGACTACGCC***GTGTTTCTCTCGGGAAATG-3′) contains a synthetic EcoRI restriction site to facilitate cloning, sequence encoding the nine-residue HA epitope (YPYDVPDYA; bold italics) immediately downstream of the α_{1a}AR start codon, and 19 bases corresponding to 4 to 22 base pairs of the α_{1a}AR (underlined). The 3′ (antisense) primer was 5′-GAGCAGCCTCACTGAGAAGTGCGT-3′, corresponding to bases 796 to 763 of the α_{1a}AR receptor. The resulting PCR product was digested with EcoRI and Eco47III and subcloned into the mammalian expression vector pcDNA3-α_{1a}AR; the final construct is called pcDNA3:HA-α_{1a} and expresses the AR-designated HA-α_{1a}. EGFP (a red-shifted variant of wild-type green fluorescent protein that has been codon optimized for expression in mammalian cells) was amplified from pcDNA3:mcsEGFP containing the NheI-XbaI EGFP cassette from pEGFP-N1 (BD Biosciences Clontech, Palo Alto, CA) using an NH_2 terminal primer designed to include both a XhoI and an NheI restriction site (ATGGCTCGAGCTAGCGATGGTGAGCAAGGGCGAG) and an SP6 primer complementary to the SP6 site of pcDNA3. The PCR product was digested with XhoI and XbaI and ligated into XhoI/XbaI-digested pcDNA3 (Invitrogen) to generate pcDNA3:EGFPneat. To eliminate the α_{1a}AR stop codon, the carboxyl terminus of the α_{1a}AR cDNA was amplified with an NH_2 terminal primer that included the naturally occurring EcoRV site of α_{1a}AR (TGGCTCGGATATCTAAACAGC) and a COOH terminal primer (GAACGCTAGCCCGACTTCCTCCCCGTTCTCACT) that replaced the stop codon with an NheI site that encodes three extra amino acids (glycine, leucine, and alanine). This PCR product was digested with EcoRV/NheI and, together with the NH_2 portion of HA-α_{1a}-digested EcoRI/EcoRV, subcloned into EcoRI/NheI-digested pcDNA3:EGFPneat, generating pcDNA3:HA-α_{1a}EGFP, which expresses the AR-designated HA-α_{1a}EGFP. PCR-generated sequences were verified by dideoxy DNA sequencing.

Cell Culture, Expression of Receptors, and α_1AR Ligand Binding. Rat-1 fibroblast cells were grown in monolayers and maintained in DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) in 5% CO_2 at 37°C. For stable expression of receptors, the plasmid pcDNA3:HA-α_{1a}EGFP was transfected into rat-1 fibroblasts cells by electroporation (Gene Pulsar II; Bio-Rad; optimal conditions were 250 mV and 950 μF capacity, resulting in a time constant of 12–15 ms) or FuGENE6 transfection reagent (Roche Diagnostics). Forty-eight hours after transfection, cells were incubated with G418 (400 μg/ml). Cells resistant to G418 were isolated and tested for receptor expression (HA-α_{1a}) or sorted by flow cytometry (HA-α_{1a}EGFP) with a FACStar Plus (BD Biosciences, San Jose, CA). HA-α_{1a}EGFP was excited using a 488-nm argon/krypton laser and detected with a 530/30-nm band pass filter. Selection of stable cells was maintained with 400 μg/ml G418. Saturation binding to determine receptor density and competition analysis of transfected cell membranes was performed with the radiolabeled α_1AR antagonist [^{125}I]HEAT (300 and 120 pmol/l for saturation and competition binding, respectively) as described previously (Schwinn et al., 1995). Resultant curves were fit using noniterative regression analysis with PRISM software (GraphPad Software Inc., San Diego, CA).

Determination of Total Inositol Phosphate Production. Cells stably expressing either HA-α_{1a} or HA-α_{1a}EGFP receptors were labeled with [^{3}H]inositol for 20 to 24 h with 2.5 μCi/ml in DMEM supplemented with 5% fetal bovine serum. After labeling, cells were washed and incubated in PBS for 15 to 30 min at 37°C; pretreated cells were exposed to various drugs during this incubation. Cells were then washed once with PBS and stimulated for 20 min in the presence of 20 mM LiCl. Inositol phosphates were ex-

tracted as described previously (Martin, 1983) and separated using Dowex AG1-X8 anion exchange (formate) columns. After washing the columns twice with water, total inositol phosphates were eluted in 1 M ammonium formate/0.1 M formic acid, combined with 15 ml of scintillation cocktail, and counted.

Confocal Laser Scanning Microscopy. For all confocal experiments, rat-1 cells expressing HA-α_{1a}EGFP were grown in media at 37°C on sterilized glass coverslips and mounted on a larger cover slip or in an Attofluor cell chamber (Molecular Probes) for analysis. Unless indicated, steps were at room temperature. Cells were fixed for 10 min with 3.7% formaldehyde in PBS and then for another 10 min with or without 0.05% Triton X-100. Fixed and fixed/permeabilized cells were then incubated with anti-HA epitope 3F10 primary antibody (1:100 dilution) for 30 min in PBS containing 3% FBS. Cells were washed with PBS and incubated with goat anti-rat IgG Texas Red conjugate (1:500 dilution) in PBS containing 10% FBS for 30 min. Cells were washed and visualized with a laser scanning confocal microscope (Zeiss Axiovert 100; Zeiss, Welwyn Garden City, UK) using a Zeiss Plan-APOCHROMAT 63× 1.40 numerical aperture oil immersion objective, pinhole of 20, and electronic zoom between 1 and 3. For all experiments, HA-α_{1a}EGFP was excited using a 488-nm argon/krypton laser and detected with a 530/30-nm band pass filter, whereas Texas Red was excited at 560 nm and detected with a 630/22-nm band pass band filter. Images were prepared with Adobe Photoshop software (Adobe Systems, Mountain View, CA). All images show a cellular cross-section removed from the "basal" membrane (next to the cover slip) but including the full width of the cell.

In antibody "pulse-chase" experiments, HA-α_{1a}EGFP on live cells was labeled with 3F10 (1:100 dilution) at 4°C for 15 min in DMEM. Cells were washed with ice-cold DMEM before the readdition of prewarmed media and incubation at 37°C with treatments as indicated in the text. Cells were quickly washed with DMEM at 4°C for the 0 time points or with PBS at 25°C for the other time points and then fixed and permeabilized as described above. Cells were treated with secondary antibody (goat anti-rat IgG Texas Red conjugate) at 1:500 dilution for 30 min in 10% FBS in PBS plus 0.05% Triton X-100. Cells were washed with PBS and used for confocal analysis as indicated above.

To label endocytotic compartments in colocalization experiments, cells were incubated with 25 μg/ml Texas Red-transferrin in media for the variable times or pulse-labeled 15 min at 4°C before incubation in media at 37°C as indicated in the text. To label lysosomal vesicles, cells were incubated overnight in media with 1 mg/ml Texas Red-dextran. Cells were fixed and used for confocal analysis as described above.

Relative Quantitation of Cell Surface and Total Receptor Populations: Quantitation by Cell Sorting. After various treatments in media at 37°C, cells expressing HA-α_{1a}AR or HA-α_{1a}EGFP were placed on ice and incubated with 3F10 antibody diluted 1:500 in DMEM for 10 min. Cells were washed with DMEM and then incubated with either Oregon Green- (for HA-α_{1a}) or Texas Red-conjugated goat anti-rat IgG for 10 min. After a brief wash with Hanks' balanced salt solution without calcium or magnesium, the cells were trypsinized by incubation with 200 μl of 0.05% trypsin on ice for 2 to 10 min. Trypsin had no effect on epitope/antibody coupling under these conditions (R. R. Price, unpublished observation). Cells were subsequently transferred to tubes containing 200 μl of 3.7% formaldehyde for fixation and analysis using flow cytometry. HA-α_{1a}EGFP and Oregon Green were excited using a 488-nm argon/krypton laser and detected with a 530/30-nm band pass filter, whereas Texas Red was excited at 560 nm and detected with a 630/22-nm band pass filter. Surface fluorescence was quantitated by measurement of secondary antibody red (for HA-α_{1a} and HA-α_{1a}EGFP) or green fluorescence (for HA-α_{1a}). Total receptor expression levels were obtained from cells expressing HA-α_{1a}EGFP by measurement of EGFP fluorescence. In each experiment, fluorescent values of treated cells were expressed as a percentage of control values (100%). Background fluorescence for both secondary antibodies without mAb 3F10 (for antibody staining) and for cells without EGFP (for total fluorescence measurements) was <10% of specific fluorescence, so data were not corrected.

To quantitate surface receptor levels after prazosin treatment, cells in 12-well plates were treated with 10^{-6} M prazosin in media at 37°C for 2 h before antibody/peroxidase surface assay. For experiments with agonist, NE was added 10 min after the addition of prazosin. After treatment, cells were washed with PBS, fixed for 10 min with 3.7% formaldehyde, washed twice with PBS, and blocked for 30 min with 5% milk in PBS, after which 3F10-horseradish peroxidase was added to 25 mU/ml (from 100× stock in PBS/milk), and the incubation continued for 30 min. Cells were washed with PBS/milk and then washed twice with PBS before incubation with ABTS at 25°C for 1 h. The solution was transferred to 48-well plates, and absorbance values were read at 405 Å. Final values were obtained by subtracting background observed for wells with blocking amounts of 3F10 (750 ng/ml) included during blocking and 3F10-horseradish peroxidase incubations. Assay incubations were at room temperature with slow rotation.

Statistical Analysis. Values are expressed as mean ± S.E.M. In cases where experimental values were normalized to 100% of untreated control or zero time, significance was assessed by determining that values were not 100% using a single value two-tailed Student's t test. For other experiments with multiple points, statistical significance was assessed using one-way analysis of variance; when significance occurred, exact p values were determined using an unpaired two-tailed Student's t test. $p < 0.05$ was considered significant.

Results

Characterization of Stable Clones Expressing HA-α_{1a}EGFP. To explore trafficking of human α_{1a}ARs, we used HA-α_{1a} and HA-α_{1a}EGFP fusion proteins expressed as stable clones in rat-1 cells at ~1.2 and ~1.0 pmol/mg total protein, respectively. A series of cell populations expressing HA-α_{1a}EGFP at levels ranging from ~400 to 1200 fmol of receptor/mg of total protein were also examined with similar results to those shown below. We have previously shown that the addition of the HA tag to the amino terminus of the α_{1a}AR has no detectable effect on receptor behavior (Price et al., 2002). Side-by-side characterization experiments with the HA-α_{1a} and HA-α_{1a}EGFP also show no difference in behavior in regard to NE binding (pK_i values of 5.1 ± 0.4 and 4.7 ± 0.1, respectively), potency (EC_{50} values of 0.24 ± 0.08 and 0.36 ± 0.033 mM, respectively), or efficacy (17 ± 1.7- and 22 ± 1.0-fold over basal levels of phosphatidylinositol hydrolysis, respectively). Furthermore, measurements of agonist-induced desensitization demonstrate that pretreatment of cells with NE reduces the HA-α_{1a}EGFP total inositol phosphate response to a second challenge with NE to 63 ± 4% of the naive receptor response, similar to reduction of the HA-α_{1a} response to 61 ± 6%. In addition, we found that basal, NE, and PMA (PKC activator)-induced phosphorylation of HA-α_{1a} and HA-α_{1a}EGFP are very similar (supplemental Fig. 1). These data indicate that salient pharmacological and signaling characteristics of α_{1a}ARs seem to be unaffected by the presence of the carboxyl-terminal EGFP.

Subcellular Localization of HA-α_{1a}EGFP. Using confocal microscopy, we examined cellular localization of HA-α_{1a}EGFP by visualization of immunofluorescent labeling of the HA epitope, EGFP fluorescence, or both. When cells expressing HA-α_{1a}EGFP are fixed but *not* permeabilized before immunofluorescent labeling (Fig. 1A), EGFP fluorescence is

enriched along some membrane edges and internally as punctate dots of various sizes, almost certainly representing vesicles containing receptor. In contrast, staining with anti-HA rat 3F10/anti-rat conjugated Texas Red antibodies shows only surface receptor in these nonpermeabilized cells (Fig. 1B). The composite in Fig. 1C shows EGFP fluorescence in green and Texas Red fluorescence in red; consequently, regions with colocalized fluorescence are colored yellow, reemphasizing the staining of the cell membrane.

When cells expressing HA-α_{1a}EGFP are fixed and then permeabilized with Triton X-100, the pattern of the EGFP fluorescence (Fig. 1D) is not significantly different from that of unpermeabilized cells (e.g., compare Fig. 1, A and D). In contrast, permeabilization of the cell now allows receptor throughout the cell to be labeled with the anti-HA rat 3F10/anti-rat-conjugated Texas Red antibodies (Fig. 1E). Most of the punctate dots within the cell that show EGFP fluorescence also stain with Texas Red. The composite image (Fig. 1F) accents colocalization of EGFP and Texas Red fluorescence, reinforcing the validity of HA-α_{1a}EGFP as a visible model of α_{1a}AR localization. There is, however, substantial artifactual Texas Red fluorescence around the nuclei of cells labeled with 1° and 2° antibodies after permeabilization (Fig. 1E). Note also that the membrane edge of most cells does not show a dramatic EGFP fluorescence (white arrows in Fig. 1, A and D). Because surface receptor is clearly present (e.g., Fig. 1B), we believe this reflects the fact that the receptor is not sufficiently enriched to create a strong signal relative to the interior of the cell that displays a diffuse green coloration.

Agonist-Mediated Internalization of HA-α_{1a}EGFP. We have previously shown that the α_{1a}AR is capable of undergoing agonist-mediated internalization (Price et al., 2002). To extend this result, we used anti-HA rat 3F10/anti-rat-conjugated Texas Red antibody labeling of intact cells expressing HA-α_{1a}AR or HA-α_{1a}EGFP to obtain quantitative flow cytometric measurements of surface receptor density. After NE exposure, levels of surface HA-α_{1a}EGFP (Fig. 2A) and HA-α_{1a}AR (minimum 78 ± 2%) were found to decrease over 1 h similar to previous results (Price et al., 2002). Cell surface receptor is maintained at this level through at least 6 h of agonist exposure, suggesting that the steady-state level of surface receptors has adjusted to a new lower level in the presence of agonist. It is interesting that complete recovery of cell surface HA-α_{1a}EGFP density was observed in only 10 min after agonist removal (Fig. 2B), suggesting that the recovery of surface receptor is from intracellular pools and not new protein synthesis. Also in agreement with previous experiments (Price et al., 2002), the PKC activator PMA does not lead to significant internalization of α_{1a}ARs (Fig. 2A), and the PKC inhibitor bisindolyl-maleimide I does not prevent agonist-mediated internalization (supplemental Fig. 2).

Constitutive Internalization of HA-α_{1a}AR and HA-α_{1a}EGFP. Although flow cytometry studies demonstrate the loss of α_{1a}ARs from the cell surface, the existence of a prom-

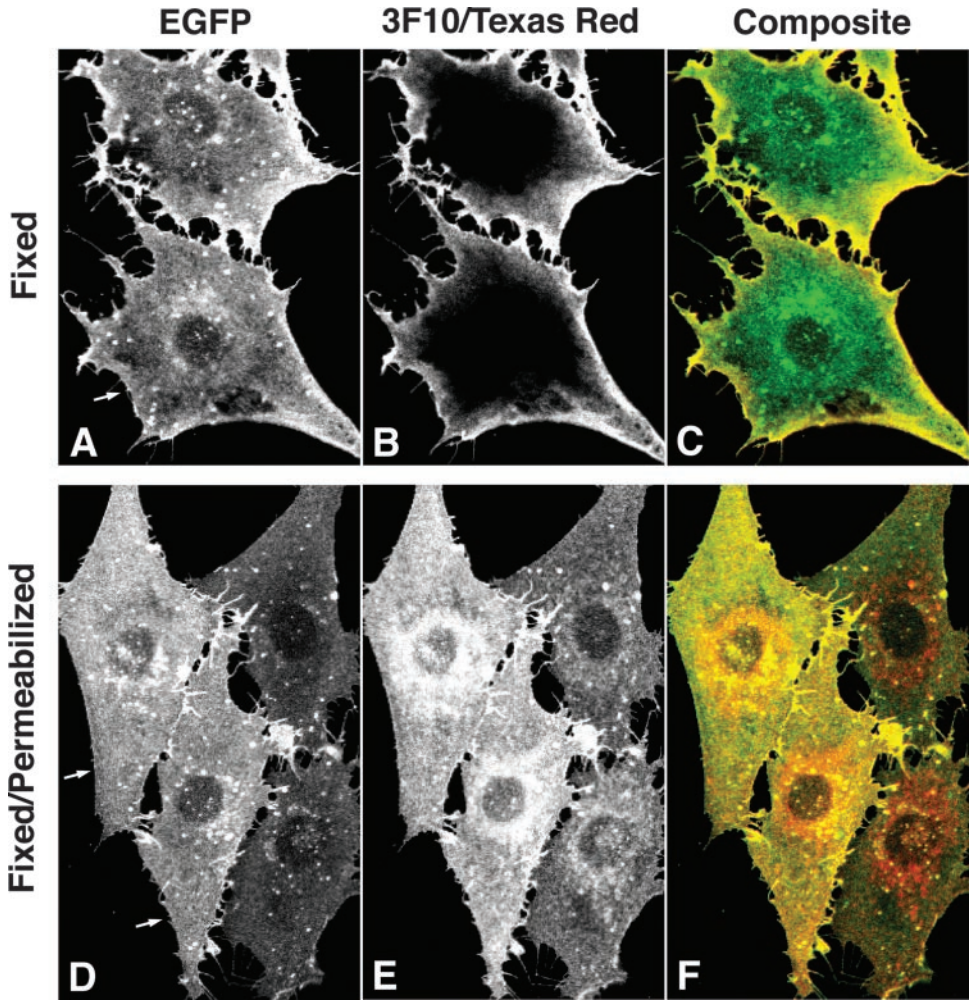

Fig. 1. Subcellular localization of HA-α_{1a}EGFP in rat-1 fibroblasts by confocal microscopy. Rat-1 fibroblasts expressing HA-α_{1a}EGFP were fixed with 3.7% formaldehyde for 10 min, after which they were incubated with 3F10 antibody against the HA epitope and then with Texas Red-conjugated goat anti-rat IgG. Images of nonpermeabilized cells show EGFP fluorescence (A), Texas Red fluorescence (B), or the composite of both (C). Regions with colocalized fluorescence appear yellow. Likewise, permeabilized cells expressing HA-α_{1a}EGFP were fixed with 3.7% formaldehyde in PBS for 10 min and then permeabilized with 0.05% Triton X-100 with 3.7% formaldehyde in PBS for 10 min before visualization of EGFP fluorescence (D), Texas Red fluorescence (E), or the composite of both (F). White arrows in A and D point at the cell edges where surface receptor is present but not obviously enriched.

inent intracellular population of α_{1a}ARs confounds the ability to readily observe agonist-mediated trafficking of receptors from the cell surface. Therefore, we used "pulse" immunofluorescent labeling of HA-tagged surface receptors to track receptor movement. Live rat-1 fibroblasts expressing HA-α_{1a}EGFP were labeled on ice with the rat 3F10 monoclonal antibody against the HA epitope followed by washing before incubation in media at 37°C. After various incubation times, cells were fixed and permeabilized, after which the 3F10 labeled receptor was stained with anti-rat IgG/Texas Red.

Initial pulse immunofluorescent experiments unexpectedly revealed that antibody-labeled receptor was internalized even in the absence of agonist. Antibody labeling of living cells at 4°C results in a diffuse membrane receptor staining that shows no obvious sequestration of antibody-labeled receptors into the interior of the cells (Fig. 3A, top). However, when unstimulated living cells were incubated at 37°C after pulse labeling, staining at the surface of the cell decreased, and intracellular punctates appeared by 30 min (Fig. 3B, top). Over 1 to 2 h, staining continues to decrease on the surface and increases intracellularly. (Fig. 3, C and D, top). In addition, the EGFP (middle) and 3F10/Texas Red (top) fluorescence patterns become similar, although in many cells only a portion of the HA-α_{1a}EGFP-containing vesicles also contain antibody-labeled receptors (Fig. 3, C and D). Although significant variations are observed in receptor EGFP fluorescence patterns (Fig. 3, middle; Figs. 1 and 4), no systematic changes were found to occur during the time course, indicating the antibody treatment had no apparent effect on HA-α_{1a}EGFP distribution. We did observe variation in overall receptor expression among these clonal cells. The cells shown represent those expressing higher levels of receptor; however, cells expressing lower levels of receptor displayed the same pattern of constitutive internalization (e.g., note the lower expressing cell at the bottom right of Fig. 3D).

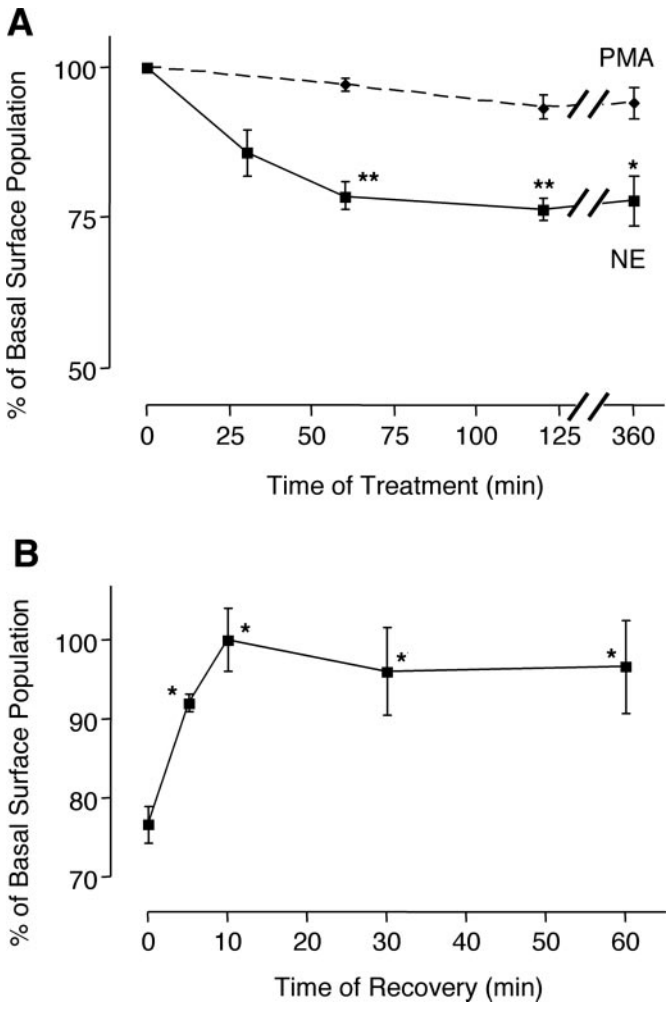

Fig. 2. Effect of agonist addition and subsequent removal on HA-α_{1a}EGFP surface density. Rat-1 fibroblasts expressing HA-α_{1a}EGFP were treated with either NE or PMA for various time intervals, after which cell surface receptors were quantitated by flow cytometry as described under *Materials and Methods* (A). To examine the ability of membrane HA-α_{1a}EGFP to recover after agonist-mediated internalization, rat-1 fibroblasts expressing the receptor were incubated for 1 h with 10^{-5}M NE and washed to remove agonist (B). Cells were then incubated for various times at 37°C and processed for cell surface and total cellular receptor quantitation by flow cytometry. Data are mean ± S.E.M. from 3 to 4 independent experiments. Cell surface populations of HA-α_{1a}EGFP at time points indicated are significantly different from cells at time 0. *, $p < 0.05$ and **, $p < 0.01$.

Effect of NE and Prazosin upon Receptor Distribution and Constitutive Internalization. Whereas cell sorting experiments indicate modest (~25%) agonist-induced internalization of HA-α_{1a}EGFP, visual analysis of surface receptor internalization after NE treatment (Fig. 4, A–D) is indistinguishable from that observed without agonist stimulation. This is not too surprising given the qualitative nature of confocal microscopy and the rapidity of constitutive receptor internalization. More unexpectedly, treatment of cells with prazosin also had no apparent effect upon constitutive internalization or HA-α_{1a}EGFP distribution (Fig. 4, E–H). If basal receptor activation was essential for constitutive internalization, then the inverse agonist activity of prazosin should have blocked internalization and led to the redistribution of HA-α_{1a}EGFP to the cell surface, which seems to be the case for α_{1d}AR (McCune et al., 2000). To determine whether this inverse agonist has any effect on receptor distribution, we quantitated surface HA-α_{1a}EGFP levels after treatment with 10^{-6} M prazosin for 2 h and observed a statistically significant 20 ± 2% ($n = 4$) increase in surface receptor. Thus, although this inverse agonist does not prevent most constitutive internalization, prazosin does increase surface receptor density, suggesting partial inhibition of constitutive internalization. These findings are not inconsistent, because small decreases in internalization can cause modest changes in surface receptor density (Szekeres et al., 1998). As expected, essentially the same increase in surface receptor (18 ± 4%, $n = 4$) was observed in the presence of 10^{-5} M NE, with blocking concentrations of prazosin (10^{-6} M) indicating agonist-mediated internalization was prevented. A complicating factor encountered during confocal analysis after NE treatment was the tendency of HA-α_{1a}EGFP expressing rat-1 cells to "round up" during incubations of 1 to 2 h, an outcome that prazosin prevented (M. Smith, unpublished observation). This behavior is not particularly surprising, because α_{1a}AR stimulation causes morphological changes in cardiomyocytes and phosphorylation of the cytoskeletal protein vimentin in rat-1 cells (supplemental Fig. 3).

Additional Evidence for Constitutive HA-α_{1a}EGFP Internalization and Recycling. Although the ability of prazosin to increase HA-α_{1a}EGFP surface receptor density

confirms the existence of constitutive internalization, we wanted to demonstrate that surface labeling of receptor will not generally cause AR internalization. When rat-1 cells stably expressing HA-α_{1b}EGFP were identically prelabeled with anti-HA 3F10 antibody as for HA-α_{1a}EGFP, incubation in media for 1 h at 37°C resulted in no obvious internalized HA-α_{1b}EGFP punctates (Fig. 5B, top), and cells appear the same as prelabeled cells at 0 min (Fig. 5A, top). An essentially complete absence of antibody internalization was also observed in two other unique HA-α_{1b}EGFP expressing clones (data not shown). In contrast, when cells expressing HA-α_{1b}EGFP are prelabeled with antibody and incubated in media plus NE for 1 h at 37°C, intracellular punctates are clearly present, and surface receptor staining is clearly reduced (Fig. 5C, top). The dramatic NE-induced internalization of total HA-α_{1b}EGFP seen with EGFP fluorescence (Fig. 5, lower) contrasts starkly with the absence of significant change in HA-α_{1a}EGFP location after agonist stimulation (Fig. 3). It needs to be noted that the antibody staining of HA-α_{1b}EGFP across the cells at 0 min (Fig. 5A, top) does not reflect intracellular receptor, because the 3F10 antibody could not enter these cells; rather, it reflects lower surface staining and consequent higher relative background near the cell center. As an additional control, we also performed the 3F10 prelabeling experiment in rat-1 cells expressing HA-α_{1a} without the EGFP motif and observed the expected receptor internalization, suggesting that the addition of the EGFP does not cause constitutive internalization (supplemental Fig. 4).

Given that surface HA-α_{1a}EGFP density is maintained, the discovery of continuous constitutive internalization implies internalized receptors must be replaced. This could occur by three possible mechanisms: 1) internalized surface receptors are immediately recycled from early endosomes, 2) replaced by receptors from an intracellular pool, or 3) replaced by newly synthesized receptors. The first two hypotheses are difficult to distinguish and potentially nonexclusive; however, the possibility of replacement by new receptor synthesis can easily be tested by growing cells with the protein synthesis inhibitor cycloheximide. Flow cytometry measurements of surface and total receptor density indicate that 6 h of exposure to cycloheximide has no effect on receptor levels (Fig. 6A). Furthermore, even in the presence of NE, total HA-α_{1a}EGFP levels are maintained, and internalization is

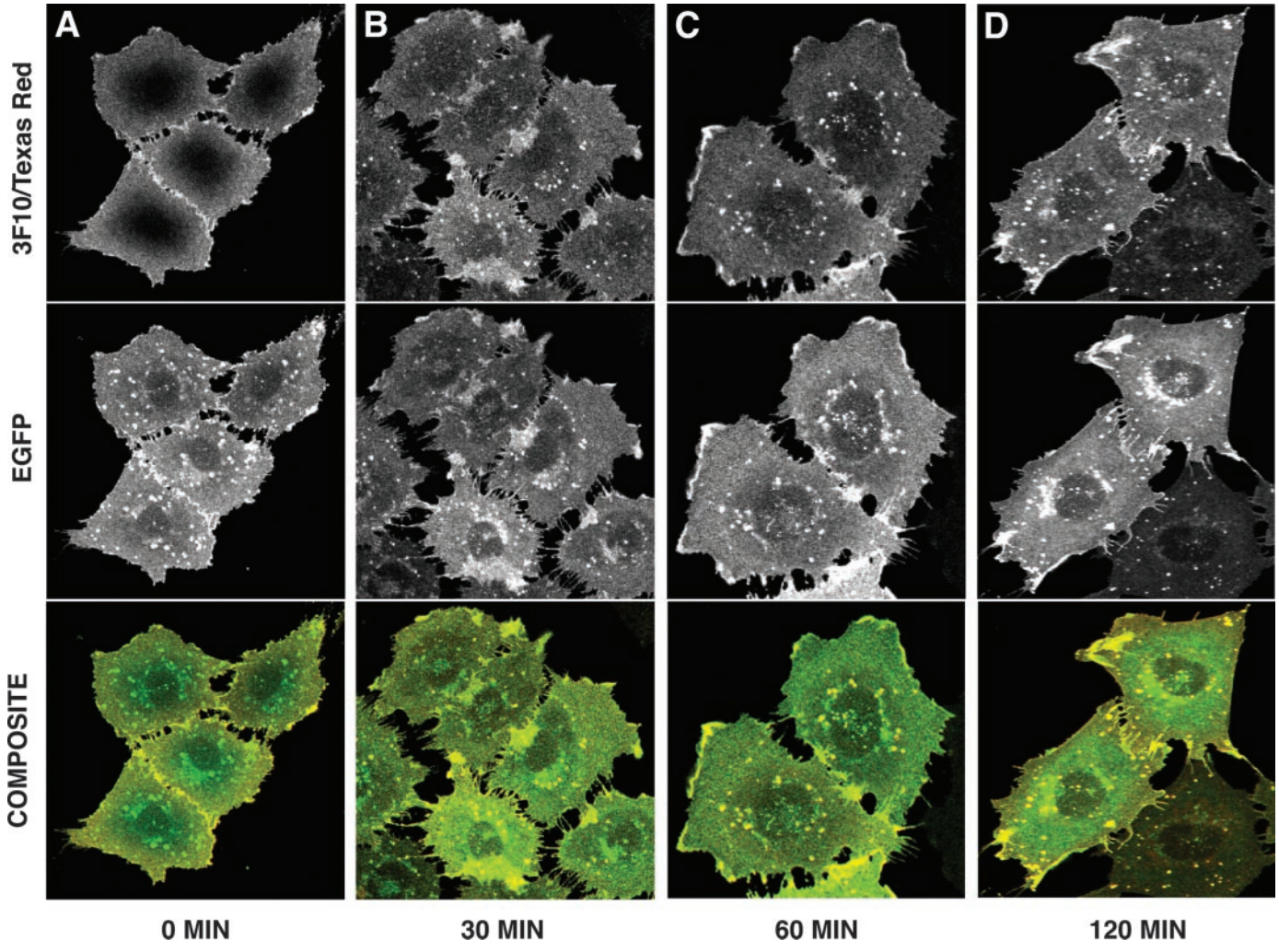

Fig. 3. Constitutive internalization of HA-α_{1a}EGFP stably expressed in rat-1 fibroblasts. Cells expressing receptor were prelabeled 15 min on ice with 3F10 antibody before incubation in media at 37°C for 0 (A), 30 (B), 60 (C), or 120 min (D). Cells were subsequently fixed, permeabilized, and stained with Texas Red-conjugated goat anti-rat IgG. The receptor in permeabilized cells was visualized using confocal microscopy: Texas Red fluorescence (top), EGFP fluorescence (middle), and composite image (bottom).

the same (Fig. 6B) as observed in the absence of cycloheximide (compare with Fig. 2A). These experiments indicate that α_{1a}ARs are relatively long-lived proteins and that de novo protein synthesis plays a minor role in maintaining total and surface receptor concentrations in the time frame of agonist-mediated and constitutive internalization. We cannot exclude the possibility that previously synthesized "new" receptors constitute a part of the intracellular pool; however, maintenance of surface receptor levels for hours in the presence of cycloheximide makes it unlikely that previously synthesized receptors constitute a significant fraction of replacement receptors.

Constitutive and Agonist-Mediated Internalization Seem to Be Additive. To address the possibility of α_{1a}ARs

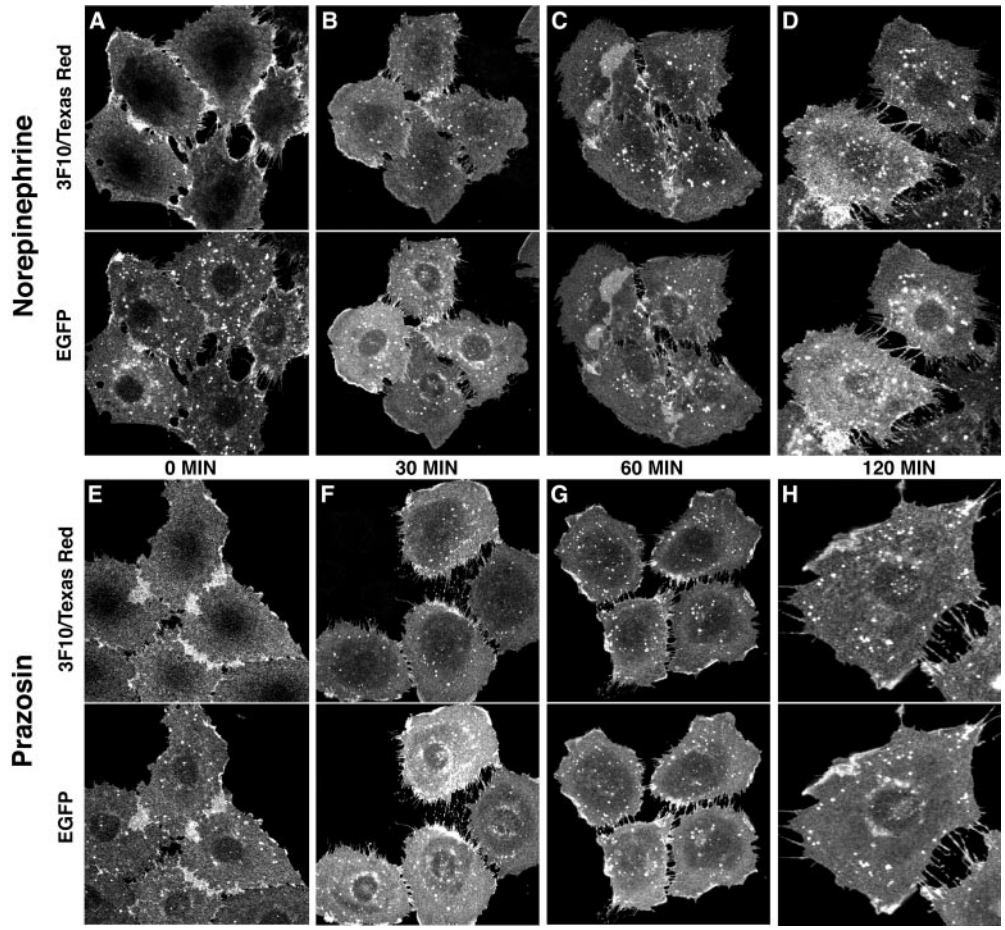

Fig. 4. Neither the α_1AR agonist NE nor the inverse agonist prazosin visually alters continuous HA-α_{1a}EGFP internalization as monitored by confocal microscopy. Cells expressing HA-α_{1a}EGFP were prelabeled on ice with 3F10 and then incubated at 37°C with 10^{-5} M NE (A–D) or 10^{-6} M prazosin (E–H) for 0, 30, 60 or 120 min in media. Cells were subsequently fixed, permeabilized, and stained with Texas Red-conjugated goat anti-rat IgG. The receptor in the permeabilized cells was visualized through antibody staining (3F10/Texas Red) and receptor fluorescence (EGFP).

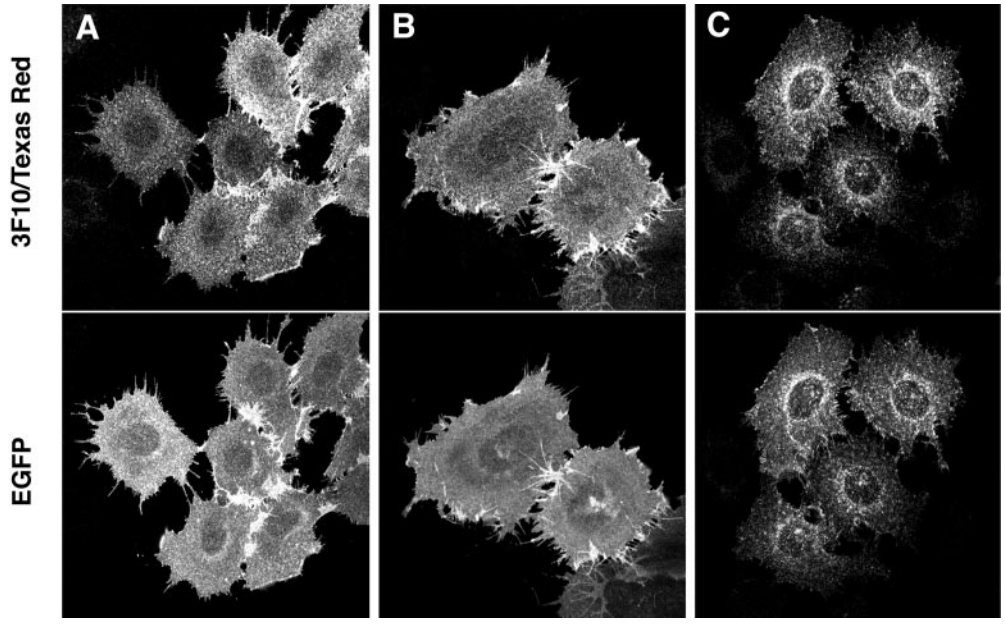

Fig. 5. Confocal analysis shows HA-α_{1b}EGFP does not constitutively internalize but does internalize upon agonist exposure. Rat-1 fibroblasts stably expressing HA-α_{1b}EGFP were prelabeled on ice with 3F10 and then incubated for 0 min (A) or incubated without (B) or with NE (C) for 60 min at 37°C in media. For analysis, cells were fixed, permeabilized, and stained with Texas Red-conjugated goat anti-rat IgG. Receptors were visualized through confocal analysis of antibody staining (top) or receptor EGFP fluorescence (bottom).

recycling through perinuclear recycling endosomes, we used the Golgi disrupting agent monensin to block endosomal acidification and subsequent HA-α_{1a}EGFP receptor recycling (Pippig et al., 1995; Rosenfeld et al., 2002). In the absence of agonist, monensin alone results in reduction of cell surface receptors to 90 ± 4% of basal levels within 30 min and to 83 ± 4% of basal values after 120 min (Fig. 7). These data are consistent with an ongoing process of constitutive internalization and suggest that basal cell surface populations of α_{1a}ARs are maintained in part through a monensin-sensitive pathway that is agonist-independent. When cells expressing HA-α_{1a}EGFP are stimulated with 10^{-5}M NE after pretreatment with monensin, the resultant loss of cell surface receptors is equal to the individual decreases caused by NE and monensin exposure combined (Fig. 7). It is interesting that concurrent prazosin treatment (10^{-6}M) of cells expressing HA-α_{1a}EGFP does not inhibit the monensin-mediated loss of cell surface receptors (supplemental Fig. 5). This is true even though NE-induced sequestration seems to be completely prevented by prazosin (see above). Thus, although both agonist-mediated sequestration and constitutive membrane receptor recycling play roles in α_{1a}AR trafficking, the two processes seem to be additive and at least partially independent.

HA-α_{1a}EGFP Is Present in Early Endosomes but Not Lysosomes. Previous work on other GPCRs (Ferguson, 2001) and the continuous presence of cycling HA-α_{1a}EGFP in vesicles suggest that HA-α_{1a}EGFPs may cycle through clathrin-coated pits to early endosomes and would therefore colocalize with cycling transferrin receptors (Rosenfeld et al., 2002). Internalization of transferrin receptors was followed using Texas Red-labeled transferrin. As shown by the yellow signal in the composite image in Fig. 8A, in less than 5 min internalized Texas Red-labeled transferrin was strongly colocalizing with HA-α_{1a}EGFP in patches and vesicles near the nucleus. Except for an increase in Texas Red signal, the overall pattern was similar at 1 h (Fig. 8B) and after 24 h (data not shown) of exposure to transferrin. Internalized transferrin colocalized similarly in the presence of NE (data not shown). Perhaps significantly, less than half of the HA-α_{1a}EGFP-containing vesicles nearer the cell periphery contain small quantities of transferrin (appear yellow), and many appear to contain none (remain green). Nevertheless, the rapid colocalization of transferrin and its receptor with HA-α_{1a}EGFP indicates the receptors are trafficked through similar populations of early endosomes (Rosenfeld et al., 2002). In contrast, almost none of the internal HA-α_{1a}EGFP-containing vesicles colocalizes with lysosomes containing internalized Texas Red-labeled dextran under agonist-free conditions (Fig. 8C), or after 6 h (data not shown) or even 24 h (Fig. 8D) of agonist stimulation. In fact, regions that contain HA-α_{1a}EGFP-containing vesicles are largely devoid of lysosomes and visa versa.

To further support the role of clathrin-coated vesicles in α_{1a}AR trafficking, immunofluorescent experiments were performed with 3F10-prelabeled HA-α_{1a}EGFP in the presence of 0.45M sucrose [known to inhibit endocytosis mediated through clathrin-coated pits/vesicles (Fonseca et al., 1995)]. To accent colocalization of internalized antibody-labeled re-

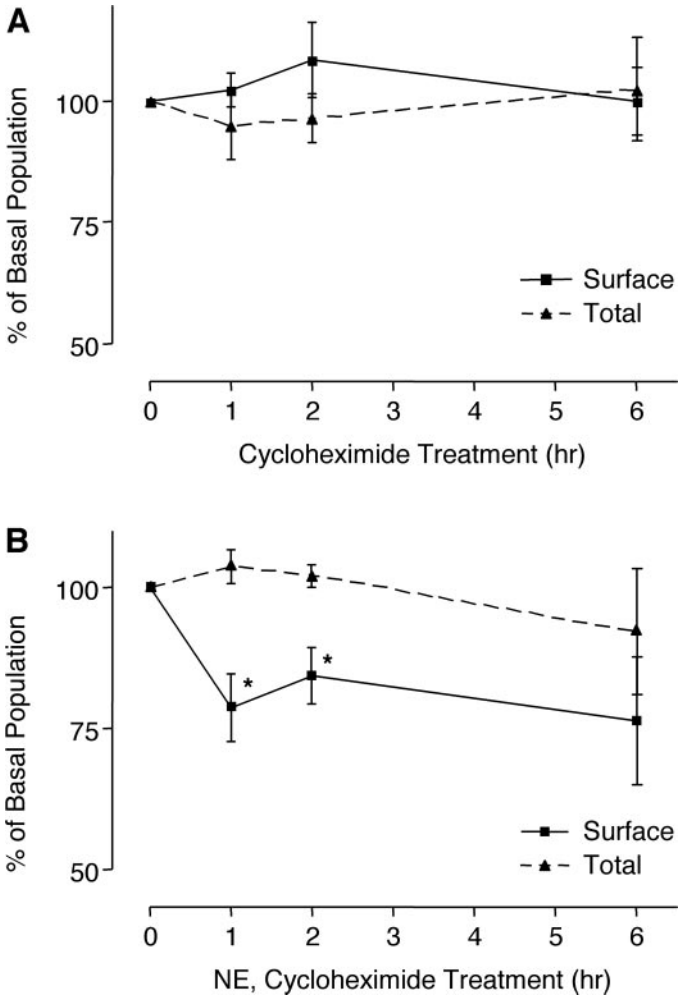

Fig. 6. Inhibition of new receptor synthesis does not alter cell surface or total receptor density in the absence or presence of agonist. A, rat-1 fibroblasts expressing HA-α_{1a}EGFP treated with 10 μg/ml cycloheximide. Cell surface (■) and total (▲) receptor levels were quantitated with flow cytometry. B, cells treated with 10 μg/ml cycloheximide for 30 min before the addition of 10^{-5}M NE for various periods of time. Cell surface (■) and total (▲) receptor levels were quantitated with flow cytometry. Data are mean ± S.E.M.; n = 3 independent experiments. HA-α_{1a}EGFP levels at the time points indicated are significantly different from cells at time 0. *, $p < 0.05$.

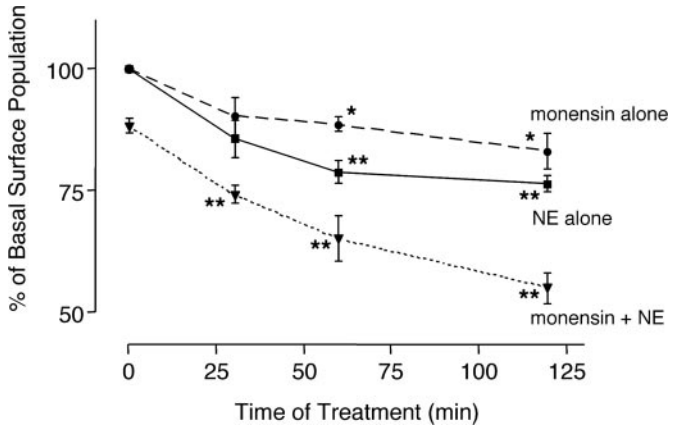

Fig. 7. Effect of treatment with receptor recycling inhibitor monensin on HA-α_{1a}EGFP trafficking. Cells expressing HA-α_{1a}EGFP were treated with 50 nM monensin (●), 10^{-5}M NE (■), or both (▼) for various intervals. Cells treated with both agents were incubated with monensin for 60 min before the addition of NE. After drug treatment, cells were processed for receptor quantitation by flow cytometry. Data are mean ± S.E.M. of 3 to 4 independent experiments. Cell surface populations of HA-α_{1a}EGFP at the time points indicated are significantly different from cells at time 0. *, $p < 0.05$ and **, $p < 0.01$.

ceptor (red) with internal pools of HA-α_{1a}EGFP (green), the composite images are presented in which colocalization appears yellow. Compared with control cells, sucrose treated cells show a clear decrease in constitutive internalization, because the cell interior remains green and free of perinuclear yellow punctates (Fig. 9). Some yellow punctates are present even in sucrose-treated cells, but these are smaller and remain close to the cell periphery, suggesting that early HA-α_{1a}EGFP entry occurs in this region. When prelabeled cells are concurrently treated with 10^{-5}M NE and hyperosmotic sucrose, agonist-induced internalization of HA-α_{1a}EGFP is similarly inhibited (data not shown). Flow cytometry measurements confirm the hyperosmotic block of NE-induced internalization (supplemental Fig. 6). These data suggest that both constitutively cycling and agonist-activated receptors are trafficked through clathrin-coated pits into endosomes and visually confirm that prolonged agonist stimulation of α_{1a}ARs does not target these receptors to lysosomes for degradation.

Discussion

We constructed a HA epitope-tagged human α_{1a}AR/EGFP fusion protein to explore agonist-mediated trafficking and regulation of α_{1a}ARs. Similar to results seen with the fusion of green fluorescent protein to the carboxyl terminus of other GPCRs (Barak et al., 1997; Tarasova et al., 1997; Drmota et al., 1998; Milligan, 1999), the addition of EGFP has no apparent effects on salient pharmacological or signaling characteristics of the human α_{1a}AR. As in other systems (Hirasawa et al., 1997; Coge et al., 1999; Chalothorn et al., 2002; Sugawara et al., 2002), visualization of HA-α_{1a}EGFPs stably expressed in rat-1 fibroblasts reveals intracellular receptor-occupied vesicles as well as cell surface expression of this membrane receptor. Cell surface α_{1a}ARs are internalized in response to agonist (but not PMA), achieving a lower steady-state level of cell surface expression after 1 h of agonist exposure with no change in total HA-α_{1a}EGFP expression. Immunolabeling of surface receptors before drug treatment and/or pharmacological manipulation of various intracellular sorting processes led to the unexpected discovery of agonist-independent constitutive internalization of α_{1a}ARs. Thus, basal surface expression of α_{1a}ARs seems to be controlled by a dynamic agonist-independent equilibrium of receptors that are constitutively internalized and returned to (or replaced at) the cell surface.

Evidence for constitutive, agonist-independent trafficking of α_{1a}ARs is strongly supported by several lines of data. First, a substantial intracellular population of α_{1a}AR-containing vesicles exists in unstimulated native smooth muscle cells (Hrometz et al., 1999; Mackenzie et al., 2000) and in transfected rat-1 fibroblasts, suggesting a population of α_{1a}ARs in motion under basal conditions. Second, and most striking, a significant portion of immunolabeled cell surface α_{1a}ARs are internalized in the absence of receptor stimulation. Third, the endosome recycling inhibitor monoesin caused a significant drop in basal surface receptor density, suggesting that surface density is con-

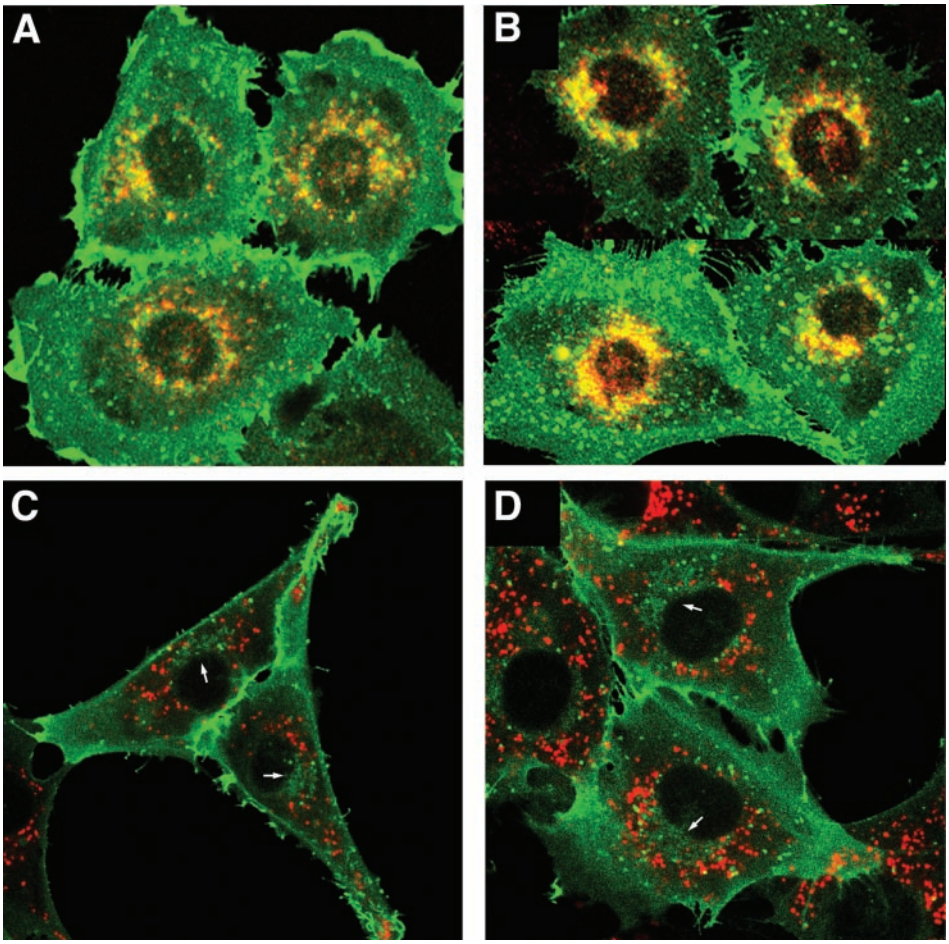

Fig. 8. HA-α_{1a}EGFP colocalizes with the endosomal marker transferrin but not with the lysosomal marker dextran. A, rat-1 fibroblasts stably expressing HA-α_{1a}EGFP prebound to Texas Red-labeled transferrin for 15 min on ice and then incubated in media for 5 min at 37°C before fixation and confocal imaging. B, cells incubated in media containing Texas Red-labeled transferrin for 1h at 37°C before fixation and confocal imaging. Colocalization of green HA-α_{1a}EGFP and Texas Red-transferrin is revealed as yellow. To label lysosomes, cells were incubated with Texas Red-labeled dextran for 24 h and treated with 10^{-5}M NE for 0 (C) or 24 h (D). The absence of colocalization of dextran (red) with HA-α_{1a}EGFP (green) containing punctates (some indicated with white arrows) is apparent even after the extended agonist treatment.

tinuously maintained at least in part by trafficking from a Golgi-associated pool of receptors. Fourth, prazosin significantly increased surface receptor density in unstimulated cells, suggesting that continuous internalization is occurring and has been partially blocked by this inverse agonist. Fifth, constitutive internalization of the α_{1b}AR does not occur, suggesting that constitutive internalization or a lack thereof is responsible for the presence of α_{1a}AR intracellular pools and the absence of α_{1b}AR intracellularly.

Constitutive trafficking is observed for numerous transport receptors (e.g., transferrin) (Stein and Sussman, 1986), low-density lipoprotein receptors, and some tyrosine kinase receptors (e.g., epidermal growth factor and insulin receptors). Although not as well characterized, there are also native and mutant GPCRs that seem to display this characteristic. For example, a truncated μ-opioid receptor that constitutively internalizes (Segredo et al., 1997) and a natural splice variant with an apparent constitutive internalization motif (Koch et al., 1998) have been identified. Multiple lines of evidence suggest that the protease-activated receptor 1 displays activation-independent internalization and recycling (Shapiro and Coughlin, 1998). In addition, a chemokine GPCR homolog (US28) constitutively internalizes (Mokros et al., 2002), as does the cholecystokinin receptor in at least one cell type (Tarasova et al., 1997). Within the adrenergic receptor family, a report by McCune et al. (2000) strongly suggests that the α_{1d}AR constitutively internalizes. In contrast, a surface receptor immunolabeling procedure similar to ours has been used to show that the predominantly intracellular α_{2c}AR does not display constitutive internalization (Daunt et al., 1997). Current data on constitutive internalization of GPCRs suggest the phenomenon is infrequent; however, published information is available on only a few receptors.

Theories of GPCR activation and signaling suggest that under basal conditions GPCRs rapidly and continuously isomerize between a predominant inactive conformation (designated R) and a minor activated conformation (designated R*) (Kenakin, 1996; Milligan, 2003). For most GPCRs, internalization is believed to be dependent on agonist binding and stabilization of the activated (R*) receptor (Szekeres et al., 1998; Ferguson, 2001). However, if the activated conformation of the receptor is sufficient to cause internalization, then the small portion of receptors in the active conformation under basal conditions could be internalized even in the absence of agonist. In this case, inverse agonists such as prazosin would be predicted to block internalization by preventing basal receptor activation. Not only has this been found to be the case for several GPCRs (Tarasova et al., 1997; Miserey-Lenkei et al., 2002) but prazosin itself has been shown to block constitutive internalization of the α_{1d}AR, leading to a clear redistribution of receptor from intracellular pools to the cell surface (McCune et al., 2000). In qualitative agreement, we find that exposure of the α_{1a}AR to prazosin does increase cell surface receptor density; however, it does not dramatically block constitutive internalization nor significantly alter receptor distribution as visualized by confocal microscopy. Thus, even though prazosin completely blocks agonist-induced inositol phosphate activity (Price et al., 2002), receptor phosphorylation (Price et al., 2002), and internalization (described above), it fails to prevent most α_{1a}AR constitutive internalization, suggesting that the α_{1a}AR has some capacity to constitutively internalize while in the inactive conformation (R). This is also implied by the fact that prazosin does not cause an obvious redistribution of the α_{1a}AR to the surface, as occurs so dramatically for the α_{1d}AR (McCune et al., 2000). Thus, our evidence suggests that constitutive internalization of the α_{1a}AR depends in part upon a mechanism that is independent of activation. We are aware of only one other example of activation-independent internalization; this example involves the cholecystokinin receptor, for which an antagonist has been identified that causes internalization without activation (Roettger et al., 1997). Another example of decoupling is observed during stimulation of the opiate receptor with morphine (but not peptide agonists), which causes activation without internalization (Keith et al., 1996).

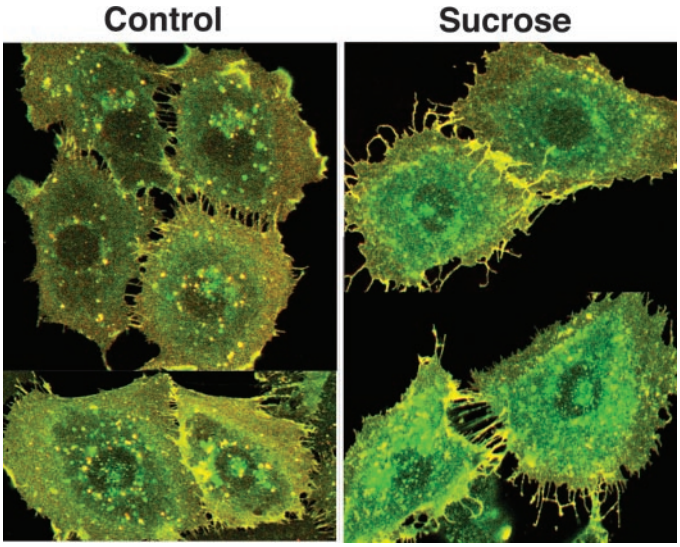

Fig. 9. Sucrose-induced hyperosmotic shock blocks HA-α_{1a}EGFP internalization. Cells expressing HA-α_{1a}EGFP were prelabeled on ice with 3F10 before incubation at 37°C in media without (Control) or with 0.45 M sucrose (Sucrose) for 60 min. Cells were subsequently fixed, permeabilized, and stained with Texas Red-conjugated goat anti-rat IgG.

In contrast to the unusual activation-independent recycling of the α_{1a}AR, agonist (activation)-induced internalization seems to follow current paradigms for many GPCRs (Price et al., 2002). Internalization of the α_{1a}AR occurs after agonist-induced receptor phosphorylation. As for many GPCRs, phosphorylation and desensitization of this receptor involve the G protein-coupled receptor kinase 2. Although the phosphorylation sites and/or sequence elements used in agonist-induced internalization of α_{1a}ARs remain to be identified, they are not found within the intracellular carboxy terminus of the receptor, because the elimination of this region does not prevent internalization or, for that matter, desensitization of the receptor (Price et al., 2002). Although our data strongly suggest that the α_{1a}AR is not subject to agonist-induced degradation or trafficking to lysosomes, it has been reported that the α_{1a}AR is down-regulated 60% after 6 h (Wise et al., 1995) or 30% after 3 h (Yang et al., 1999) of phosphatidylethanolamine exposure. The reason for these discrepancies remains unclear, but it is worth noting that the later report found no decrease in α_{1a}AR levels from 3 to 24 h.

Despite evidence of alternative mechanisms, both constitutive and agonist-induced internalization of the α_{1a}AR appar-

ently share at least some common functional pathways. Both internalization pathways probably involve clathrin-coated pits, because hyperosmotic sucrose blocks internalization. This is supported by the fact that HA-α_{1a}EGFP colocalizes with the transferrin receptor in both agonist-treated and unstimulated cells, because this receptor is trafficked through clathrin-coated pits. Indeed, the behavior of the α_{1a}AR is remarkably similar to that of the transferrin receptor (Stein and Sussman, 1986). In addition to the common use of clathrin-coated pits, both receptors display constitutive and agonist-induced internalization, and in each case constitutive trafficking can be partially blocked by the Golgi-disturbing agent monensin. This implies not only continuous recycling through perinuclear endosomes but also that each receptor uses a second trafficking system. In the case of the transferrin receptor and perhaps the α_{1a}AR, this second system uses rapid recycling from early endosomes (Rosenfeld et al., 2002). On the other hand, several recent reports suggest that internalization via clathrin-coated vesicles may be a more diverse set of processes than has been recognized (Conner and Schmid, 2003; Motley et al., 2003), suggesting that trafficking pathways need not be identical. Much more work will have to be done to elucidate the trafficking systems involved in α_{1a}AR internalization as well as the molecular details of this process.

In addition, the physiological consequence of continuous α_{1a}AR recycling remains to be investigated. It seems clear that a continuous recycling process could prevent complete desensitization of the α_{1a}AR by continually replenishing the supply of α_{1a}AR on the cell surface that can be activated. More generally, continuous α_{1a}AR recycling will almost certainly have functional consequences for resistance vessels and other organs (e.g., heart and prostate) in which this receptor plays important physiologic and regulatory roles and may provide key insights into associated human diseases.

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