Important role for the V-type H^+-ATPase and the Golgi apparatus in the recycling of PTH/PTHrP receptor

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Tawfeek, Hesham A. W., and Abdul B. Abou-Samra. Important role for the V-type H^+-ATPase and the Golgi apparatus in the recycling of PTH/PTHrP receptor. Am J Physiol Endocrinol Metab 286: E704–E710, 2004. First published December 23, 2003; 10.1152/ajpendo.00404.2003.—Our previous studies demonstrated that a green fluorescent protein-tagged parathyroid hormone (PTH)/PTH-related peptide (PTHrP) receptor stably expressed in LLC-PK-1 cells undergoes agonist-dependent internalization into clathrin-coated pits. The subcellular localization of the internalized PTH/PTHrP receptor is not known. In the present study, we explored the intracellular pathways of the internalized PTH/PTHrP receptor. Using immunofluorescence and confocal microscopy, we show that the internalized receptors localize at a juxtanuclear compartment identified as the Golgi apparatus. The receptors do not colocalize with lysosomes. Furthermore, whereas the internalized receptors exhibit rapid recycling, treatment with proton pump inhibitors (bafilomycin-A1 and concanamycin A) or brefeldin A, Golgi disrupting agents, reduces PTH/PTHrP receptor recycling. Together, these data indicate an important role for the V-type hydrogen-ATPase and the Golgi apparatus in postendocytic PTH/PTHrP receptor recovery.

PARATHYROID HORMONE (PTH)/PTH-related peptide (PTHrP) receptor mediates the PTH effects on mineral ion homeostasis and the multiple actions of PTHrP on different tissues in adult and fetus (26, 29, 32). G protein-coupled receptors (GPCRs) undergo agonist-induced endocytosis or internalization. Receptor internalization is an important process for receptor regulation (50, 53). Identifying the postendocytic targeting of the internalized receptor is important for understanding the receptor fate and consequently receptor regulation. Internalized GPCRs follow two classical intracellular pathways; some receptors recycle to the cell membrane, and others are sorted for degradation (50, 53). Two major cell organelles, the lysosomes and the Golgi apparatus, which are involved in receptor degradation and processing, respectively, are potential intracellular destinations for GPCRs. Several GPCRs were shown to be targeted to lysosomes and to undergo lysosomal degradation (3, 30, 45, 49, 51). Other GPCRs were reported to localize, after internalization, into perinuclear recycling endosomes (5, 8, 13, 22, 24, 44). Specific proton pump inhibitors [bafilomycin-A1 (20, 35, 39, 52) and concanamycin A (42, 54)] and brefeldin A (2, 9, 14) are Golgi-disrupting agents and are useful tools for studying the role of the Golgi apparatus in receptor recycling.

The PTH/PTHrP receptor undergoes internalization into clathrin-coated pits (48). Major advances have been made in understanding the mechanisms and the role of PTH/PTHrP receptor internalization in receptor signaling (10, 47, 48). Postendocytic localization and trafficking of the PTH/PTHrP receptor are, however, poorly investigated. The goal of the present study is therefore to explore the postendocytic pathways of a GFP-tagged PTH/PTHrP receptor stably expressed in LLC-PK-1 cells. Using immunofluorescence and confocal microscopy, we demonstrate that the internalized PTH/PTHrP receptor colocalizes with the Golgi apparatus and does not colocalize with lysosomes. The data using bafilomycin-A1, concanamycin A, and brefeldin A suggest that the vacuolar-type hydrogen-ATPase [V-type H^+-ATPase] plays a major role in PTH/PTHrP receptor recycling and that this role is partly through the Golgi apparatus.

MATeRIALS AND METHODS

Materials. [Nle^8,18,Tyr^34]bPTH(1–34)NH2 (PTH) was synthesized by a solid-phase method purified by HPLC and characterized by amino acid hydrolysis, NH2-terminal sequencing, and mass spectrometry (Peptide Synthesis Core, Endocrine Unit, Massachusetts General Hospital, Boston, MA). All chemicals were of the highest grade available and were obtained from either Sigma Chemicals (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). Tissue culture media were prepared by the Massachusetts General Hospital media facility; fetal bovine serum (FBS), streptomycin, and penicillin were from Sigma. Flasks, plates, and other tissue culture supplies were from Corning (Oneonta, NY). Concanamycin A was purchased from Sigma. Bafilomycin-A1, brefeldin A, and cycloheximide were purchased from Biomol Research Laboratories (Butler Pike, Plymouth Meeting, PA). Rabbit anti-sheep IgG was from KPL (Gaithersburg, MD). Radioiodinated goat anti-rabbit IgG was from Perkin Elmer (Boston, MA). Monoclonal anti-cis-Golgi 58K protein antibody (27, 40) and monoclonal anti-lysosomal antibody (6, 17), which recognizes a principal lysosomal membrane protein [lysosomal-associated membrane protein (LAMP-1)], were purchased from Sigma and Transduction Laboratories (Lexington, KY), respectively. Lysotracker red (13) was from Molecular Probes (Eugene, OR). Cy3-labeled goat anti-mouse antiserum was purchased from Amersham (Arlington Heights, IL). Vectashield was from Vector Laboratories (Burlingame, CA).

Cell culture. LLC-PK-1 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS. All media contained 1 μg/ml streptomycin and 100 U/ml penicillin. The cells were incubated in a humidified atmosphere containing 95% air-5% CO2 at 37°C. Media were replaced every other day.

Cell surface receptor quantification. Cell surface expression of the PTH/PTHrP receptor was assessed using the G48 antibody, an anti-rat PTH/PTHrP receptor antiserum (38), as described previously (47). Cells, grown in 24-well plates, were treated as indicated, rinsed (3×) with ice-cold PBS, pH 7.4, and then incubated on ice for 2 h with the G48 antibody.
antibody or nonimmune IgG in PBS/5% heat-inactivated FBS (PBS/FBS). The cells were then rinsed (3×) with PBS and incubated at room temperature for 90 min with a rabbit anti-sheep antibody (Sigma) in PBS/FBS and rinsed (3×) with PBS, and an 125I-labeled goat anti-rabbit IgG (200,000 cpm/well) was then added for another 90 min at room temperature. The incubation was terminated by removing the supernatant and rinsing the cells (3×) with PBS. The cells were solubilized in 1 N NaOH, and the radioactivity was counted.

Confocal microscopy and colocalization studies. Cells seeded on coverslips in six-well plates were grown as a monolayer until they reached 60–80% confluence. Cells were rinsed (2×) with PBS and were then treated with PTH or drugs (as indicated) in serum-free DMEM containing 20 mM HEPES buffer and 0.1% BSA at 37°C in a CO2 incubator. The medium was removed, and the cells were rinsed (2×) with ice-cold PBS and fixed with 4% paraformaldehyde (in PBS) for 15 min at room temperature. Fixed cells were rinsed (3×) with PBS, each rinse lasting 5 min, and then mounted using Vectashield. The coverslips were sealed on the slides with nail polish.

For colocalization experiments, cells were further permeabilized at room temperature with 0.1% SDS (5 min) for Golgi staining or 100% methanol (10 min) for lysosomal staining and then rinsed with PBS (3× 5 min). The permeabilized cells were incubated with the primary antibody (monoclonal anti-Golgi or LAMP-1, diluted 1:100) in PBS for 3 h at room temperature. The unbound antiserum was removed, and the cells were rinsed (3×) with PBS. A secondary Cy3-conjugated goat anti-mouse IgG (1:1,000) in PBS was added for 2 h at room temperature. At the end of the incubation, the cells were rinsed (3×) with PBS, mounted, and sealed.

The confocal microscopy images were obtained, as described previously (48), by use of a Radiance 2000 Laser Scanning system configured with a 40× 1.30 oil objective. Green and red images were captured simultaneously at 1.024/1.024 resolutions with 2 optical zoom, iris 2, gain 20, and offset 0. XY horizontal planes (Z-series) were performed from basolateral to apical direction with 0.2-μm Z-step. The micrographs presented are XY sections performed midway between basolateral and apical cell surfaces after determination of the Z-start and Z-stop. The images were processed using LaserSharp 2000 software and assembled and labeled using Adobe Photoshop and Microsoft PowerPoint software. To ensure consistency of the results, each experiment was repeated at least three times, and the fields were selected blindly and randomly.

Statistical analysis. The results are representative of the means ± SD of at least three experiments. Each experimental condition was carried out in triplicate.

RESULTS

The PTH/PTHrP receptor colocalizes with the Golgi apparatus but not with the lysosomes. As reported previously (48), treatment of LLCPK-1 cells stably expressing green fluores-

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Fig. 1. No colocalization of parathyroid hormone/parathyroid hormone-related peptide (PTH/PTHrP) receptor with lysosomes. LLCPK-1 cells stably expressing green fluorescent protein-tagged PTH/PTHrP receptors (GFP-PPR), plated on coverslips, were treated with PTH (100 nM for 40 min at 37°C) in DMEM containing 20 mM HEPES + 0.1% BSA. At the end of the incubation, cells were placed on ice, rinsed (3×) with ice-cold PBS, fixed, permeabilized, and incubated with a monoclonal anti-lysosomal antibody (LAMP-1) followed by a Cy3-conjugated goat anti-mouse IgG. GFP-PPRs (green) and Cy3-labeled lysosomes (red) were scanned simultaneously by confocal microscope in the same planes. Merged pictures do not show yellow or orange staining, indicating absence of colocalization of the receptors with lysosomes. Images are taken at the bottom third (L) from the basolateral surface and the middle third (M) at the XY planes.
cent protein (GFP)-tagged PTH/PTHrP receptors (GFP-PPR) with PTH (100 nM for 40 min) causes receptor internalization; most of the receptors become localized in a perinuclear zone (Fig. 1; PPR). To determine the subcellular localization of the internalized receptors, we performed immunofluorescence analysis using specific antibodies that recognize the cellular organelles, Golgi apparatus, and lysosomes. The cells were scanned for green and red fluorescence simultaneously in the same planes and visualized using the confocal microscope. The internalized GFP-PPRs (Fig. 1; green) do not colocalize with lysosomes (Fig. 1; red) as evidenced by lack of overlap between the green and the red colors (Fig. 1; merged). Absence of colocalization of the receptors and lysosomes was also observed at shorter (10 min) and longer PTH treatment (120 min) (data not shown) and at different cell planes (Fig. 1; L and M).

Fig. 2. Colocalization of PTH/PTHrP receptor with the Golgi apparatus. LLCPK-1 cells stably expressing GFP-PPR, plated on coverslips, were treated with PTH (100 nM for 40 min at 37°C) in DMEM containing 20 mM HEPES + 0.1% BSA. At the end of the incubation, cells were placed on ice, rinsed (3×) with ice-cold PBS, fixed, permeabilized, and incubated with a monoclonal anti-Golgi antibody followed by a Cy3-conjugated goat anti-mouse IgG. GFP-PPRs (green) and Cy3-labeled Golgi apparatus (red) were scanned simultaneously by confocal microscope in the same plane. Merged pictures show yellow and orange staining, indicating colocalization of receptors with the Golgi apparatus. Images are taken at the top third from the basolateral surface at the XY planes.

Fig. 3. Effects of PTH removal on internalized GFP-tagged PTH/PTHrP receptors. LLCPK-1 cells stably expressing GFP-PPR, plated on coverslips, were treated with PTH (100 nM for 40 min at 37°C) in DMEM containing 20 mM HEPES + 0.1% BSA. PTH was removed, and cells were rinsed with PBS and incubated at 37°C in PTH-free medium for 0, 5, 20, and 60 min. Cells were then fixed, mounted, and examined at the middle third at the XY planes with the confocal microscope.
On the other hand, colocalization of a large fraction of the green fluorescence of the internalized GFP-PPRs (Fig. 2; green) and of the red fluorescence of the Golgi apparatus (Fig. 2; red) is observed; areas of colocalization appear in yellow and orange (Fig. 2; merged).

Effects of PTH removal on internalized PTH/PTHrP receptors. LLCPK-1 cells stably expressing GFP-PPR were treated with PTH (100 nM for 40 min) to stimulate receptor internalization, PTH was then removed, and the cells were rinsed and incubated in PTH-free medium for 0, 5, 20, or 60 min at 37°C in a CO₂ incubator. The cells were then fixed and examined under the confocal microscope. As we showed previously, under basal conditions, GFP-PPRs are mostly localized to the plasma membrane (Fig. 3; vehicle). The intracellular accumulation of the PTH/PTHrP receptor as a result of PTH stimulation of receptor internalization (Fig. 3; 0 min) starts to gradually disappear after PTH removal (Fig. 3; 0, 5, 20, and 60 min). Disappearance of the internalized receptors is observed as early as 20 min (Fig. 3; 20 min) and is almost complete after 60 min of PTH removal (Fig. 3; 60 min).

Effects of bafilomycin-A1 and brefeldin A on recycling of the PTH/PTHrP receptor. To examine the role of receptor recycling in the disappearance of the internalized GFP-PPRs after PTH removal and whether Golgi is involved in this process, we used bafilomycin-A1 (20, 35, 39, 52) and brefeldin A (2, 9, 14), two chemically and mechanistically distinct Golgi-disrupting agents.

Bafilomycin-A1 (50 nM) or brefeldin A (10 μg/ml) was added 60 min before and was kept during PTH treatment (100 nM, 40 min). Neither bafilomycin-A1 nor brefeldin A causes an observable effect on receptor accumulation in the Golgi area (Fig. 4; V, P, BP, and DP). PTH was then removed, and the cells were either incubated in the presence of vehicle (Fig. 4; P + V) or maintained in bafilomycin-A1 or brefeldin A (Fig. 4; BP + B and DP + D) for 60 min.

As shown above, when the cells were incubated with vehicle for 60 min after PTH removal, most of the receptors recycle to the cell membrane (Fig. 4, P + V). In contrast, when maintained after PTH removal, bafilomycin-A1 or brefeldin A decreases receptor recycling (Fig. 4; BP + B and DP + D); most of the internalized receptors are retained in the Golgi area (Fig. 4; BP + B and DP + D). Brefeldin A alone does not affect receptor distribution (data not shown). Bafilomycin-A1 alone causes small increase in intracellular accumulation of the receptors (data not shown). Similar effects to those of bafilomycin-A1 were also observed using concanamycin A (20 nM), another specific V-type H⁺-ATPase inhibitor (42, 54) (data not shown).

To ensure that none of these agents interferes with other trafficking pathways, we examined the effects of these drugs on the uptake and distribution of Lysotracker red, a live lysosomal marker (13). No major differences were observed when Lysotracker red (1:1,000) was added to cells treated under the same conditions as those causing receptor retention after PTH removal (Fig. 5). Cells examined under the confocal microscope show similar distribution of the lysosomal marker added (40 min at 37°C) to cells treated with vehicle (Fig. 5; P + V).

Fig. 4. Effects of bafilomycin-A1 and brefeldin A on recycling of GFP-tagged PTH/PTHrP receptors. LLCPK-1 cells stably expressing the GFP-PPR, plated on coverslips, were treated with PTH (100 nM for 40 min at 37°C) in the absence or presence of 50 nM bafilomycin-A1 or 10 μg/ml brefeldin A (added 60 min before PTH and kept in the presence of PTH) in DMEM containing 20 mM HEPES + 0.1% BSA. PTH was removed, and cells were rinsed with PBS and incubated at 37°C in presence of vehicle or maintained in 50 nM bafilomycin-A1 or 10 μg/ml brefeldin A for 60 min. Cells were then fixed, mounted, and examined at the middle third at XY planes with the confocal microscope. V, cells treated with vehicle during the whole experimental time; P, cells treated with PTH for 40 min; BP, cells treated with bafilomycin-A1 + PTH; DP, cells treated with brefeldin A + PTH; P + V, cells treated with PTH for 40 min and then PTH was removed and the cells were incubated in the presence of vehicle for 60 min; BP + B, cells treated with bafilomycin-A1 and PTH and then PTH was removed and the cells were maintained in bafilomycin-A1 for 60 min; DP + D, cells treated with brefeldin A and PTH and then PTH was removed and the cells were maintained in brefeldin A for 60 min.
Effects of bafilomycin-A1 on cell surface PTH/PTHrP receptor immunoreactivity after PTH removal. To further confirm the effects of PTH and bafilomycin-A1 on receptor recycling, we measured cell surface PTH/PTHrP receptor immunoreactivity.

As demonstrated previously (47, 48), cell surface PTH/PTHrP receptor immunoreactivity decreases after PTH treatment (40 min, 100 nM) due to receptor internalization (Fig. 6; V and P). Treatment with bafilomycin-A1 before PTH does not affect PTH-induced loss of cell surface PTH/PTHrP receptor immunoreactivity (Fig. 6; P, BP, and B).

PTH was then removed, and the cells were either incubated in the presence of vehicle (Fig. 6; P + V) or maintained in bafilomycin-A1 (Fig. 6; BP + B) for 60 min.

Most of the cell surface PTH/PTHrP receptor immunoreactivity in cells treated with vehicle after PTH removal is regained after 60 min (Fig. 6; P + V). Cell surface PTH/PTHrP receptor immunoreactivity is, however, only partially recovered when the cells are maintained in bafilomycin-A1 after PTH removal (Fig. 6; BP + B). The effects of bafilomycin-A1 are dose dependent, with a maximal effect at 50 nM (data not shown). The decrease in cell surface PTH/PTHrP receptor immunoreactivity in cells treated with bafilomycin-A1 alone (Fig. 6; B) probably results from inhibition of constitutive receptor recycling; this is consistent with the confocal microscopy observation that bafilomycin-A1 causes a small increase in the intracellular receptors.

Cycloheximide (60 μM for 60 min), an inhibitor of protein synthesis, alone or in the presence of PTH, had no significant effects on receptor internalization or recycling (data not shown).

DISCUSSION

The cellular responses to effectors are regulated, at least in part, by the movement and compartmentalization of the receptors and their effectors within the cell. Identifying the subcellular trafficking and the fate of the internalized GPCR receptors is important for understanding the physiological response to a variety of hormones and transmitters (3, 4, 16, 19, 41, 49).

Several GPCRs, such as thrombin receptor (19), ETB receptor (5), calcitonin receptor-like receptor (28), and neuropeptide neurotensin 1 receptor (51), are targeted to lysosomes after internalization.
Our previous studies have shown that a GFP-PPR stably expressed in LLCPK-1 cells undergoes agonist-dependent internalization into clathrin-coated pits (48). A major fraction of the internalized receptors localizes into a perinuclear compartment(s) (48). To identify the postendocytic pathways of the PTH/PTHrP receptor and their role in cell surface receptor recovery, we utilized both immunofluorescence staining and pharmacological approaches. By immunostaining of the lysosomes using LAMP-1 antibody, we show that the internalized GFP-PPRs do not colocalize with lysosomes. The data suggest that the internalized PTH/PTHrP receptor does not undergo lysosomal degradation. This conclusion is further supported by the finding that the loss of cell surface PTH/PTHrP receptor as a result of receptor internalization is rapidly recovered after PTH removal and despite treatment with cycloheximide, a de novo protein synthesis inhibitor.

Other GPCRs, such as β2-adrenergic receptor (19), cholecystokinin receptor type A (46), and ETα receptor (5), recycle to the cell membrane without degradation. ETα receptor follows a recycling pathway after colocalization with transferrin in the pericentriolar recycling compartment (5). Although this localization has been reported for several other internalized GPCRs (5, 8, 13, 22, 24, 44), the identity of this compartment has not been determined. By use of anti-cis-Golgi 58K protein, our combined analysis using immunofluorescence and confocal microscopy reveals that a large number of the internalized PTH/PTHrP receptors in a juxtanuclear area localize to a compartment of the Golgi apparatus. To determine whether localization of the receptors into the Golgi apparatus is important for recycling, we used specific proton pump inhibitors [bafilomycin-A1 (20, 35, 39, 52) and concanamycin A (42, 54)], which disrupt Golgi function.

Treatment with the proton pump inhibitors results in retention of the internalized receptor in the area of Golgi after PTH removal. The data are further confirmed by the finding that bafilomycin-A1 treatment markedly reduces cell surface receptor recovery after PTH removal. The fact that bafilomycin-A1 does not completely prevent cell surface receptor recovery suggests the presence of an unidentified bafilomycin-A1-insensitive recycling compartment(s). This observation is consistent with the confocal microscopy data that some receptors do not localize in the Golgi apparatus or the lysosomes. A similar finding was also observed by Mueller et al. (34) that recovery of chemokine receptor CCR5 does not involve recycling of the internalized receptor from the Golgi apparatus or new protein synthesis; the chemokine receptor was thought to recycle from early endosomes (34). Because the proton pump inhibitors may inhibit receptor recycling through effects on organelle V-type H+-ATPase other than Golgi (31), we utilized another chemically and mechanistically distinct Golgi-disrupting agent, brefeldin A (2, 9, 14). Similar to proton pump inhibitors, brefeldin A causes perinuclear receptor retention after PTH removal.

Although some GPCRs, such as the α2c-adrenoceptor, were shown to colocalize under basal state with markers for both endoplasmic reticulum and the cis/medial Golgi compartments (23), no postendocytic localization of GPCRs with the Golgi apparatus has been demonstrated. Our immunofluorescence results, however, demonstrate that the internalized PTH/PTHrP receptors localize to the Golgi apparatus. The pharmacological data using specific inhibitors of the V-type H+-ATPase and brefeldin A suggest that the V-type H+-ATPase plays a major role in postendocytic trafficking and recycling of PTH/PTHrP receptor and that this role is, at least in part, through the Golgi apparatus. The data imply a role for receptor targeting to the Golgi apparatus in receptor processing before recycling. It is conceivable that Golgi is the site of receptor dephosphorylation and/or dissociation of the receptor/ligand/β-arrestin complex to deliver a resensitized receptor to the cell surface (1, 7, 11, 12, 15, 18, 21, 24, 25, 33, 36, 37, 43). This notion is, however, yet to be investigated.

GRANTS

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REFERENCES


