Downregulation of the vasopressin type 2 receptor after vasopressin-induced internalization: involvement of a lysosomal degradation pathway

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Downregulation of the vasopressin type 2 receptor after vasopressin-induced internalization: involvement of a lysosomal degradation pathway. Am J Physiol Cell Physiol 288: C1390–C1401, 2005. First published January 26, 2005; doi:10.1152/ajpcell.00353.2004.—Vasopressin (VP) increases urinary concentration by signaling through the vasopressin receptor (V2R) in collecting duct principal cells. After downregulation, V2R reappears at the cell surface via an unusually slow (“recycling”) pathway. To examine this pathway, we expressed V2R-green fluorescent protein (GFP) in LLC-PK1a cells. V2R-GFP showed characteristics similar to those of wild-type V2R, including high affinity for VP and adenylyl cyclase stimulation. V2R-GFP was located mainly in the plasma membrane in unstimulated cells, but it colocalized with the lysosomal marker Lysotracker after VP-induced internalization. Western blot analysis of V2R-GFP showed a broad 57- to 68-kDa band and a doublet at 46 and 52 kDa before VP treatment. After 4-h VP exposure, the 57- to 68-kDa band lost 50% of its intensity, whereas the lower 46-kDa band increased by 200%. The lysosomal inhibitor chloroquine abolished this VP effect, whereas lactacystin, a proteasome inhibitor, had no effect. Incubating cells at 20°C to block trafficking from the trans-Golgi network reduced V2R membrane fluorescence, and a perinuclear patch developed. Cycloheximide reduced the intensity of this patch, showing that newly synthesized V2R-GFP contributed significantly to its appearance. Cycloheximide also inhibited the reappearance of cell surface V2R after downregulation. We conclude that after downregulation, V2R-GFP is delivered to lysosomes for degradation (13, 14, 20). Furthermore, the β-opioid receptor appears to be degraded by the proteasome (54).

The V2R is a “slow recycling” GPCR that regulates water reabsorption by renal collecting duct epithelial cells. V2R is phosphorylated upon activation by vasopressin (VP) binding. Activated V2R then binds to β-arrestin, and the complex is internalized via clathrin-mediated endocytosis (6, 41). Unlike the β2AR, internalized V2R fails to recycle rapidly and V2R forms a stable complex with β-arrestin throughout the internalization pathway (23, 40). Inamorari et al. (26) showed that prolonged association of β-arrestin with the V2R could be responsible for intracellular retention but not the final destination of the receptor, in contrast to the idea that stable binding of β-arrestin directs internalized receptors to lysosomes (8). Although mechanisms involving agonist-induced GPCR endocytosis have been characterized extensively, less is known about the intracellular pathways and proteins involved in fast and slow GPCR recycling. An earlier study (34) showed that the VP ligand is delivered to lysosomes after V2R binding, as are many other ligands that are internalized by receptor-
mediated endocytosis; but the fate of the actual V2R was not followed in that earlier report.

Therefore, to study the endocytosis and recycling pathways followed by the V2R itself, we established stably transfected LLC-PK1a epithelial cell lines expressing a V2R-green fluorescent protein (V2R-GFP) chimera. Our immunofluorescence, biochemical, and ligand binding data show that much of the V2R that is internalized after VP addition to cells enters a lysosomal degradation compartment. The reestablishment of baseline levels of VP binding sites (V2R) at the cell surface requires de novo protein synthesis, providing a partial explanation for the slow recycling pathway previously reported for this receptor.

MATERIALS AND METHODS

Reagents. Unless otherwise stated, all chemicals were purchased from Sigma (St. Louis, MO), and all cell culture reagents were obtained from Gibco-BRL (Grand Island, NY). Primary antibodies were obtained from BD Transduction Laboratories (San Diego, CA), and secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA).

Construction of wild-type V2R-GFP. GFP was attached to the carboxy terminus of the V2R. The TGA stop codon after the carboxy-terminal serine was replaced using site-directed mutagenesis with an in-frame CGG sequence. The entire V2R-containing cassette was subcloned into the 5'-XhoI and 3'-BamHI sites of the pEGFP-N1 vector (Clontech, Palo Alto, CA). The fidelity of the construct was confirmed by performing sequence analysis at the Massachusetts General Hospital Core DNA Sequencing Facility.

Cell culture and transfection. LLC-PK1a cells, a variant of the native LLC-PK1 cell line, which expresses only very low levels of endogenous V2R (6), were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). This cell line was provided by Dr. Steven Krane (Arthritis Unit, Massachusetts General Hospital, Boston, MA). To obtain stable cell lines expressing wild-type V2R-GFP (LLC-V2R-GFP) or GFP alone (LLC-GFP), LLC-PK1a cells were plated at a density of 150,000 cells/60-mm dish 20 h before transfection. For transfection, Lipofectamine (15 μl) with 4 μg of plasmid DNA was added to the cells, incubated at 37°C for 4 h, and washed once with serum-free DMEM. After 14–20 days of selection in medium containing 1 mg/ml geneticin (G418), resistant colonies were isolated with cloning rings and transferred to separate culture dishes for expansion and analysis of their [3H]-VP binding abilities. Several clones were isolated, and their [3H]-VP binding activities were characterized. All clones shared similar characteristics in terms of V2R biology, although the absolute number of receptors expressed differed among the different clones.

[3H]-VP binding to LLC-V2R-GFP cells. [3H]-VP binding assays were performed in 48-well plates. LLC-V2R-GFP cells were plated at a density of 30,000 cells 48 h before the binding assay. Briefly, 0.25 ml of ice-chilled phosphate-buffered saline (PBS), pH 7.4, containing 0.9 mM CaCl2, 0.9 mM MgCl2, 3.5 mM KCl, 1 mg/ml glucose, 1 mM tyrosine, 1 mM phenylalanine, and 0.5% BSA containing the appropriate ligand site for VP (1 μM) was diluted in DMEM. After ligand removal by three acid washes (in mM: 50 sodium citrate, 0.2 NaH2PO4, and 90 NaCl, pH 5), the pH was neutralized by three washes with cold PBS, pH 7.4, and then the cold medium was replaced by warmed cell culture medium (DMEM supplemented with 10% heat-inactivated FBS). Cells were incubated for different times at 37°C before binding assays as described above. Briefly, after the recovery incubation, cells were incubated for 3 h at 4°C with [3H]-VP (9 nM) on the basolateral side. Nonspecific binding was determined in the presence of 1 μM unlabeled VP. Incubations were stopped by rinses with ice-cold PBS at pH 7.4. The bound radioactivity in solubilized cells in NaOH (0.1 N) was determined using a liquid scintillation analyzer.

CAMP assays. Briefly, LLC-V2R-GFP cells were grown in 96-well plates until confluence was reached. The cells were pretreated for 15 min with the phosphodiesterase inhibitor IBMX (1 μM), followed by incubation with different concentrations of VP for 10 min at 37°C. The intracellular levels of cAMP were measured with the BioTrak kit (Amersham Pharmacia Biotech, Piscataway, NJ) as previously described (5). Each intracellular cAMP assay was performed in triplicate.

Immunofluorescence. LLC-V2R-GFP cells were plated on 12 × 12-mm glass coverslips (Fisher Scientific, Pittsburgh, PA). The cells were incubated with or without VP (1 μM) at either 37°C or 20°C for either 1 or 4 h, respectively. Most of the experiments were performed in duplicate with or without cycloheximide (10 μg/ml) present in the medium to determine the potential contribution of newly synthesized vs. recycling V2R-GFP. After treatment, cells were fixed in PBS containing 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA), and 5% sucrose for 20 min at room temperature. The cells were washed three times in PBS and then used for immunocytochemistry.

Immunocytochemistry. To examine the recovery of cell surface V2R-GFP fluorescence on LLC-V2R-GFP cells, the cells were incubated for 1 h at 37°C in the presence or absence of VP (1 μM) diluted in DMEM. After incubation, the cells were washed three times with a solution containing (in mM) 50 sodium citrate, 0.2 NaH2PO4, and 90 NaCl, pH 5, and then washed three more times with cold PBS, pH 7.4. The cold medium was replaced by warmed cell culture medium and incubated for 7 h at 37°C before being fixed as described above and used for immunocytochemistry.

Immunocytochemistry was performed using several antibodies that recognize different intracellular compartments. Primary antibodies were applied to cells permeabilized with 1% SDS for 4 min at room temperature as an antigen retrieval step (11). Golgi cisternae and associated vesicles were identified using an anti-β-subunit of coat protein coatamer (anti-β-COP) antibody (0.01 μg/ml; Sigma), and the trans-Golgi network (TGN) was labeled using either an anti-clathrin antibody (5 μg/ml) or an anti-P230 (a TGN protein marker) antibody (5 μg/ml). A secondary antibody, Cy3-conjugated donkey anti-mouse (1.5 μg/ml), was applied for 1 h at room temperature. Coverslips were mounted on slides with Vectashield medium (Vector Laboratories, Burlingame, CA). Localization of both GFP fusion proteins and compartments marked by antibodies were visualized using a Bio-Rad Radiance 2000 confocal microscope.

In addition to these antibodies, we also used a fluorescent tracer to study the intracellular localization of V2R-GFP. Cells were preincubated for 30 min with Lysotracker (500 nM; Molecular Probes, Eugene, OR), a lysosomal marker, before the VP or cold treatment. Vesicles containing GFP-V2R were then compared with the distribution of Lysotracker-labeled vesicles. After incubation, the cells were fixed and visualized as described above.
The effect of cycloheximide on another protein trafficking exocytosis pathway was also examined using LLC-AQP2 cells, an LLC-PK1 cell line that stably expresses c-myc-tagged aquaporin 2 (28). These cells were incubated for 6 h in the presence or absence of cycloheximide (10 µg/ml). After incubation, VP (1 µM) and forskolin (10 µM) were added to the incubation medium for 10 min to induce AQP2 plasma membrane expression as previously described (28). Treated cells were fixed and stained with a monoclonal anti-c-myc antibody as previously reported (5).

Fluorescence intensity of the perinuclear path that appeared after incubation at 20°C was quantified using IP Lab Spectrum software (Scanalytics, Vienna, VA) on fluorescence microscopic images. The patch was outlined using the freehand drawing tool available as part of the software package, and the average pixel intensity of the resulting region of interest was obtained for each cell. The mean pixel intensity is the average of values from 30 different cells in each condition. This quantification is representative of at least of three independent experiments. Statistical analyses were performed using the unpaired or paired Student’s t-test when applicable. Difference were considered significant at P < 0.05.

Protein extraction. Confluent LLC-V2R-GFP and LLC-GFP cells were incubated at 37°C for 6 h in the absence or presence of VP (1 µM) with or without preexposure for 30 min to the protein synthesis blocker cycloheximide (10 µg/ml); a lysosomal protein degradation inhibitor, chloroquine (10 µM); or lactacystin (6 µM), a proteasome inhibitor. After treatment, cells were lysed for 20 min at 4°C in radioimmunoprecipitation assay (RIPA) buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS supplemented with protease inhibitors (Roche, Mannheim, Germany). Protein concentrations were measured by performing a bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL) before Western blot analysis.

Endoglycosidase digestion of solubilized material. Proteins from either LLC-V2R-GFP or LLC-GFP cells were extracted in RIPA buffer as described above. Solubilized proteins (650 µg) were incubated in the presence of either N-glycosidase F (PNGase F, 25 U; New England Biolabs, Boston, MA) or a mixture containing both neuraminidase (10 mU) and O-glycosidase (0.5 mU) (Roche, Indianapolis, IN). Some solubilized protein aliquots were incubated simultaneously with all enzymes. Endoglycosidase digestion was performed at 37°C for 24 h in a final volume of 250 µl. It was terminated by addition of denaturing buffer and incubation at 70°C for 10 min. The resulting material was immediately analyzed using SDS-PAGE.

SDS-PAGE and Western blot analysis. Protein samples were separated using 12% Bis-Tris-PAGE (Invitrogen, Carlsbad, CA) and then transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA), followed by Western blot analysis. Membranes were blocked by incubation overnight with blotting solution (PBS, pH 7.4, 0.05% Tween 20, and 5% nonfat dry milk). Membranes were first incubated for 1 h with a polyclonal rabbit anti-GFP antibody (0.4 µg/ml; Molecular Probes), then with Amdex goat anti-rabbit IgG-horseradish peroxidase (1:100,000 dilution; Amersham, Little Chalfont, UK). Signals were detected using the protocol described in the Western Lightning Chemiluminescence Reagent Plus system (PerkinElmer Life Sciences, Boston, MA). Identified protein bands were quantified using a video densitometer and Kodak 1D software (Kodak, New Haven, CT).

RESULTS

[3H]-VP binding and adenyl cyclase activity. The agonist binding properties of LLC-V2R-GFP cells were studied using increasing amounts of [3H]-VP. Saturation binding assays showed that specific binding sites were progressively occupied at increasing concentrations of the tritiated, labeled radioligand, reaching a maximal binding capacity of 174,000 ± 14,000 sites/cell. Scatchard analysis of the binding data revealed only one class of [3H]-VP binding site in LLC-V2R-GFP cells, with a Kd of 4.8 ± 0.3 nM (n = 3). The EC50 for cAMP stimulation was 0.10 ± 0.08 nM VP (n = 3; mean ± SE), and a maximum cAMP level of 17 ± 3 pmol/10^6 cells was stimulated by 1 µM VP. The number of [3H]-VP binding sites was reduced by 68 ± 3.8% (n = 3; mean ± SE) when cells were exposed to 1 µM VP for 1 h, and <20% of the lost binding sites were recovered within 2 h after the agonist was removed (see Fig. 10). Binding assays on polarized LLC-V2R-GFP cells showed an apical-to-basolateral distribution of [3H]-VP binding sites (33 ± 3% vs. 67 ± 3%, respectively) similar to that observed in nontransfected LLC-PK1 cells (6). These results show that the V2R-GFP fusion protein behaves similarly to the wild-type V2R with respect to VP binding, down-regulation, and signaling in LLC-PK1 cells.

Localization of V2R-GFP in LLC-PK1a cells: VP treatment induces internalization and delivery to lysosomes. The localization of V2R-GFP in LLC-PK1a cells was observed directly using confocal microscopy. In the steady state, V2R-GFP was found in the plasma membrane and some intracellular vesicles in the perinuclear region also contained the V2R-GFP protein (Fig. 1A). V2R-GFP distribution changed dramatically after the addition of VP to the cells at 37°C. V2R-GFP disappeared from the membrane and was almost completely internalized into numerous intracellular vesicles that were concentrated in the perinuclear region within 1 h after hormone addition (Fig. 1B). To examine the involvement of the lysosomal compartment in V2R-GFP trafficking, cells were incubated with Ly-
sootracker, a vital lysosomal marker, for up to 4 h at 37°C with and without VP treatment. Under baseline conditions, V2R-GFP was located predominantly at the cell surface, whereas Lysotracker was located in large vesicles in the cytoplasm (Fig. 2, A–C). In the presence of VP, V2R-GFP and Lysotracker fluorescence overlapped in many intracellular structures after VP addition (Fig. 2, D–F). At this 2-h time point, most of the V2R-GFP had been internalized and many of the Lysotracker-positive structures within the cell also contained V2R-GFP fluorescence as shown by the yellow color in the merged image (Fig. 2F).

VP modifies the V2R-GFP band pattern observed using Western blot analysis, and this effect is inhibited by a lysosomal protease inhibitor, chloroquine. Because the localization data strongly suggested that V2R-GFP was delivered to lysosomes after VP binding, we examined the biochemical characteristics of the chimeric receptor protein by performing Western blot analysis to detect its possible fragmentation and/or degradation. LLC-V2R-GFP cells were incubated with VP (1 μM) for 4 h and examined using Western blot analysis. Anti-GFP antibody revealed several bands in the LLC-V2R-GFP cell lysates that were not detected in LLC-GFP cells that expressed a soluble GFP, a 31-kDa protein (Fig. 3A, lane 7), confirming the specificity of the antibody for GFP and showing that the bands detected in the LLC-V2R-GFP cells were due to the presence of the chimeric V2R-GFP receptor protein. In 30 separate experiments, several protein bands were detected in LLC-V2R-GFP cells in the absence of VP (time 0), including a broad smear between 57 and 68 kDa as well as two distinct lower bands at 52 and 46 kDa (Fig. 3A, lane 1). In the presence of VP, the intensity of the higher molecular mass bands was greatly reduced, while the intensity of the lower molecular mass, 46-kDa band was greatly increased (Fig. 3A, lane 2). The change in band intensity was time dependent (data not shown). After 4 h, the density of the 57- to 68-kDa bands was reduced, on average, by 53 ± 6%, while that of the 46-kDa band was

![Fig. 2. Effect of VP treatment on Lysotracker and V2R-GFP distribution in LLC-V2R-GFP cells. At 37°C, Lysotracker, a lysosome marker, is located in large vesicles in the cytoplasm (A; red), and V2R-GFP is located mainly at the cell surface (B; green). The merged image (C) shows little or no overlap in labeling. In the presence of VP (1 μM) at 37°C for 2 h, Lysotracker staining also appears in vesicles (D; red), many of which contain V2R-GFP (E, green; F, yellow). These images are representative of six independent experiments. Bar, 5 μm.](http://ajpcell.physiology.org/ by 10.220.33.1 on June 21, 2017)
increased by $197 \pm 27\%$ ($n = 6$; mean $\pm$ SE). β-actin antibody staining was used as a loading control (Fig. 3A, bottom).

This modification in the band intensities after VP exposure also was observed when cells were incubated with cycloheximide to block de novo protein synthesis (Fig. 3A, lanes 5 and 6), suggesting that modification of the band intensities did not involve newly synthesized receptors but reflected degradation of existing protein. Newly synthesized receptors were associated with the 57 to 68 kDa bands. The residual bands observed in the region of the gel after 24 h of incubation in the presence of VP (Fig. 3A, lane 4) disappeared completely in the presence of VP and cycloheximide (Fig. 3A, lane 6). This result suggests a downregulation of the total cellular complement of V2R-GFP as expected in a pathway leading to a lysosomal degradation compartment. The upper bands shifted down to 57 kDa ($n = 11$) when digested with PNGase F (Fig. 3B, lane 1 vs. lane 3) and showed a smaller and variable shift to 61 kDa in the presence of neuraminidase and O-deglycosidase ($n = 4$) (Fig. 3B, lane 4). Digestion in the presence of all three enzymes produced a strong deglycosylated band of 53 kDa, but the lower 46-kDa band of the doublet was not affected. After exposure of extracts from VP-treated cells to PNGase F, the 57-kDa deglycosylated band was reduced in intensity, and the lower molecular mass 46-kDa band was increased. Similar results were obtained with all combinations of deglycosylation enzymes (Fig. 3B, lanes 7–9). There was no change in the band pattern of GFP itself when digested with the three glycosidases (Fig. 3B, lanes 10 and 11). These results suggest that modification of the band pattern in the presence of VP is not explained by a change in the glycosylation state of the affected bands. Deglycosylation of the V2R does result in a downshifting of the major higher molecular mass bands, as expected, but the disappearance of these bands and the parallel increase in intensity of the lower molecular mass 43-kDa band is dependent on VP treatment. After VP treatment, an increase in the intensity of the 53-kDa band was also observed, but only in the absence of PNGase F. This finding implies that the 53-kDa band that increased after deglycosylation alone contained not only deglycosylated V2R but also a degradation fragment after VP treatment of cells. After N-deglycosylation of the VP-treated extracts, an increase in the 53-kDa fragment was no longer detectable, presumably because of a marked shift in its molecular mass as a result of glycosylation (Fig. 3B, lanes 7 and 9), whereas the 46-kDa fragment still increased in intensity.

Two protein degradation inhibitors were incubated with VP to study this degradation hypothesis further. Incubation with lactacystin, a proteasome degradation inhibitor, showed no inhibitory effect on V2R-GFP degradation in VP-stimulated cells (Fig. 4, lanes 3 and 4). In contrast, inhibition of V2R-GFP degradation was observed in the presence of chloroquine, a lysosomal degradation inhibitor (Fig. 4, lanes 5 and 6). Den-sitometric analysis showed that in the presence of both VP and lactacystin or with VP alone, the 57- to 68-kDa upper band was reduced by 78 ± 8% ($n = 3$; mean ± SE). No reduction in band intensity was detectable when VP was added together with chloroquine ($n = 3$). These data indicate that receptor degradation that occurred during VP stimulation, represented by the relative change in the 57- to 68-kDa vs. 46-kDa band intensities, was abolished by lysosomal degradation inhibitors.
Dissection of the V2R-GFP trafficking pathway using a 20°C temperature block: accumulation of V2R-GFP in the TGN at low temperature. Previous data, including those from our laboratory (21, 38), have shown that exposing cells to 20°C for 1–4 h results in a progressive block in the intracellular protein trafficking at the level of the TGN, resulting in the appearance of a bright perinuclear patch when specific antibodies are applied to cells or when GFP-tagged proteins are monitored. This procedure can be used to estimate the relative amount of newly synthesized vs. recycling protein trafficked through the TGN for ultimate delivery to the cell surface. After 4 h of incubation at 20°C without VP stimulation, plasma membrane V2R-GFP fluorescence was reduced but still present, and, in addition, a dense accumulation of V2R-GFP was observed as a bright patch in the perinuclear area compared with cells incubated at 37°C (Fig. 5A). The brightness and size of the perinuclear patch increased greatly after VP treatment in parallel with the almost complete disappearance of V2R-GFP from the plasma membrane (Fig. 5B), which is similar to observations in cells incubated at 37°C (Fig. 1). Densitometric analysis showed that the perinuclear patch of V2R-GFP in cells exposed to VP at 20°C was 2.4 ± 0.5 times larger and 34 ± 6% brighter than patches in cells incubated at 20°C only (mean ± SE; n = 4) (P < 0.05).

Newly synthesized V2R-GFP contributes to perinuclear patch formation at 20°C. The difference in the size and intensity of the perinuclear patches formed at 20°C in the presence or absence of VP suggests that both internalized and newly synthesized V2R-GFP contribute to the formation of the fluorescent patch. The patch is larger in the presence of VP because it contains V2R that is internalized after ligand binding in addition to newly synthesized V2R that is in transit through the TGN before being packaged into export vesicles. Cycloheximide, a protein synthesis inhibitor, was used to study the contribution of newly synthesized receptor to patch formation (Fig. 6). LLC-V2R-GFP cells were incubated at 20°C for 3 and 5 h (Fig. 6, B and C, respectively). Cycloheximide pretreatment of the cells had no significant effect on baseline V2R-GFP fluorescence in the presence or absence of VP (Fig. 6, H and I). This result supports the idea that newly synthesized V2R-GFP contributed to patch formation. In the presence of VP, V2R is internalized into the perinuclear patch and the brightness of the spot in cycloheximide-treated cells was correspondingly increased compared with control cells not treated with VP (Fig. 6, H and I).

The effect of cycloheximide on V2R-GFP patch formation in cold-treated LLC-V2R-GFP cells was quantified using densitometric analysis (Fig. 7). The total area occupied by V2R-GFP reflects the intracellular distribution and/or spread of the protein. Concentration of V2R-GFP into the perinuclear patch is reflected by a decrease in the total area in which fluorescence is detected. During cold treatment, the intracellular area containing the perinuclear fluorescence signal decreased during a period of time from ~600 μm² to reach a steady state of ~200 μm² in control cells and 300 μm² with cycloheximide, a difference that was not significant between the control and cycloheximide-treated cells. The pixel intensity (brightness) of the patch gradually increased over time to a maximum value after 3 h of cold treatment (Fig. 7). This gradual increase in fluorescence intensity was completely inhibited by cycloheximide, suggesting that newly synthesized V2R-GFP is mainly responsible for the augmentation of the fluorescence intensity in the perinuclear patch in the absence of VP treatment.

Colocalization of V2R-GFP and different intracellular compartment markers in cells incubated at 20°C. V2R-GFP is located in both the plasma membrane and the perinuclear region as a dense patch after incubation of cells at low temperature (Fig. 5). The distribution of perinuclear V2R-GFP partially overlapped with clathrin and P230, which are both localized in the TGN (Fig. 8, A and B, respectively). In contrast, less overlap (indicated by less yellow costaining) was observed between V2R-GFP and β-COP, a Golgi cisterna- and Golgi vesicle-associated marker (Fig. 8C). After 4 h at low temperature, Lysotracker was localized mainly in the perinuclear body (Fig. 8D).

Fig. 4. Western blot analysis using anti-GFP antibodies showing V2R-GFP degradation in lysosomes at 37°C. LLC-V2R-GFP cells were incubated for 6 h in the presence or absence of 1 μM VP. A predominant 57- to 68-kDa band was considerably reduced in intensity after VP treatment of cells (lanes 1 and 2), whereas a lower molecular mass band at 46 kDa was greatly increased in intensity (lanes 1 and 2). This pattern was identical in cells pretreated with the proteasome inhibitor lactacystin (lanes 3 and 4) but was completely inhibited upon incubation of cells with the lysosomal protease inhibitor chloroquine (lanes 5 and 6).

Fig. 5. Effect of low temperature on V2R-GFP localization in LLC-V2R-GFP cells. After 4-h incubation at 20°C, V2R-GFP was located in the plasma membrane and in a perinuclear patch (arrows in A). In the presence of VP, added at 20°C, the intensity of the perinuclear patch was increased (arrows in B) and the amount of plasma membrane fluorescence was greatly reduced because of ligand-induced receptor internalization. Images are representative of six independent experiments. Bar, 10 μm.
clear area, and V2R-GFP was located both in plasma membrane and in the perinuclear region. The merged image (Fig. 9A) shows that V2R-GFP and Lysotracker staining demonstrated only minimal overlap in the perinuclear region, suggesting that while both lysosomes and vesicles containing V2R-GFP accumulate in the same cellular region at low temperature, V2R-GFP is not preferentially delivered to lysosomes under these incubation conditions, which is in contrast to the situation at 37°C (Fig. 2, D–F). In support of this finding, no loss of the intensity (degradation) of the 57- to 68-kDa V2R-GFP band was detectable when cells were incubated at 20°C to induce the formation of a perinuclear patch, in either the presence or absence of cycloheximide (Fig. 9B; compare with Fig. 4).

Cycloheximide treatment inhibits restoration of plasma membrane V2R levels after downregulation. The data described above indicate that much of the V2R-GFP that is internalized after VP-induced downregulation is degraded in lysosomes and that there is a significant traffic of newly synthesized V2R through the TGN. To test whether the restoration of prestimulation levels of V2R at the cell surface depends on de novo protein synthesis, cell surface VP binding was quantified by performing [3H]-VP binding assays followed by fluorescence microscopy in the presence and absence of cycloheximide (Fig. 9B; compare with Fig. 4).

Cycloheximide treatment inhibits restoration of plasma membrane V2R levels after downregulation. The data described above indicate that much of the V2R-GFP that is internalized after VP-induced downregulation is degraded in lysosomes and that there is a significant traffic of newly synthesized V2R through the TGN. To test whether the restoration of prestimulation levels of V2R at the cell surface depends on de novo protein synthesis, cell surface VP binding was quantified by performing [3H]-VP binding assays followed by fluorescence microscopy in the presence and absence of cycloheximide. As shown in Fig. 10A, VP treatment resulted in the usual disappearance of 80% of the VP binding sites from the cell surface, both in the presence and in the absence of cycloheximide. After VP washout for 2 h, the number of cell surface binding sites had recovered to ~40% of the initial level (representing an additional 20% increase over the post-VP baseline level because only 80% of the VP binding was lost after hormone exposure) in both the presence and

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Fig. 6. Effect of 20°C incubation on V2R-GFP localization in LLC-V2R-GFP cells under different conditions. LLC-V2R-GFP cells were incubated for 0 min (A), 3 h (B), and 5 h (C) at 20°C. At time 0, V2R-GFP was located in the plasma membrane and in vesicles loosely scattered around the nucleus, whereas at longer incubation times, V2R-GFP immunoreactivity appeared as a dense spot adjacent to the nucleus (arrows in B and C) and plasma membrane staining was reduced but not absent. A similar qualitative V2R-GFP distribution was observed when cells were incubated at 20°C for 0 min (D), 3 h (E), or 5 h (F) in the presence of cycloheximide (Cyc; 10 μg/ml). However, the intensity of the perinuclear patch was greatly reduced at 5 h (arrows in E) and 5 h (arrows in F) time points in the absence of protein synthesis compared with the respective controls (B and C). In the presence VP and cycloheximide for 0 min (G), 3 h (H), or 5 h (I), V2R-GFP was internalized and the intracellular vesicles were more loosely scattered around the nucleus (H and I) than observed in cells not treated with VP. Plasma membrane staining was virtually absent after VP exposure. These images are representative of six independent experiments. Bar, 10 μm.

Fig. 7. Quantification of the time course of V2R-GFP translocation to the perinuclear spot of the LLC-V2R-GFP cells after incubation at 20°C in the absence (top trace) or in the presence (bottom trace) of cycloheximide. Fluorescence intensity of the perinuclear patch was quantified using IP Lab Spectrum software with fluorescence microscopic images such as those shown in Fig. 5. The patch was outlined to define a region of interest, and the mean pixel intensity of the patch was measured using the image analysis software. In control cells (top trace), the 20°C incubation resulted in a time-dependent increase in mean pixel intensity within the patch. The increase in intensity was completely inhibited by cycloheximide treatment (bottom trace). This result is representative of three independent experiments, and each point is the average of 50 different cells (means ± SE).

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absence of cycloheximide. Subsequently, however, cell surface binding showed a progressive time-dependent increase under control conditions but no further increase in the presence of cycloheximide. Fluorescence microscopy confirmed only partial recovery of plasma membrane V2R-GFP after 7 h under control conditions; less plasma membrane V2R was detectable after cycloheximide treatment (Fig. 10, Ac vs. Af).

To confirm that other cellular trafficking processes were still intact after several hours of protein synthesis inhibition, we have repeated our previously published result (27) that VP was able to stimulate plasma membrane insertion of AQP2 in LLC-PK1 cells exposed to cycloheximide for 6 h (Fig. 10 C). Furthermore, we treated LLC-V2R-GFP cells for 10 h with cycloheximide after VP treatment and then washed out the drug. After a further 12-h incubation in the absence of VP, plasma membrane levels of the receptor were completely restored to control values (data not shown). These data indicate that cycloheximide treatment for several hours does not irreversibly damage protein trafficking pathways in LLC-PK1 cells.

DISCUSSION

The major conclusion to emerge from these studies is that after VP-induced receptor internalization, much of the internalized V2R is trafficked to lysosomes in LLC-PK1 cells, where it is fragmented or degraded. The cell surface level of V2R returns only partially to its prestimulated value in the presence of cycloheximide, implying a significant contribution of newly synthesized receptors to the restoration process. Thus the slow recycling (several hours) pathway followed by the V2R after ligand binding can be explained mainly by the fact that much of the protein is not actually recycled. Instead, internalized and degraded V2R is gradually replaced by insertion into the cell surface of the newly synthesized receptor in the hours after downregulation. Entry into a lysosomal degradation pathway was previously implicated in the slow recycling of the EGF receptor protein (47).

To dissect the intracellular pathways and reveal fate of the V2R after ligand-induced internalization, a V2R-GFP chimeric construct was stably expressed in LLC-PK1a epithelial cells, a subtype of LLC-PK1 cells that expresses a very low amount of

Fig. 8. Colocalization of V2R-GFP and intracellular compartment markers in LLC-V2R-GFP cells incubated at low temperature (20°C) for 3 h. At 20°C, V2R-GFP (green) was localized in the plasma membrane and in a dense perinuclear patch. A partial overlap in staining was observed between V2R-GFP (green) and both trans-Golgi network (TGN) markers, clathrin (A) and P230 (B). In contrast, much less apparent overlap (yellow color) was observed with the Golgi vesicle and the cisternal marker β-subunit of coat protein coatomer (β-COP) (C). This result is representative of six independent experiments. Bar, 5 μM.

Fig. 9. Localization of V2R-GFP and Lysotracker in cells incubated for 4 h at 20°C. Only a limited amount of overlap can be detected with the lysosomal marker under these low-temperature conditions (A). B: LLC-V2R-GFP cells were incubated for 4 h in the presence or absence of cycloheximide (10 μg/ml) at 37°C (lanes 1 and 2) or at 20°C (lanes 3 and 4). No modification in the 70-kDa band intensity was observed under any conditions. These data indicate that the intracellular, perinuclear accumulation of V2R-GFP that occurred at 20°C did not result in significant delivery to lysosomes and subsequent degradation. This result is representative of three different experiments. Bar, 5 μM.
endogenous V2R (6). The introduction of GFP at the carboxy terminus of V2R produces a fully functional V2R-GFP chimera as found with other GPCRs (8, 17, 50). In contrast, an NH2 terminus GFP-V2R chimera was not inserted into the plasma membrane (data not shown). Western blot analysis using anti-GFP antibodies allowed us to detect protein bands between 57 and 68 kDa corresponding to the chimeric protein, similar to those described in Madin-Darby canine kidney or human embryonic kidney (HEK)-293 cells (3, 22), as well as two additional bands at ~46–52 kDa that were much weaker than the 57- to 68-kDa smear under baseline conditions. The 57- to 68-kDa smear corresponds to the intact N- and O-glycosylated V2R-GFP chimeric protein (4, 24, 44), the band at 52 kDa is a nonglycosylated form of V2R-GFP, and the lower molecular mass 46-kDa band probably represents a nonglycosylated degradation product (see below). This 46-kDa band was detectable at low levels even in the absence of VP stimulation, reflecting a low level of intracellular V2R trafficking and degradation observed under nonstimulated conditions.

The chimeric V2R-GFP binds VP normally, initiates a cAMP signaling response, and internalizes into a perinuclear location after agonist stimulation. The resulting reduction of cell surface [3H]-VP binding sites in LLC-V2R-GFP cells is expected (data not shown) and ultimately becomes predominantly colocalized with the lysosomal marker Lysotracker. In parallel, Western blot analysis data showed that the intensity of the 57- to 68-kDa band representing intact V2R-GFP was considerably reduced 4 h after exposure of cells to VP, while the intensity of the lower molecular mass 46-kDa band was significantly increased. This VP-induced shift in band density was completely prevented by the lysosomal inhibitor chloroquine but was not affected by lactacystin, a proteasome inhibitor. Taken together, these data suggest that after internalization, a significant quantity of V2R is trafficked to lysosomes for degradation. However, the degradation process may be initiated in late endosomes, which also contain proteases and are a site of significant proteolysis (9, 51). Furthermore, a potential contribution of previously described cell surface proteases to this cleavage event cannot be excluded (32).

This interpretation leads to the conclusion that complete restoration of cell surface V2R levels after downregulation would require de novo receptor synthesis. This hypothesis is supported by two distinct but convergent sets of data from our study. When cells were exposed to VP in the presence of cycloheximide to block protein synthesis, the rate of recovery of the cell surface receptor pool was inhibited significantly between 2 and 6 h after VP washout. After VP washout for 2 h, an additional 20% of the prestimulation level of cell surface receptor was restored, despite the presence of cycloheximide. However, no subsequent increase in this level was detected.
after inhibition of protein synthesis, in contrast to untreated cells, in which levels were restored to 75% of the nonstimulated value after 6 h. These data indicate that while the early recovery (20% of total recovery) could be due to recycling V2R or to the membrane insertion of a pool of preexisting intracellular V2R, the bulk (~80%) of the restoration process is due to the insertion of newly synthesized V2R into the cell surface. The more rapidly inserted pool may be important for maintaining at least a partial VP response of collecting duct principal cells under physiological conditions of fluctuating VP levels.

An earlier study in isolated rat collecting ducts concluded that the V2R recycles rapidly, in contrast to several other reports that it is a slow recycler (30). Furthermore, the reappearance of cell surface VP binding sites was not inhibited by cycloheximide in this earlier report (30). However, in addition to the differences in cell type and the use of a V2R-GFP construct in the present study, there are several other methodological differences between the studies that make a direct comparison of the results difficult. First, the exposure to cycloheximide was brief and would not have inhibited the processing and packaging of V2R that had already been synthesized. Approximately 30 min are required for newly synthesized proteins to be transported through post-endoplasmic reticulum compartments, including the Golgi, and for an effect of cycloheximide on membrane protein insertion to become apparent. Thus the packaging and export to the cell surface of this presynthesized pool of V2R could explain the reappearance of cell surface binding sites that were measured in the previous study. The presence of a rapidly inserted pool of the V2R was also detected in the present study in LLC-PK1 cells. Approximately 20% of total cell surface binding sites were restored within 60 min of VP washout, and this reappearance was not inhibited by cycloheximide. Second, the molecular mass and $B_{\text{max}}$ of the collecting duct receptors examined by Kim et al. (30) are similar to those of smooth muscle and hepatocyte vasopressin V$_1$ receptors, which are known to be expressed in collecting duct principal cells (1, 18, 55) and to which the VP ligand would also bind. Cycloheximide has been reported to have no effect on rapid V$_1$ receptor recycling in smooth muscle cells (10) or in hepatocytes (16). Third, the internalization studies of Kim et al. (30) were performed at 24°C. In the present study, we were unable to detect V2R degradation when cells were incubated at 20°C, implying that this process is temperature dependent. Lysosomal degradation of the EGF receptor is also inhibited by incubation at 25°C (48).

The de novo synthesis hypothesis is also supported by our data derived from the use of a 20°C temperature block to dissect the pathway of intracellular V2R trafficking. Under these conditions, V2R-GFP accumulates during a 4-h period in a compact perinuclear patch even in the absence of VP stimulation. This accumulation is significantly reduced by cycloheximide, indicating that de novo protein synthesis is required for its appearance. Previous studies from several laboratories, including our own, have shown that this low-temperature treatment causes a block in the export of proteins from the TGN (27, 38). In the present study, V2R-GFP in the perinuclear patch colocalized with the TGN markers clathrin and P230, but less so with the Golgi vesicle/cisternal marker β-COP. Thus both newly synthesized proteins and any proteins that recycle through the TGN could contribute to the formation of the patch. It is known, for example, that the TGN protein furin recycles between the plasma membrane and the TGN via a recycling endosome compartment, indicating that these two compartments are interconnected at least in some recycling pathways (35, 49). After VP treatment at 20°C, the patch was larger and brighter than it was in the absence of VP, indicating that it contains internalized V2R in addition to newly synthesized V2R-GFP. The level of resolution of our images was not sufficient to determine whether both newly synthesized and internalized V2R were located in the same vesicles within the patch, however. The internalized V2R in the patch showed only a very partial colocalization with Lysotracker and was not degraded under low-temperature conditions, indicating that it had been blocked in a prelysosomal compartment. Whether internalized V2R-GFP is blocked in the TGN or in another closely related, temperature-sensitive compartment such as the recycling endosome is unclear.

The conclusion that the lysosome appears to be the final destination of much of the internalized V2R-GFP contrasts with the results of a previous study showing that internalized receptors remained intact for several hours in a transferrin, Rab11-positive recycling endosome compartment in the perinuclear region of HEK-293 cells (25). The difference in cell types may be one explanation for this difference, but it is also probable that in contrast to V2R-GFP, the degraded V2R was not detectable in the previous study. Furthermore, the amount of degradation product may be proportional to the duration and strength of the agonist incubation, which also differed between the two studies. An earlier report showed that the VP ligand is directed to lysosomes after internalization but that trafficking of the V2R itself was not directly followed (34). The appearance of an internalized fluorescent VP analog in large, lysosome-like intracellular vesicles in collecting duct principal cells has also been described (31), although no definite identification of the vesicles was attempted. Clear identification of the nonlysosomal perinuclear compartment that contains internalized V2R, and newly synthesized V2R is complicated by the dynamic interaction between the numerous vesicular compartments involved in the trafficking of the receptor. Furthermore, many “specific” markers, including Rab11, may be found in more than one compartment (12, 46, 53). Further dissection of the juxtanuclear compartments involved in V2R processing will require extensive double-labeling studies at the electron microscopy (EM) level.

The information for receptor targeting to either recycling or lysosomal degradation pathways may reside in the cytoplasmic tail of the receptor (52). A recent report suggests that the presence of a cluster of serines in the tail of a GPCR appears to mediate stable binding of arrestin and to dictate trafficking of the internalized receptor to the lysosome (40). The hypothesis that arrestin may target GPCRs to lysosomes is also supported by the subcellular localization of β-arrestin in multivesicular bodies (2). Furthermore, VP stimulation leads to rapid, β-arrestin-dependent ubiquitination of the V2R and increased degradation (37). Whether these mechanisms are also involved in the lysosomal targeting of internalized V2R remains to be determined. One question that is unanswered by our present data is the physiological reason for V2R receptor degradation in its slow recycling pathway. It is possible that the association between VP and its receptor has evolved to with-
stand the unusually harsh environment that exists in much of the renal medulla. Not only is the interstitium in which receptor-ligand binding occurs hypertonic with respect to urea and NaCl but also its pH is significantly more acidic than plasma (29). Thus V2R-ligand binding may be at least partially resistant to the pH-induced dissociation that normally occurs for many receptor-ligand pairs in the acidic endosomal compartment (39). After receptor-ligand dissociation, receptors are often recycled back to the plasma membrane, while the ligand is targeted to lysosomes for degradation. If this uncoupling does not occur in the case of the VP-V2R interaction, both may then be subject to lysosomal degradation. Thus the apparently inefficient process of V2R degradation, rather than recycling, that occurs after internalization may be the price that the kidney pays to maintain body fluid homeostasis under the influence of the antidiuretic hormone VP. While the cortical environment of the kidney is more “friendly” with respect to pH and toxicity, the need for the V2R to function in the medulla may have contributed to the evolution of its binding characteristics.

**REFERENCES**


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