Organic Anion Transporter OAT1 Undergoes Constitutive and Protein Kinase C-regulated Trafficking through a Dynamin- and Clathrin-dependent Pathway*

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Organic anion transporter 1 (OAT1) mediates the body disposition of a diverse array of environmental toxins and clinically important drugs. Therefore, understanding the regulation of this transporter has profound clinical significance. We previously demonstrated that OAT1 activity was down-regulated by activation of protein kinase C (PKC), kinetically revealed as a decrease in the maximum transport velocity \( V_{\text{max}} \) without significant change in the substrate affinity \( K_m \) of the transporter. In the current study, we showed that OAT1 constitutively internalized from and recycled back to the plasma membrane, and PKC activation accelerated OAT1 internalization without affecting OAT1 recycling. We further showed that treatment of OAT1-expressing cells with concanavalin A, depletion of K+ from the cells, or transfection of dominant negative mutants of dynamin-2 or Eps15 into the cells, all of which block the clathrin-dependent endocytotic pathway, significantly blocked constitutive and PKC-regulated OAT1 internalization. We finally showed that OAT1 colocalized with transferrin, a marker for clathrin-dependent endocytosis, at the cell surface and in the EEA1-positive early endosomes. Together, our findings demonstrated for the first time that (i) OAT1 constitutively traffics between plasma membrane and recycling endosomes, (ii) PKC activation down-regulates OAT1 activity by altering already existent OAT1 trafficking, and (iii) OAT1 internalization occurs partly through a dynamin- and clathrin-dependent pathway.

The organic anion transporter (OAT) family mediates the body disposition of a diverse array of environmental toxins, and clinically important drugs, including anti-HIV therapeutics, anti-tumor drugs, antibiotics, anti-hypertensives, and anti-inflammatories (1–3). Therefore, understanding the regulation of these transporters has profound clinical significance.

Seven OATs (OAT1–7) have been cloned, and their expressions were identified in distinct tissues and cell membranes (4–16). OAT1 and OAT3 are predominantly expressed at the basolateral membrane of kidney proximal tubule cells and the apical membrane of brain choroid plexus. OAT4 is expressed at the apical membrane of kidney proximal tubule cells and the basolateral membrane of placental trophoblast. OAT2 is expressed at the basolateral membrane of hepatocytes and is expressed in the kidney. The cellular localization of OAT2 in the kidney is still controversial. OAT5 is expressed only in the kidney. OAT6 is expressed in the olfactory mucosa, and OAT7 was identified in the liver. The cellular localization of OAT5–7 has not been defined.

In the kidney, OAT1 and OAT3 utilize a tertiary transport mechanism to move organic anions across the basolateral membrane into the proximal tubule cells for subsequent exit across the apical membrane into the urine for elimination. Through this tertiary transport mechanism, \( \text{Na}^+/\text{K}^+\)-ATPase maintains an inwardly directed (blood-to-cell) \( \text{Na}^+ \) gradient. The \( \text{Na}^+ \) gradient then drives a sodium dicarboxylate cotransporter, sustaining an outwardly directed dicarboxylate gradient that is utilized by a dicarboxylate/organic anion exchanger, namely OAT, to move the organic anion substrate into the cell. This cascade of events indirectly links organic anion transport to metabolic energy and the \( \text{Na}^+ \) gradient, allowing the entry of a negatively charged substrate against both its chemical concentration gradient and the electrical potential of the cell.

All of the cloned OATs share several common structural features (4–16), including 12 transmembrane domains flanked by intracellular N and C termini; multiple glycosylation sites localized in the first extracellular loop between transmembrane domains 1 and 2, and multiple potential phosphorylation sites present in the intracellular loop between transmembrane domains 6 and 7, and in the C terminus. Recent investigation from our laboratory on the structure-function relationship of OATs revealed that glycosylation is necessary for the targeting of these transporters to the plasma membrane (17, 18).

Studies from our laboratory and from others have shown that PKC activation resulted in a down-regulation of organic anion transport in intact kidney tubules and in cells transfected with OAT1, OAT2, and OAT4 (19–29). Kinetic analysis (27, 29) revealed that the decreased transport activity of OAT1 and
OAT4 caused by activation of PKC resulted from a decreased maximal transport velocity $V_{\text{max}}$, without significant change in the substrate affinity $K_m$ of the transporter.

There are several mechanisms by which PKC could modulate OAT activity. PKC-induced direct phosphorylation has been reported for other membrane proteins (30–32). We therefore asked whether PKC activation inhibited OAT activity through direct phosphorylation of the transporter. Our previous results showed (29) that PMA and other PKC activators failed to elevate the phosphorylation level of OAT1 under various experimental conditions. This suggests that direct phosphorylation is unlikely to be the cause for PKC-induced inhibition of OAT1 activity. This was further confirmed by Wolff et al. (23) showing that PKC-induced inhibition of OAT1 activity is independent of the conserved canonical PKC consensus sites in OAT1.

Alternatively, PKC activation may induce an internalization of membrane transporters and/or inhibit the recruitment of preformed transporters into plasma membrane (a “trafficking” mechanism). Certain plasma membrane proteins are static in the cell surface but internalize only in response to activated cellular signaling. For example, G-protein-coupled receptors exhibit very little basal internalization, but rapidly internalize during agonist-induced desensitization (33, 34). In contrast, GLUT4, the predominant glucose transporter of muscle and adipose cells, recycles constitutively between the cell surface and intracellular compartments, with the steady-state distribution favoring the latter. Insulin shifts the subcellular distribution of GLUT4 resulting in a new steady state where a large fraction of GLUT4 resides at the plasma membrane. This shift occurs primarily through the stimulation of GLUT4 recycling without significant inhibition of GLUT4 internalization (35). PKC modulation on both internalization and recycling has recently been described for other transporters/receptors. For example, brain dopamine transporter and Na⁺/K⁺ exchanger constitutively traffic but are retained intracellularly by accelerating internalization and attenuating recycling in response to PKC activation (36, 37).

In the present studies, we examined (i) whether OAT1 undergoes constitutive trafficking between cell surface and intracellular compartments, (ii) whether PKC-modulated inhibition of OAT1 activity occurs by altering the trafficking of this transporter, and (iii) the pathway through which OAT1 traffics.

**MATERIALS AND METHODS**

$p$-[³H]Aminoohippuric acid (PAH) was from NEN Life Science Products (Hercules, CA). Membrane-impermeable biotinylation reagent NHS-SS-biotin and streptavidin-agarose beads were purchased from Pierce. COS-7 cells and Dynamic-2/K44A mutant were purchased from American Type Culture Collection (Manassas, VA). Eps15 mutant (D95/295) was generously provided by Dr. Jennifer Lippincott-Schwartz from NICHD, National Institutes of Health (Bethesda, MD). Mouse anti-c-Myc antibody (9E10) was from Hybridoma Center, Mount Sinai School of Medicine (New York, NY). Mouse anti-EEA1 antibody was from BD Biosciences. Transferrin-tetramethylrhodamine conjugate, Alexa Fluor® 488 goat anti-mouse IgG (H+L), and Alexa Fluor® 550 goat anti-rabbit IgG (H+L) were from Molecular Probes (Eugene, OR). Protease inhibitor mixture, rabbit anti-c-Myc antibody, and all other reagents were purchased from Sigma.

**Generation of COS-7 Cells Stably Expressing OAT1**—Parental COS-7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin/streptomycin (100 units/ml), and glucose (100 mg/ml) in a 5% CO₂ atmosphere at 37 °C. Cells were seeded at 5 × 10⁵/well of 6-well plate cluster plate 24 h before transfection. cDNA plasmid encoding human OAT1-tagged with myc at its C terminus (38) was transfected into COS-7 cells using a Lipofectamine 2000 reagent, following the manufacturer’s instruction. After 7–8 days of selection in medium containing 0.5 mg/ml Geneticin (G418, Invitrogen), resistant colonies were replated to 96 wells for cloning, expansion, and analyzing positive clones.

**Transport Measurements**—For each well, uptake solution was added. The uptake solution consisted of phosphate-buffered saline (PBS)/CM (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, 0.1 mM CaCl₂, and 1 mM MgCl₂, pH 7.3) and [³H]PAH (20 μM). At the time points indicated in the figure legends, the uptake was stopped by aspirating the uptake solution off and rapidly washing the cells with ice-cold PBS solution. The cells were then solubilized in 0.2 N NaOH, neutralized in 0.2 N HCl, and aliquotted for liquid scintillation counting. The uptake count was standardized by the amount of protein in each well.

**Cell Surface Biotinylation**—Cell surface expression level of OAT1 was examined using the membrane-impermeable biotinylation reagent, NHS-SS-biotin. Cells were plated in 6-well plates. Each well of cells was incubated with 1 ml of NHS-SS-biotin (1 mg/ml in PBS/CM) in two successive 20-min incubations on ice with very gentle shaking. The reagent was freshly prepared for each incubation. After biotinylation, each well was briefly rinsed with 3 ml of PBS/CM containing 100 mM glycine then incubated with the same solution for 30 min on ice, to ensure complete quenching of the unreacted NHS-SS-biotin. The cells were then dissolved in ice for 1 h in 400 μl of lysis buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100 with 1/100 protease inhibitor mixture). The cell lysates were cleared by centrifugation at 16,000 × g at 4 °C. 50 μl of streptavidin-agarose beads was then added to the supernatant to isolate cell membrane proteins. OAT1 was detected in the pool of surface proteins by SDS-PAGE and immunoblotting.

**Internalization Assay**—We followed the procedure described by Loder et al. (36) and Zhao et al. (59). OAT1-expressing cells underwent biotinylation with 1.0 mg/ml sulfo-NHS-SS-biotin at 4 °C. Following biotinylation, one set of cells was washed with PBS and kept at 4 °C to determine the total initial surface OAT1 and stripping efficiencies. To initiate internalization, cells in the duplicate plate were washed repeatedly with prewarmed (37 °C) PBS containing either 1 μM PMA or vehicle and incubated with the same solutions for 5, 10, 15, and 30 min at 37 °C. Residual cell surface biotin was stripped by incubating cells three times for 20 min with freshly prepared 50 mM MesNa in NT buffer (150 mM NaCl, 1 mM EDTA, 0.2% bovine serum albumin, 20 mM Tris, pH 8.6). Stripping efficiency was determined for each experiment on biotinylated cells kept in parallel at 4 °C. Cells were lysed in lysis buffer with protease inhibitor.
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FIGURE 1. Characterization of OAT1 in COS-7 cells. a, kinetic analysis of OAT1-mediated PAH uptake. Kinetic characteristics were determined at substrate concentration ranging from 10 to 600 μM (3-min uptake). The data represent uptake into OAT1-expressing cells minus uptake into mock cells. Values are mean ± S.E. (n = 3). Inset, transport kinetic values were calculated using the Eadie–Hofstee transformation. b, activation of PKC by PMA inhibits OAT1 activity. COS-7 cells stably expressing OAT1 were incubated for 30 min with or without 1 μM PMA added directly to the culture media. After washing the cells, 3-min uptake of [3H]PAH (20 μM) was measured. Uptake activity was expressed as a percentage of the uptake measured in cells without treatment with PMA. Values are mean ± S.E. (n = 3).

mixture. Biotinylated proteins were separated from non-biotinylated proteins by streptavidin pull down from equivalent amounts of cellular proteins, and samples were analyzed by SDS-PAGE and Western blotting. Relative OAT1 internalized was calculated as % of the total initial cell surface OAT1 pool.

Recycling Assay—We followed the procedure described by Loder et al. (36) and Zhao et al. (59). OAT1-expressing cells underwent biotinylation with 1.0 mg/ml sulfo-NHS-SS-biotin at 4 °C for 30 min to label cell surface pool of OAT1. Subsequently, one set of cells was continuously biotinylated at 4°C. Cells in the duplicate plate were warmed to 37 °C and continuously biotinylated at 37 °C. At the time points indicated in the figures, biotinylation was stopped and biotin-labeled OAT1 was analyzed by SDS-PAGE and Western blotting as described above. Relative OAT1 recycled was calculated as the difference between OAT1 biotin-labeled at 37 °C and OAT1 biotin-labeled at 4 °C.

Electrophoresis and Immunoblotting—Protein samples (100 μg) were resolved on 7.5% SDS-PAGE minigels and electroblotted onto polyvinylidene difluoride membranes. The blots were blocked for 1 h with 5% nonfat dry milk in PBS-0.05% Tween 20, washed, and incubated for 1 h at room temperature with appropriate primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies. The signals were detected by SuperSignal West Dura Extended Duration Substrate kit (Pierce). Nonsaturating, immunoreactive protein bands were quantified by scanning densitometry with the FluorChem 8000 imaging system (Alpha Innotech Corp., San Leandro, CA).

Immunofluorescence Analysis—OAT1-expressing COS-7 cells were grown on coverslips (22 mm) for 24 h, washed three times in PBS, and incubated with 1 μM transferrin-tetramethylrhodamine conjugate in Dulbecco’s modified Eagle’s medium at 4°C for 1 h to allow the binding of transferrin to transferrin receptor, followed by incubation at 37 °C to allow the internalization of transferrin-transferrin receptor complex. The cells were then fixed with 3% paraformaldehyde for 20 min at room temperature, permeabilized with 0.01% Triton X-100 for 5 min three times, and incubated with 5% dry milk at room temperature for 1 h. Afterward, the cells were incubated with rabbit anti-c-Myc antibody (1:300) to label c-myc hOAT1 and with mouse anti-EEA1 antibody (1:250) at 4 °C overnight. The coverslips were then incubated with Alexa Fluor® 488 goat anti-mouse IgG (H+L) (1:500) or Alexa Fluor® 350 goat anti-rabbit IgG (H+L) (1:500) at room temperature for 2 h. After washing, the coverslips were mounted on slides for image acquisition and analysis. Samples were visualized with a Zeiss LSM-510 laser-scanning microscope (Carl Zeiss Inc., Thornwood, NY).

Data Analysis—Each experiment was repeated a minimum of three times. The statistical analysis was from multiple experiments. Statistical analysis was performed using Student’s paired t tests. A p value of <0.05 was considered significant.

RESULTS

Characterization of OAT1 in COS-7 Cells—To study the mechanisms underlying regulation of OAT1-mediated drug transport, we established COS-7 cells stably expressing OAT1. The OAT1-mediated transport of PAH, a prototypical organic anion, across the cell membrane was saturable (Fig. 1a). Based on Eadie-Hofstee plot analysis (Fig. 1a, inset), the K_m value for PAH was 112.7 μM and V_max was 1070 pmol/mg/3 min. Given the many reports that OAT1 activity was down-regulated by acute OAT1 down-regulation through PKC-induced Cellular Redistribution of OAT1—A decrease in the V_max of PAH transport in response to PKC activation by PMA may reflect either a decrease in transporter density at the cell surface or a decrease in transport turnover rates. To distinguish between these possibilities, we used cell surface biotinylation approach to examine the cellular distribution of OAT1 in cells treated with or without PMA. We showed (Fig. 2) that treatment of COS-7 cells with PMA resulted in a decrease of OAT1 at the cell surface (Fig. 2a) with a concomitant increase of OAT1 in the intracellular compartments (Fig. 2b). Total
OAT1 signals were not significantly changed after PMA treatment (Fig. 2c), suggesting that reduction in cell surface OAT1 is not caused by degradation. Therefore, activation of PKC results in a redistribution of OAT1 from the cell surface to intracellular compartments. The loss of OAT1 from the cell surface is responsible for the decrease in $V_{\text{max}}$ observed after PMA treatment.

**Constitutive OAT1 Trafficking**—A sizable intracellular OAT1 pool under basal condition (Fig. 2b) suggests that OAT1 may be subject to constitutive internalization from and recycling back to the cell surface. To test this hypothesis, we took a biotinylation-based strategy. We first investigated whether OAT1 constitutively internalizes. OAT1-expressing COS-7 cells were biotinylated with cell-impermeable biotinylation reagent sulfo-NHS-SS-biotin under trafficking-impermissive condition (4 °C). The labeled cells were then rewarmed back to trafficking-permissive condition (37 °C) to allow internalization to occur. At indicated time points after initiation of internalization, biotin from biotinylated proteins remaining on the surface was removed by treatment with MesNa, a nonpermeant reducing agent that cleaves disulfide bond and liberates biotin from biotinylated proteins at the cell surface. The amount of biotinylated proteins resistant (inaccessible) to MesNa treatment was defined as “the amount of protein internalized.” Our result (Fig. 3, a and c) showed that 0 min after initiation of internalization, there was no surface-labeled OAT1 internalized. However, 5, 10, or 15 min after initiation of internalization, ~10%, 20%, or 30% of surface-labeled OAT1 was detectable in the intracellular compartments. Therefore, OAT1 undergoes constitutive internalization in COS-7 cells. Similar results were also observed in OAT1-expressing LLC-PK1 cells (Fig. 4, a and c), suggesting that OAT1 internalization is not cell type-specific, but is rather a general feature of this transporter.

We next investigated whether OAT1 constitutively recycles back to the cell surface (Fig. 5). For such purpose, OAT1-expressing cells first underwent biotinylation with 1.0 mg/ml sulfo-NHS-SS-biotin at 4 °C for 30 min to completely label the cell surface pool of OAT1. Subsequently, one set of cells was continuously biotinylated at 4 °C. Cells in the duplicate plate were warmed to 37 °C and continuously biotinylated at 37 °C (36, 59). The rationale was that if OAT1 constitutively traffics between the cell surface and the intracellular compartments, then biotinylation under
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Trafficking-permissive conditions (37 °C) should significantly increase the amount of biotinylated OAT1 as compared with biotinylation performed under trafficking-restrictive conditions (4 °C). The experimental conditions were first tested with two key control proteins, transferrin receptor, which is known to participate in membrane recycling and the α subunit of the Na⁺/K⁺-ATPase, which is a negative control for recycling (36, 59). As shown in Fig. 5, the amount of transferrin receptor biotin-labeled 30 min after the initiation of recycling (37 °C) was much greater than that before the initiation of recycling (4 °C) (Fig. 5a, top panel), whereas the amount of Na⁺/K⁺-ATPase biotin labeled was almost the same before and after the initiation of recycling (Fig. 5b, top panel). Under the same experimental conditions, we further showed that the amount of OAT1 biotin-labeled at 37 °C was much greater than that biotin-labeled at 4 °C (Fig. 5c, top panel and Fig. 5d). These results demonstrated OAT1 constitutively recycles back to the cell surface. To rule out the contribution of newly synthesized OAT1 to the cell surface pool, we examined total and surface OAT1 while protein synthesis was blocked with cycloheximide. Cycloheximide treatment (10 μM) over a time span of 2 h had no significant effect on either OAT1 surface level or total OAT1 level (data not shown). Therefore, the biosynthetic contribution of OAT1 to the cell surface pool is not significant in the time frames in which our experiments were performed.

PKC-modulated OAT1 Trafficking—PKC-induced redistribution of OAT1 from cell surface to intracellular compartments (Fig. 2) could result from an increase in OAT1 internalization, a decrease in OAT1 recycling, or a combination of both. Using the same biotinylation approach as we used to investigate constitutive OAT1 trafficking, we examined whether activation of PKC modulates OAT1 trafficking. We observed (Fig. 6, a and c) that the amount of surface-labeled OAT1 internalized in the presence of PMA was much greater than that in the absence of PMA, suggesting that activation of PKC by PMA accelerated OAT1 internalization into the intracellular compartments. However, PMA treatment had no significant effect on OAT1 recycling back to the plasma membrane (Fig. 7).

Clathrin-dependent OAT1 Internalization—A requisite step for transporter internalization is their interaction with the cellular internalization machinery. So far, three different internal-
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To determine whether OAT1 internalizes via clathrin-dependent pathway, the constitutive and PKC-modulated OAT1 internalization were determined under the manipulations that specifically block clathrin-dependent internalization (42–45): (i) treatment of the cells with concanavalin A (ConA) (Fig. 8a, top panel and Fig. 8b), (ii) depletion of $K^+\$ from the cells (Fig. 9a, top panel and Fig. 9b), and (iii) transfection of dominant negative mutant of dynamin-2 into the cells (Fig. 10a, top panel, an Fig. 10b). Our results showed that the amount of surface-labeled OAT1 internalized both in the presence and in the absence of PMA was significantly reduced under these manipulations as compared with that of the control. The effect of reduced OAT1 internalization on OAT1 cellular distribution and function in cells transfected with dominant negative mutant of dynamin-2 was further analyzed. A reduced OAT1 internalization resulted in an increase in cell surface expression of OAT1 with concomitant decrease in its intracellular expression without significant change in its total expression (Fig. 10c). As a result, OAT1 transport activity was increased (Fig. 10d).

Like the effect of dynamin-2 mutant, OAT1 internalization was also reduced in cells transfected with dominant surface proteins such as transporters for internalization (39). During internalization, transporter-containing clathrin-coated pits pinch off from the membrane and move as internalization vesicles into the cytoplasm. Dynamin plays an essential role in this process. Three dynamin isoforms have been identified: dynamin-1 is exclusively expressed in neurons, dynamin-2 is ubiquitously expressed, and dynamin-3 is expressed in testes and to a lesser extent in neurons and lung. Caveolea-mediated internalization is an alternative to clathrin-mediated internalization (40, 41). Caveolea are flask-shaped invaginations of plasma membrane of many cell types, rich in cholesterol and sphingolipids as well as a structural protein called caveolin. During internalization, transporter-containing caveolea detach from the membrane and move as internalization vesicles into the cytoplasm.

Clathrin-dependent internalization nor-
malization pathways have been described for other transporters: (i) clathrin-mediated internalization, (ii) caveolea-mediated internalization, and (iii) clathrin- and caveolea-independent internalization. Clathrin-dependent internalization normally occurs at specialized membrane sites, where a complex structure, called a coated pit, is assembled to concentrate
transferrin was first incubated with cells at 4°C for 1 h to allow the formation of transferrin-transferrin receptor complex followed by incubation at 37°C to allow the internalization of transferrin-transferrin receptor complex into the cells. The fluorescence images were taken at various time points after 37°C incubation. Images taken 10 min after 37°C incubation (Fig. 12c) showed that OAT1 partially colocalized with transferrin both at the cell surface (shown as arrows) and in the intracellular compartments. Images taken 45 min after 37°C incubation showed that OAT1 partially colocalized with transferrin in EEA1-positive endosomes (Fig. 12, d–l). There appeared to be no significant colocalization of OAT1 with lysosome marker LAMP1 (not shown).

**DISCUSSION**

The organic anion transporter (OAT) family mediates the body disposition of a diverse array of environmental toxins, and clinically important drugs, including anti-HIV therapeutics, anti-tumor drugs, antibiotics, anti-hypertensives, and anti-inflammatory agents. Therefore, understanding the regulation of these transporters has profound clinical significance.

We previously showed (29) that OAT1 activity is down-regulated by activation of PKC, kinetically revealed as a decrease in the maximum transport velocity $V_{\text{max}}$ without significant change in the substrate affinity $K_m$ of the transporter, and that such down-regulation of OAT1 activity is not due to direct phosphorylation of the transporter. These observations let us hypothesize that PKC-mediated down-regulation of OAT1 activity may result from internalization of the transporter and/or inhibit the recruitment of the preformed transporter into membrane (a “trafficking” mechanism). PKC may phosphorylate a protein that is associated with OAT1 in the trafficking process. Recently, our laboratory has isolated several hOAT1-interacting proteins.3 Whether any of these proteins is a substrate for PKC-induced phosphorylation is under investigation.

In the current study, we examined hOAT1 trafficking in COS-7 cells stably expressing hOAT1. COS-7 cells offer several advantages for the study of the trafficking process of OAT1

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3 G. You, unpublished results.
The transport kinetics of OAT1 in COS-7 cells and its inhibition by PKC activation (Fig. 1) were in a good agreement with that observed in other systems (19–29). Using these cells, we obtained new data (Fig. 2) showing that, under basal condition, OAT1 was distributed between cell surface and intracellular compartments, with the steady-state distribution favoring the former. Activation of PKC by PMA shifted the cellular distribution of OAT1 resulting in a new steady state where a large fraction of OAT1 residues in the intracellular compartments. Therefore, PKC-induced down-regulation of OAT1 activity, reflected as a reduced maximum transport velocity $V_{\text{max}}$, resulted from a redistribution of OAT1 from cell surface to the intracellular compartments.

A sizable intracellular OAT1 pool under basal condition (Fig. 2) suggests that OAT1 may be subject to constitutive trafficking between cell surface and intracellular compartments. Membrane transporters were once considered to be static at the cell surface. However, a growing body of evidence demonstrated that many transporters undergo internalization from and recycling back to cell surface constitutively or in response to stimuli. Dopamine transporter, glucose transporter and water channel aquaporin-2 are such examples (34–36, 44). The direct evidence on constitutive OAT1 trafficking came from our biotinylation study. We showed that, under basal condition, OAT1 robustly internalized from (Fig. 3) and recycled back (Fig. 5) to the cell surface to maintain a steady-state cell surface level of the transporter. The rates for both internalization and recycling were $\sim 10\% / \text{min}$, similar to that observed for transferrin receptor, a protein that undergoes rapid internalization and recycling (50). The biosynthetic contribution of OAT1 to the cell surface pool can be ruled out in the time frames in which our experiments were performed, because treatment with cycloheximide, an inhibitor for protein synthesis, over a time span of 2 h had no significant effect on either OAT1 surface levels or total OAT1 levels.

Our biotinylation study further showed that PKC modulated OAT1 activity by accelerating OAT1 internalization (Fig. 6) without significantly affecting OAT1 recycling (Fig. 7). Similar
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PKC effects on internalization and recycling have been described for other transporters such as brain dopamine transporter (36) and Na+/H+ exchanger (37).

What are the cellular machineries governing the constitutive and PKC-regulated OAT1 internalization? So far, three different internalization pathways have been described for other transporters: (i) clathrin-dependent internalization, (ii) caveola-dependent internalization, and (iii) clathrin- and caveola-independent internalization. Evidence from our studies pointed that both the constitutive and the PKC-modulated OAT1 internalization occurred partly through a dynamin- and clathrin-dependent pathway. Indeed, treatment of OAT1-expressing cells with ConA, depletion of K+ from the cells, or transfection of dominant negative mutants of dynamin-2 into the cells, all of which blocked clathrin-dependent pathway (42–45), significantly blocked OAT1 internalization (Figs. 8–10). OAT1 internalization was also significantly reduced in cells transfected with dominant negative mutant of Eps15, another component of clathrin-coated pits (58) (Fig. 11). Until very recently, dynamin was thought to be specifically involved in clathrin-mediated endocytic pathways. Recent studies (60) suggested that dynamin might also be involved in clathrin-independent pathways as well. Therefore, the use of Eps15 mutant is a new powerful tool to specifically study clathrin-mediated endocytosis. Interestingly, although these manipulations completely prevented the internalization of transferrin (not shown), which has been shown to internalize through clathrin-dependent pathway (52), OAT1 internalization could only be partially blocked by these manipulations, suggesting that other internalization pathways may also contribute to OAT1 trafficking. This is worth further investigation.

OAT1 internalization, occurring partly through a dynamin- and clathrin-dependent pathway, was further supported by our immunofluorescence studies. As mentioned above, transferrin is known to enter cells, after binding to its receptor, via clathrin-mediated endocytosis (52). After internalization, transferrin-transferrin receptor complex is then targeted to EEA1-positive early endosomes and recycled back to the cell surface (52). We showed (Fig. 12) that OAT1 partially colocalized with transferrin both at the cell surface and in the EEA1-positive early endosomes, suggesting that OAT1 traffics, at least in part, through the same pathway as that of transferrin. In addition, the partial colocalization of hOAT1 with transferrin and EEA1 could also arise from other possibilities. It has been shown that recycling endosomes are heterogeneous in their biochemical compositions, ion transport properties, and pH values (51). Therefore, it may be possible that some of hOAT1 resides in a different subpopulation of recycling endosomes from that enriched in transferrin. It is also worth to note that hOAT1 is overexpressed in COS-7 cells. In such an overexpression system, it is not surprising to see that hOAT1 does not completely overlap with transferrin and EEA1. In conclusion, in the current study, we demonstrated for the first time that (i) OAT1 constitutively traffics between cell membrane and recycling endosomes, (ii) PKC activation down-regulates OAT1 activity by altering already existent OAT1 trafficking: accelerating OAT1 internalization without significantly affecting OAT1 recycling, and (iii) OAT1 internalization occurs partly through a dynamin- and clathrin-dependent pathway. We have observed that other members of OAT family, such as OAT3 and OAT4, also undergo trafficking like OAT1 (not shown).

Why would the kidney expend energy to constantly cycle transporters? Perhaps the best explanation is that the transporter in a dynamic rather than a static state is more primed for the input to initiate trafficking and, therefore, is capable of providing quick and efficient fine-tuning in body response to environmental changes.

Abnormal OAT1 trafficking may contribute to the impaired drug elimination in bilateral ureteral obstruction (BUO). BUO is a serious and common clinical condition, and an important cause of acute renal failure (53, 54). It is shown (54) that in BUO rats, elimination of drugs was impaired partly due to a redistribution of OAT1 from cell surface to intracellular compartment. In BUO, angiotensin II has an elevated level of expression (53, 55, 56). It was shown that angiotensin II exerts its effect through activation of PKC (57). Therefore, angiotensin II may affect OAT1 trafficking through such a signaling pathway. Our current studies may provide important insight into the molecular, cellular, and clinical bases underlying BUO.

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