

# Protein Kinase C Activation Downregulates Human Organic Anion Transporter 1-Mediated Transport through Carrier Internalization

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**Abstract.** Organic anion transport in intact renal proximal tubule cells in animal model systems is downregulated by treatments that activate protein kinase C (PKC). How this downregulation is achieved is not yet known. Stimulation of PKC with *sn*-1,2-dioctanoylglycerol resulted in strong inhibition of *p*-aminohippurate transport mediated by the cloned human organic anion transporter 1 (hOAT1) expressed in *Xenopus* oocytes and HEK293 cells, as well as hOAT1 internalization in both expression systems. The *sn*-1,2-dioctanoylglyc-

erol-induced transport inhibition was partially prevented by staurosporine. It was independent of the conserved canonical PKC consensus sites in hOAT1, however, and was unaffected by agents that destabilize actin filaments or microtubules, which altered baseline hOAT1-mediated *p*-aminohippurate uptake activity in oocytes. It is concluded that PKC-induced hOAT1 downregulation is achieved through carrier retrieval from the cell membrane and does not involve phosphorylation of the predicted classic hOAT1 PKC consensus sites.

The renal secretory organic anion transport pathway plays an important role in the elimination of a variety of potentially toxic environmental chemicals, such as phenoxyacetate herbicides and fungicides, as well as anionic drugs, including  $\beta$ -lactam antibiotics, cytostatic agents, diuretics, and antiviral drugs (1,2). The importance of this pathway is exemplified by the increased toxicity of methotrexate when it is coadministered with nonsteroidal anti-inflammatory drugs (3), which compete for the same elimination pathway (4) and thus lead to higher serum methotrexate concentrations (3). In a number of non-mammalian and mammalian renal proximal tubule model systems, peritubular uptake and/or transepithelial secretion of prototypical organic anions have been demonstrated to be inhibited by agents that stimulate protein kinase C (PKC) activity. The nonselective PKC activator phorbol-12-myristate-13-acetate (PMA) inhibited cellular and luminal fluorescein accumulation in isolated nonperfused killifish renal tubules (5), net secretion of 2,4-dichlorophenoxyacetic acid across winter flounder proximal tubule monolayer cultures (6), peritubular uptake and transepithelial fluorescein flux in isolated nonperfused (7) and perfused (8) rabbit proximal tubule S2 segments, respectively, and basolateral uptake and transcellular move-

ment of *p*-aminohippurate (PAH) in opossum kidney cells (9,10).

Because a number of agents, such as parathyroid hormone (PTH) (11),  $\alpha_1$ -adrenergic agonists (12), angiotensin II (13), and bradykinin (14), modulate renal proximal tubule function via activation of PKC, these agents might be expected to impair renal elimination of organic anions. Indeed, bradykinin and phenylephrine were observed to inhibit basolateral uptake (7) and transepithelial secretion (8) of fluorescein in isolated rabbit proximal tubule S2 segments, and these effects were suppressed by the PKC inhibitor bisindolylmaleimide.

On the basis of their localization, two of the cloned organic anion transporters (OAT), namely OAT1 (15) and OAT3 (1,16), might be involved in the basolateral uptake of small organic anions into renal proximal tubule cells. When heterologously expressed in oocytes or mammalian cells, both OAT1 and OAT3 have been demonstrated to be inhibited by more or less selective PKC activators (17–20). However, little is known regarding the mechanism of PKC-mediated OAT downregulation. The only detailed investigation of OAT1 regulation (using the mouse orthologue) suggested, on the basis of the absence of a mouse OAT1-sized phosphoprotein, that at least mouse OAT1 might not be phosphorylated (19). No information regarding the mode of human OAT1 (hOAT1) downregulation is available. In this study, we investigated whether PKC-dependent inhibition of hOAT1 involves any of the conserved potential PKC phosphorylation sites of hOAT1 and whether it is mediated by carrier internalization. We demonstrate that the PKC activators PMA and *sn*-1,2-dioctanoylglycerol (DOG) lead to rapid redistribution of hOAT1, expressed in *Xenopus* oocytes or HEK293 cells, to submembra-

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nous compartments and that this process is independent of the PKC sites of the carrier.

## Materials and Methods

### Construction of a hOAT1 Clone for Expression in *Xenopus laevis* Oocytes and Mammalian Cells by PCR and Ligation of the Restricted Products

The hOAT1 coding region, cloned as previously described (21), was subcloned into the untranslated regions (UTR) of the flounder sodium/dicarboxylate cotransporter (fNaDC-3), which can be well expressed in oocytes (22). For this, the *Bam*HI and *Xba*I sites of the fNaDC-3 vector (pSport1) were disrupted by site-directed mutagenesis (see below). The hOAT1 reading frame and the fNaDC-3 UTR separated by the vector were then amplified by using Powerscript polymerase (PAN Systems, Aidenbach, Germany), according to the instructions provided by the manufacturer, with 30 cycles of 94°C for 20 s, 58°C for 20 s, and 72°C for 4 min, followed by a final extension period of 10 min at 72°C. The primers (obtained from IBA Nucleic Acids Product Supply, Göttingen, Germany) used in this amplification incorporated *Bam*HI and *Xba*I sites (underlined) for hOAT1 as close as practical to the start and stop codons, respectively, to enable subsequent subcloning, as follows: hOAT1: hR5'+*Bam*HI, GGATCCATGGCCTTTAATGACCTCCTGCARC; hR3'+*Xba*I, TCTAGACCTCAAAATCCATTCTTCTCTGTGTC; fNaDC-3 UTR in pSport1: 5'fNaDC-3-UTR+*Bam*HI, CCACA-GAGATTTGGATCCCCAGAGGAAAG; 3'fNaDC-3-UTR+*Xba*I, GCTCAATGCGACTCTCTAGACCTCGC.

The hOAT1 coding region flanked by *Bam*HI and *Xba*I restriction sites, which had been subcloned into pPCR-Script (Stratagene, La Jolla, CA), and the fNaDC-3 UTR/pSport1 amplicon were then sequentially digested with *Bam*HI and *Xba*I and were ligated together with T4 DNA ligase. For expression in mammalian cells, hOAT1 was subcloned into the *Kpn*I and *Not*I sites of pcDNA3.1(+) (Invitrogen, Calsbad, CA).

### Site-Directed Mutagenesis and Sequencing

Single- and double-base mutations were introduced into pSport1 and hOAT1 by using the QuikChange site-directed mutagenesis kit (Stratagene), according to the instructions provided by the manufacturer, with the following primer pairs (obtained from MWG Biotech, Ebersberg, Germany) (the positions of the mutations are underlined): pSport *Bam*HI/*Xba*I disrupt (sense), CGTAAGCTTGGATCGACTAGAGCGGCCGC; pSport *Bam*HI/*Xba*I disrupt (antisense), GCGGCCGCTCTAGTCGATCCAAGCTTACG; hOAT1-S129A (sense), GGACCTTGTGTGCGCTCAGACGGCC; hOAT1-S129A (antisense), GGCCCTGTGAGCGCACACAAGGTCC; hOAT1-S271A (sense), GGTTCTTCATTGAGGCGCCCGCTGG; hOAT1-S271A (antisense), CCA-GCGGGCCGCTCAATGAAGAACC; hOAT1-S271E (sense), GGTTCTTCATTGAGGAGGCCCGCTGGCACTC; hOAT1-S271E (antisense), GAGTGCCAGCGGGCCCTCCTCAATGAAGAACC; hOAT1-S271C (sense), CTGGTTCTTCATTGAGTGTGCCCGCTGGCACTC; hOAT1-S271C (antisense), GAGTGCCAGCGGGCACTCAATGAAGAACCAG; hOAT1-S278A (sense), GCACTCCTCCGCGGAGGAGTGC; hOAT1-S278A (antisense), CAGCCTCCCAGGCGGAGGAGTGC; hOAT1-T284A (sense), GGCTGGACCTCGCCCTGAGGGC; hOAT1-T284A (antisense), GCCCTCAGGGCGAGGTCCAGCC; hOAT1-T334A (sense), GCGCTGCCCGCCCTCCGCCAC; hOAT1-T334A (antisense), GTGGCGGAGGGCGGGCAGCGC; hOAT1-S521A (sense), GTGCAGGACCTGGAGGCCAGGAAAGGAAAC; hOAT1-S521A (antisense), GTTC-CCCTTTCCTGCGCTCCAGGTCTGCAC.

The FLAG epitope was introduced either carboxy-terminally or in the large first extracellular loop between amino acid residues 107 and 108, with primers containing the FLAG sequence flanked by regions complementary to adjacent regions of hOAT1 (MWG Biotech) (the FLAG sequence is underlined): hOAT1-C-FLAG (sense), CAGCACAAGAG-AAGAATGGATTTGACTACAAGGACGACGATGACAAGTG-AGGTCTAGACCCTCGCAGAGG; hOAT1-C-FLAG (antisense), CC-TCTGCGAGGGTCTAGACCTCACTTGTTCATCGTCGCTCCTTGTA-GTCAAATCCATTCTTCTTGTGCTG; hOAT1-N+107-FLAG (sense), CCACAGAGCCCTGCACCGATGACTACAAGGACG-ACGATGACAAGGGCTGGATCTACGACAACAGCACC; hOAT1-N+107-FLAG (antisense), GGTGTGTTGTCGTAGATCCAGCCC-TTGTTCATCGTCGCTCCTTGATGTCATCGGTGCAGGGCTCTGTGG.

For verification of the mutated clones, both strands of the entire coding region were sequenced by dye terminator cycle sequencing (ABI Prism automatic sequencer; Applied Biosystems, Foster City, CA). The sequence was assembled and analyzed by using the Genetics Computer Group software package (Genetics Computer Group [GCG], Madison, WI).

### cRNA Synthesis, Oocyte Injection, and Transport Experiments

Stage V/VI oocytes were defolliculated by overnight incubation at 18°C with collagenase (0.5 mg/ml, type CLSII; Biochrom, Berlin, Germany) in oocyte Ringer's solution (ORI) (90 mM NaCl, 3 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5 mM HEPES-Tris, pH 7.6) and subsequent washing for 10 min in Ca<sup>2+</sup>-free ORI. Oocytes were then injected with 23 or 46 nl of water or, in an equivalent volume, 35 ng (unless otherwise stated) of cRNA synthesized from *Not*I-linearized plasmid (mMessage mMachine-T7 *in vitro* transcription kit; Ambion, Austin, TX). After injection, the oocytes were incubated for 3 d at 18°C in modified Barth's solution containing 12 μg/ml gentamicin, as described previously (23). Uptake of *p*-[glycyl-2-<sup>3</sup>H]aminohippurate (<sup>3</sup>H]PAH) (5 μCi/ml, 1 to 5 Ci/mmol; NEN, Boston, MA) was assayed at room temperature in ORI. Oocytes were then washed with ice-cold ORI and dissolved, and their <sup>3</sup>H contents were determined by liquid scintillation counting.

Pretreatments with PMA (up to 1 μM), 4α-phorbol-12-myristate-13-acetate (4α-PDD) (1 μM), or DOG (up to 5 μM), in the absence or presence of 10 μM staurosporine, in ORI were performed at room temperature for up to 1 h. Control oocytes were preincubated in the presence of an equal amount of DMSO, which was used as the solvent for PMA, 4α-PDD, DOG, and staurosporine. After three washes of the oocytes with ORI at room temperature, <sup>3</sup>H]PAH uptake was assayed in the absence of the test agents. Colchicine and cytochalasin D stock solutions were prepared in ethanol, and nocodazole stock solution was prepared in DMSO. Oocytes were preincubated with colchicine (20 μM) for 5 h or cytochalasin D (20 μM) for 1 h at room temperature; cells to be treated with nocodazole were first precooled on ice for 60 min and then treated with nocodazole (20 μM) for 1 h at 18°C. The oocytes were then washed three times with ORI at room temperature, incubated without or with DOG for 1 h at room temperature, and washed three times before <sup>3</sup>H]PAH uptake measurements. In testing for brefeldin A (dissolved in ethanol) inhibition of the cytochalasin D effect, oocytes were first incubated without or with brefeldin A (10 μg/ml) for 1 h at room temperature, washed, and then treated with cytochalasin D in the absence or presence of brefeldin A, respectively. <sup>3</sup>H]PAH uptake was then assayed in the absence of the test agents. PMA, DOG, 4α-PDD, staurosporine, colchicine, nocodazole, cytochalasin D, and brefeldin A were all obtained from Sigma Chemical Co. (St. Louis, MO).

### Expression of hOAT1 in HEK293 Cells and Transport Measurements

HEK293 cells (kindly provided by Dr. H. Koepsell, Department of Anatomy, Julius Maximilians University, Würzburg, Germany) were maintained in low-glucose Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (all from Sigma). For transient expression of hOAT1, cells were seeded in 24-well plates at  $3 \times 10^5$  cells/well and were transfected by using Lipofectamine 2000 reagent (Invitrogen), according to the instructions provided by the manufacturer, with 2  $\mu$ l of Lipofectamine 2000 and 1  $\mu$ g of the pcDNA3.1(+)-hOAT1 construct (see above) per well. Forty-eight hours after transfection, uptake of [ $^3$ H]PAH (5  $\mu$ Ci/ml) was assayed for 30 min at 37°C in mammalian Ringer's solution (130 mM NaCl, 4 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgSO<sub>4</sub>, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, 18 mM glucose, 20 mM HEPES-Tris, pH 7.4), in a humidified atmosphere. Cells were then washed three times with 0.5 ml/well of ice-cold mammalian Ringer's solution, air-dried, and dissolved in 0.5 ml/well of 1 N NaOH. After neutralization with 1 N HCl, the  $^3$ H contents of the cells were determined by liquid scintillation counting. [ $^3$ H]PAH uptake was normalized to protein contents, as assayed with Coomassie brilliant blue G-250 (Serva, Heidelberg, Germany) according to the method described by Bradford (24), using bovine serum albumin as a standard.

Pretreatment of cells with 10  $\mu$ M colchicine was performed for 5 h, pretreatment with 10  $\mu$ M cytochalasin D for 1 h, and pretreatment with 5  $\mu$ M DOG for 1 h, all in mammalian Ringer's solution at 37°C in a humidified atmosphere. Uptake was then assayed after three washes with mammalian Ringer's solution, in the continuous presence of 5  $\mu$ M DOG for cells that had been pretreated with DOG.

### Immunocytochemical Analyses

To study hOAT1 trafficking in oocytes, immunocytochemical assays were performed as described previously (25). Oocytes expressing carboxy-terminally FLAG-tagged hOAT1 were pretreated without or with PMA or DOG on day 3 after injection, incubated for 5 to 10 min in 200 mM potassium aspartate, manually devitellinized, and fixed in Dent's solution overnight at -20°C. Oocytes were stained first with mouse anti-FLAG M2 IgG monoclonal antibody (1:2000 dilution; Sigma) at 4°C for 12 h and then with Alexa 546-goat anti-mouse IgG antibody (1:200 dilution; Molecular Probes, Eugene, OR) at room temperature for 1 h. Stained oocytes were postfixed with 3.7% paraformaldehyde for 30 min and embedded in acrylamide (Technovit 7100), according to the instructions provided by the manufacturer (Heraeus Kulzer, Inc., Hanau, Germany). Embedded oocytes were cut into 5- $\mu$ m sections and analyzed with a fluorescence microscope (Optiphot; Nikon, Langen, Germany).

hOAT1-expressing HEK293 cells for immunocytochemical analyses were seeded in 24-well plates as before but, in this case, the wells were fitted with sterile, 12-mm-diameter coverslips that had been pretreated for 5 min with polylysine (0.2 mg/ml in phosphate-buffered saline [PBS]). On day 2 after transfection with the extracellularly FLAG-tagged hOAT1 (as described above), primary mouse anti-FLAG M2 antibody (1:50 dilution in mammalian Ringer's solution) was allowed to bind for 1 h at 4°C, excess antibody was washed off, and the cells were then incubated for 1 h in the absence or presence of 5  $\mu$ M DOG, as for the transport experiments. The cells were then fixed for 8 min (3.7% formaldehyde in PBS), permeabilized for 5 min (50 mM sodium phosphate, pH 7.4, 0.5% NaCl, 0.3% Triton X-100), blocked for 30 min (33% goat serum, 0.1% Triton X-100 in PBS), and incubated for 1 h with secondary Alexa 488-goat anti-mouse IgG

antibody (1:200 dilution in PBS with 0.1% bovine serum albumin; Molecular Probes), all at room temperature and with several washes with PBS after each step. Finally, the specimens were mounted in 80% glycerol, 20 mM NaHCO<sub>3</sub>, in water and were examined with a laser scanning confocal microscope (LSM 510; Zeiss, Oberkochen, Germany). Serial optical sections were recorded as three-dimensional stacks. For illustrations, three intersecting orthogonal planes through individual cells, generated with the LSM-510 software (Zeiss), were imported into Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA) and then equally processed to enhance contrast only.

### Statistical Analyses

Statistical analyses were performed with the GraphPad InStat program (version 3.00 for Windows 95; GraphPad Software, San Diego, CA), and apparent  $K_{0.5}$  values were determined with GraphPad Prism 3.03. Unless otherwise stated, for oocyte experiments,  $n$  represents the number of independent experiments with cells from different donor animals, each performed with at least seven oocytes per treatment. For experiments with HEK293 cells,  $n$  represents the number of independent experiments with cells of different passages, each performed in triplicate or quadruplicate.

## Results

### Functional Characterization of the hOAT1 Construct

To functionally verify hOAT1 (21), which was made expressible in oocytes by insertion between the fNaDC-3 UTR, we first assayed for several typical OAT1 characteristics, *i.e.*, high-affinity PAH transport sensitive to glutarate,  $\alpha$ -ketoglutarate, and probenecid *cis*-inhibition (1,26). In *Xenopus* oocytes, hOAT1 mediated uptake of 10  $\mu$ M PAH, which was nearly completely inhibited by 1 mM  $\alpha$ -ketoglutarate, glutarate, and probenecid ( $92 \pm 3\%$  inhibition,  $n = 3$ ;  $96 \pm 1\%$ ,  $n = 4$ ; and  $99 \pm 0.4\%$ ,  $n = 2$ , respectively). In three independent experiments, the apparent  $K_{0.5}$  for PAH averaged  $11.6 \pm 1.6$   $\mu$ M (one representative experiment is presented in Figure 1). Similar to the PAH transport characteristics observed in renal cortical basolateral membranes (27,28), PAH uptake in hOAT1-expressing oocytes was strongly Cl<sup>-</sup>-dependent; replacement of Cl<sup>-</sup> in the medium with gluconate inhibited uptake by 79.8% (one experiment with 12 oocytes/group). Therefore, the hOAT1 construct was suitable for studies of hOAT1 regulation.

### Downregulation of hOAT1-Mediated Transport by Agents Stimulating PKC

hOAT1 expressed in *X. laevis* oocytes was inhibited by PMA preincubation in a time- and concentration-dependent manner, with an IC<sub>50</sub> of approximately 20 nM PMA (Figure 2). Preincubation of the oocytes with 1  $\mu$ M PMA for 1 h before the transport assay resulted in  $82.6 \pm 10.3\%$  inhibition of hOAT1-mediated PAH transport (five independent experiments). This effect could not be mimicked by the inactive phorbol ester 4 $\alpha$ -PDD, which slightly increased rather than decreased PAH uptake in hOAT1-expressing oocytes ( $122.7 \pm 10.2\%$  of uptake in the absence of 4 $\alpha$ -PDD, three independent experiments). Similar to PMA, the more PKC-specific agent DOG, which is thought to lack the artifactual effects on *Xenopus* oocytes observed for phorbol esters (29,30), strongly re-

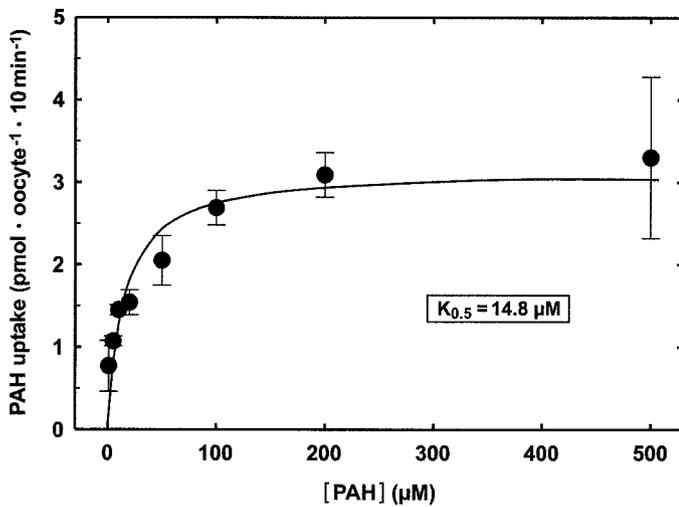


Figure 1. *p*-Aminohippurate (PAH) affinity of human organic anion transporter 1 (hOAT1). Oocytes were injected with cRNA for the construct of the hOAT1 reading frame in the fNaDC-3 untranslated regions, generated for expression in oocytes. On day 3 after injection, 10-min uptake of [<sup>3</sup>H]PAH at the given concentrations was assayed. Results are expressed as means  $\pm$  SEM of seven to 11 oocytes, in one representative experiment of three.

duced hOAT1-mediated PAH uptake, by an average of  $82.7 \pm 2.9\%$  ( $n = 17$ ). The time course of DOG inhibition was similar to that observed for PMA (Figure 3A), whereas the  $IC_{50}$  was higher for DOG (average of  $1 \mu\text{M}$ ) (Figure 3B). Inhibition by  $5 \mu\text{M}$  DOG (60 min) could be significantly, albeit only partially, suppressed by the simultaneous presence of  $10 \mu\text{M}$  staurosporine (DOG alone,  $80.3 \pm 3.3\%$  inhibition,  $n = 12$ ; DOG plus staurosporine,  $58.0 \pm 4.4\%$  inhibition,  $n = 12$ ;  $P < 0.001$ ). Neither preincubation first with staurosporine alone nor injection of staurosporine ( $4.2 \text{ pmol/oocyte}$ ) 1 h before DOG administration significantly enhanced the staurosporine effect (data not shown). By itself, staurosporine preincubation consistently inhibited hOAT1-mediated transport ( $35 \pm 19\%$  inhibition in three independent experiments).

#### Subcellular Localization of hOAT1 after PMA or DOG Treatment

To allow immunocytochemical detection of hOAT1 in oocytes, a carboxy-terminally FLAG-tagged construct, which was similar to untagged hOAT1 with respect to PAH transport activity (tagged,  $3.6 \pm 0.5 \text{ pmol/oocyte}$  per 15 min; untagged,  $4.3 \pm 0.4 \text{ pmol/oocyte}$  per 15 min;  $n = 3$  experiments), was generated. In hOAT1-expressing oocytes treated for 60 min with solvent (DMSO) only, the carrier was clearly visible at the cell surface (Figure 4). In contrast, oocytes preincubated with  $1 \mu\text{M}$  PMA or  $5 \mu\text{M}$  DOG demonstrated redistribution of much of the hOAT1 staining to submembranous compartments. Water-injected oocytes demonstrated minimal nonspecific reactivity.

#### Dependence of DOG-Induced hOAT1 Internalization on Cytoskeletal Elements

Several studies reported the involvement of cytoskeletal elements in carrier internalization (31,32). We therefore as-

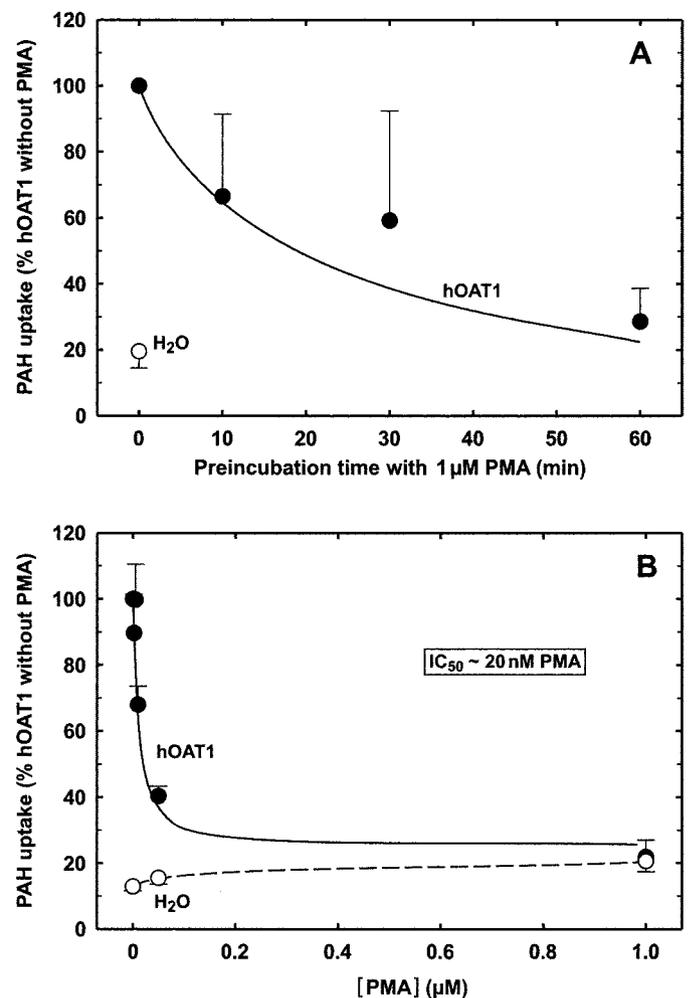
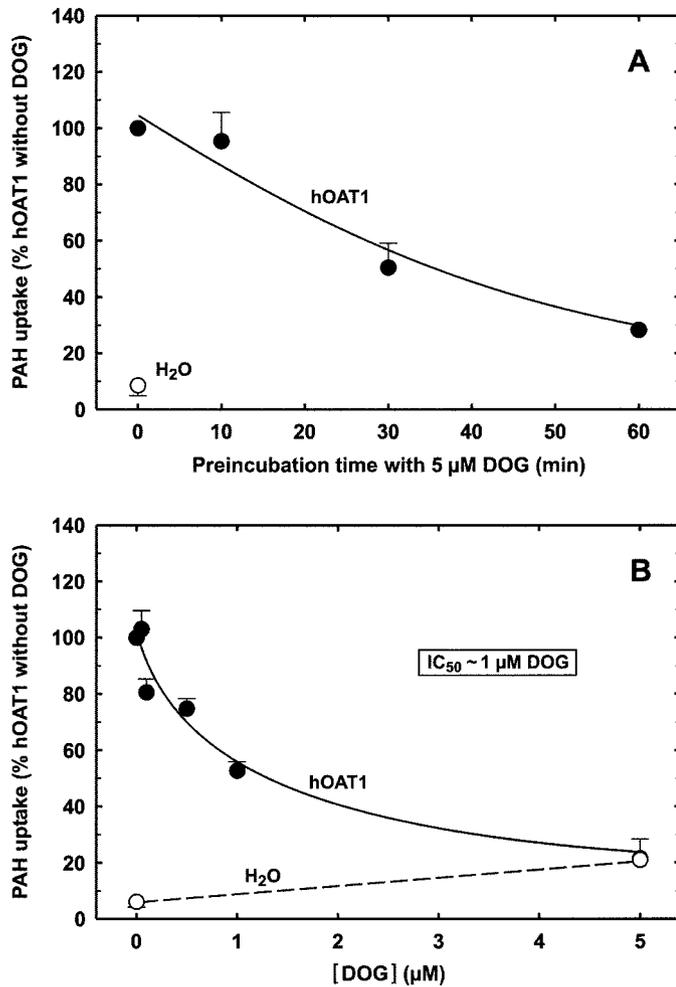


Figure 2. Time (A) and concentration (B) dependence of phorbol-12-myristate-13-acetate (PMA) inhibition of hOAT1-mediated PAH uptake. Oocytes were injected with hOAT1 cRNA (●) or an equivalent volume of water (○). On day 3 after injection, they were preincubated for a total of 1 h at room temperature in the presence of DMSO but with  $1 \mu\text{M}$  PMA for the indicated times only (A) or were preincubated for 1 h at room temperature in the absence (DMSO alone) or presence of various PMA concentrations (B). Fifteen-minute uptake of  $10 \mu\text{M}$  [<sup>3</sup>H]PAH was then assayed. Results are expressed as percentages of the uptake in hOAT1-expressing oocytes without PMA. Data are means  $\pm$  SEM of two independent experiments (A) or means  $\pm$  SEM of 10 or 11 oocytes in one of two experiments (B).

essed the effects of colchicine (or nocodazole) and cytochalasin D (destabilizing agents for microtubules and actin filaments, respectively) on DOG-induced hOAT1 inhibition. None of the agents significantly altered the relative magnitude of DOG inhibition in individual sets of experiments ( $71.9 \pm 1.8\%$  with colchicine versus  $75.6 \pm 4.3\%$  without colchicine,  $n = 4$ ;  $62.7 \pm 7.2\%$  with nocodazole versus  $85.0 \pm 3.7\%$  without nocodazole,  $n = 2$ ;  $88.0 \pm 4.1\%$  with cytochalasin D versus  $89.4 \pm 17.0\%$  without cytochalasin D,  $n = 4$ ) (Figure 5). Interestingly, cytochalasin D alone led to significant inhibition of hOAT1-mediated PAH transport ( $40.1 \pm 5.2\%$  inhibition), whereas colchicine consistently stimulated uptake by  $42.4 \pm$

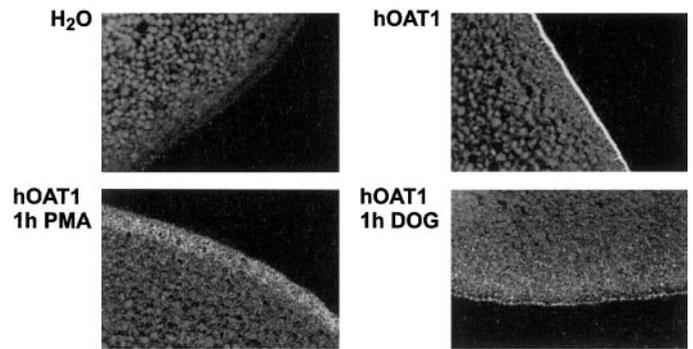


**Figure 3.** Time (A) and concentration (B) dependence of *sn*-1,2-dioctanoylglycerol (DOG) inhibition of hOAT1-mediated PAH uptake. Oocytes were injected with hOAT1 cRNA (●) or an equivalent volume of water (○). On day 3 after injection, they were preincubated for a total of 1 h at room temperature in the presence of DMSO but with 5 μM DOG for the indicated times only (A) or were preincubated for 1 h at room temperature in the absence (DMSO alone) or presence of various DOG concentrations (B). Fifteen-minute uptake of 10 μM [<sup>3</sup>H]PAH was then assayed. Results are expressed as percentages of the uptake in hOAT1-expressing oocytes without DOG. Data are means ± SEM of four (A) or three (B) independent experiments.

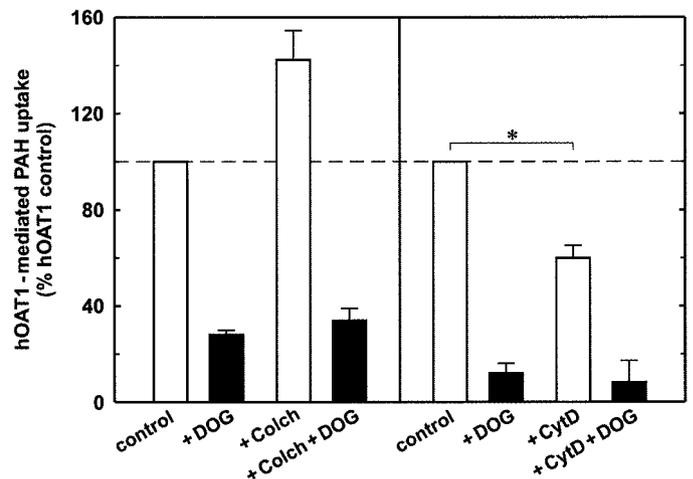
12.2%, suggesting some actin filament-dependent incorporation of hOAT1 into the membrane and microtubule-dependent internalization in unstimulated oocytes. The cytochalasin D effect on PAH uptake by hOAT1 was not attenuated by brefeldin A, an agent that reversibly blocks anterograde vesicular transport from the endoplasmic reticulum (30.9 ± 16.1% decrease without brefeldin A versus 37.4 ± 9.6% decrease in the presence of brefeldin A, *n* = 4).

**Dependence of DOG-Induced hOAT1 Downregulation on Potential hOAT1 PKC Sites**

To determine whether the DOG-induced downregulation of hOAT1 was attributable to PKC-dependent phosphorylation of



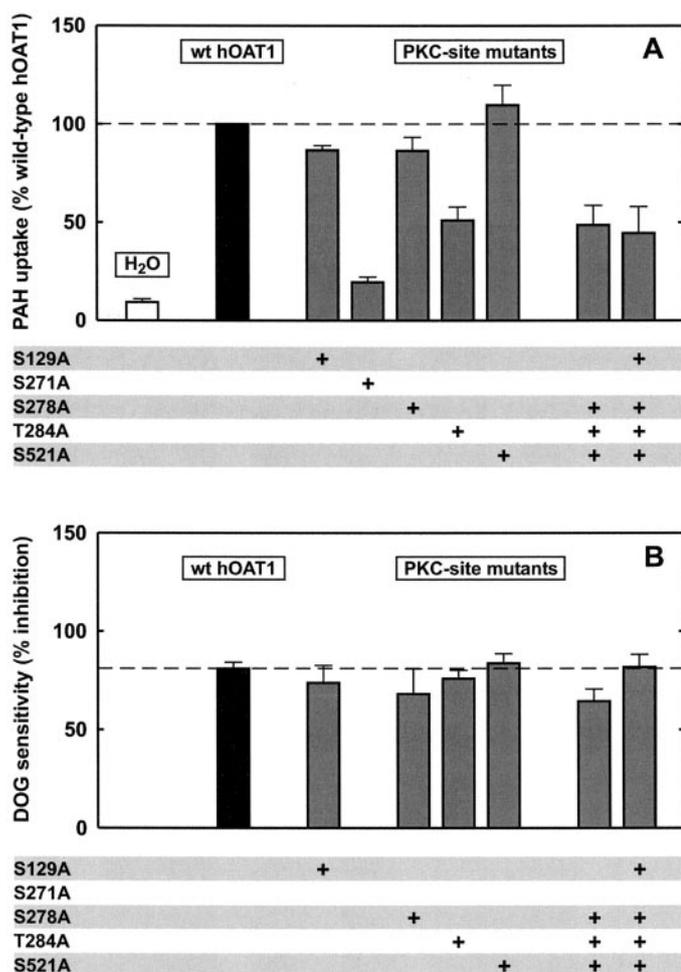
**Figure 4.** Immunocytochemical detection of hOAT1 localization after PMA or DOG treatment. Devitellinized oocytes expressing carboxy-terminally FLAG-tagged hOAT1 or injected with water were preincubated for 1 h at room temperature in the presence of 1 μM PMA, 5 μM DOG, or an equivalent amount of solvent alone. Oocytes were then immunostained with anti-FLAG M2 IgG, followed by secondary Alexa 546-goat anti-mouse IgG. Oocytes were embedded in acrylicamide, and 5-μm sections were analyzed with fluorescence microscopy. Magnification, ×250.



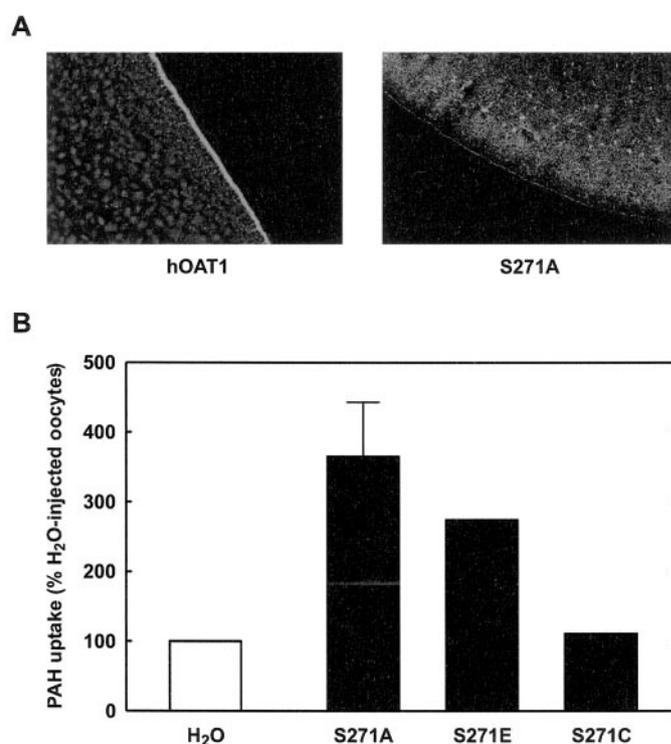
**Figure 5.** Effects of colchicine and cytochalasin D on DOG-induced downregulation of hOAT1. In two sets of experiments, oocytes were injected with hOAT1 cRNA or an equivalent volume of water. On day 3 after injection, they were pretreated at room temperature, first in the absence (ethanol only) or presence of colchicine (Colch) (20 μM, 5 h) (left) or cytochalasin D (CytD) (20 μM, 1 h) (right) and then for 1 h without (DMSO only) or with 5 μM DOG. Control cells were treated with solvent alone for the same time periods. Uptake of 10 μM [<sup>3</sup>H]PAH was then assayed for 1 h (left) or 15 min (right). Results are expressed as percentages of the cRNA-dependent uptake in the respective hOAT1-expressing control cells. Data are means + SEM of four independent experiments each. The significance of changes in PAH uptake induced by colchicine or cytochalasin D alone (two-tailed *t* test, Welch correction applied) is indicated (\**P* < 0.05).

hOAT1 itself, we removed potential PKC phosphorylation sites, both individually and in groups. Of the six canonical PKC sites of hOAT1, we initially mutated the five sites conserved between hOAT1 and the rabbit OAT1 orthologue, because it was previously demonstrated that PKC activation

downregulated basolateral organic anion transport in isolated rabbit proximal tubules (7,8). The mutants were analyzed for both their transport activity, relative to that of wild-type hOAT1, and their sensitivity to DOG inhibition. The disruption of PKC sites did not result in a loss of function (although transport activity was somewhat lower when mutation T284A was included) except in the case of S271A (Figure 6A), which was not correctly targeted to the oocyte membrane (Figure 7A). This finding seems to indicate a requirement for serine itself at this position, because neither mimicking of a phosphoserine by replacement with glutamate nor substitution with the structurally similar cysteine restored function (Figure 7B).



**Figure 6.** Transport activity (A) and DOG sensitivity (B) of protein kinase C (PKC) site mutants of hOAT1. Oocytes were injected with cRNA for wild-type (wt) hOAT1, one of the PKC site mutants (as indicated), or an equivalent volume of water. On day 3 after injection, they were preincubated for 1 h at room temperature in the absence (DMSO only) (A) or presence (B) of 5  $\mu$ M DOG. Uptake of 10  $\mu$ M [<sup>3</sup>H]PAH was then assayed for 15 min. Results are expressed as percentages of the uptake in wild-type hOAT1-expressing oocytes (A) or percentages of DOG inhibition of the respective cRNA-dependent uptake without DOG (B). Data are means  $\pm$  SEM of three (S278A/T284A/S521A and S129A/S278A/T284A/S521A), four (S129A and S278A), five (T284A), seven (S521A), eight (S271A), or 23 (water and wild-type hOAT1) independent experiments.

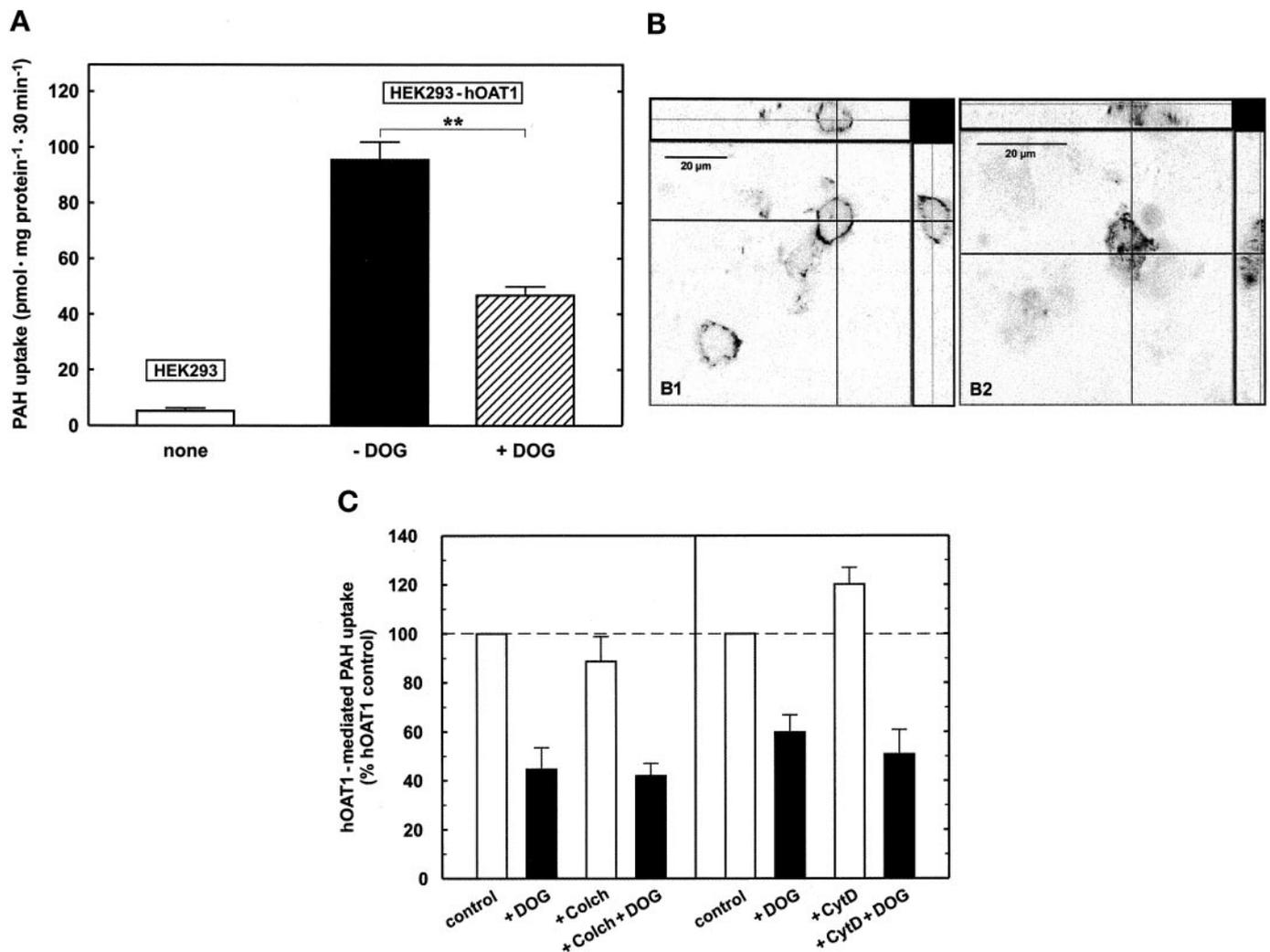


**Figure 7.** Immunocytochemical detection of hOAT1 mutant S271A (A) and mutant S271A transport activity, compared with mutants S271E and S271C (B). (A) Devitalized oocytes expressing the carboxy-terminally FLAG-tagged hOAT1 mutant S271A or injected with water were immunostained with anti-FLAG M2 IgG, followed by secondary Alexa 546-goat anti-mouse IgG. Oocytes were embedded in acrylamide, and 5- $\mu$ m sections were analyzed with fluorescence microscopy. Magnification,  $\times$ 250. (B) Oocytes expressing mutant S271A, S271E, or S271C or injected with water were assayed for 15-min uptake of 10  $\mu$ M [<sup>3</sup>H]PAH. Data are expressed as percentages of the uptake of water-injected oocytes, as the means (S271E and S271C,  $n = 2$ ) or means  $\pm$  SEM (S271A,  $n = 4$ ) of  $n$  independent experiments.

None of the mutants that retained function, however, exhibited significantly altered sensitivity to DOG (Figure 6B). Similarly, the T334A mutation at the only nonconserved site did not affect DOG sensitivity, compared with that of the wild-type protein ( $68.8 \pm 7.0\%$  inhibition for T334A *versus*  $59.3 \pm 0.3\%$  inhibition for wild-type hOAT1,  $n = 2$ ).

#### DOG-Induced Downregulation of hOAT1 Expressed in HEK293 Cells

To confirm that the DOG-induced downregulation of hOAT1 was not an oocyte-specific phenomenon, we also assessed the effects of DOG on hOAT1-mediated PAH uptake when hOAT1 was expressed in HEK293 cells. As demonstrated in Figure 8A, hOAT1 induced  $>20$ -fold greater PAH uptake in HEK293 cells, compared with nontransfected control cells, and this hOAT1-mediated uptake was significantly inhibited ( $53.9 \pm 2.3\%$  inhibition,  $n = 5$ ) by a 1-h preincubation with 5  $\mu$ M DOG. The PKC site mutant S129A/S278A/T284A/S521A induced PAH uptake of a similar magnitude, compared



**Figure 8.** DOG sensitivity of hOAT1 expressed in HEK293 cells. (A) Nontransfected or hOAT1-transfected cells were preincubated for 1 h at 37°C in the absence (DMSO only) or presence of 5 μM DOG. Uptake of 1.1 μM [<sup>3</sup>H]PAH was then assayed for 30 min at 37°C, in the absence or presence of 5 μM DOG as before. Data are means + SEM of five independent experiments, each performed in quadruplicate. The significance of DOG inhibition of PAH uptake in hOAT1-expressing cells (two-tailed *t* test, Welch correction applied) is indicated (\*\**P* < 0.01). (B) Cells transfected with extracellularly FLAG-tagged hOAT1 were live-stained with anti-FLAG M2 IgG, followed by treatment for 1 h at 37°C in the absence (DMSO only) (B1) or presence (B2) of 5 μM DOG. Cells were then fixed and permeabilized, incubated with secondary Alexa 488-goat anti-mouse IgG, mounted, and examined with confocal laser scanning microscopy. (C) In two sets of experiments, hOAT1-transfected cells were preincubated at 37°C, first without (ethanol only) or with colchicine (Colch) (10 μM, 5 h) (left) or cytochalasin D (CytD) (10 μM, 1 h) (right) and then for 1 h in the absence (DMSO only) or presence of 5 μM DOG. Control cells were treated with solvent alone for the same time periods. Uptake of 1.1 μM [<sup>3</sup>H]PAH was then assayed for 30 min at 37°C, in the absence or presence of 5 μM DOG as before. Data are expressed as percentages of DOG inhibition of hOAT1-mediated PAH uptake and are means + SEM of three independent experiments, each performed in triplicate.

with wild-type hOAT1 ( $100.2 \pm 6.1$  pmol/mg protein per 30 min *versus*  $88.0 \pm 1.6$  pmol/mg protein per 30 min;  $n = 4$  determinations in one experiment), and was equally sensitive to DOG inhibition (108% of the inhibition observed with wild-type hOAT1). As in *Xenopus* oocytes, 10 μM staurosporine only partially suppressed the DOG effect (*i.e.*, by approximately 35%; data not shown). DOG-induced hOAT1 redistribution in HEK293 cells was assayed by immunolocalization of primary antibody bound to expressed hOAT1 that had been extracellularly FLAG-tagged between amino acid residues 107 and 108. In this position, the FLAG epitope did not signifi-

cantly alter the transport activity of hOAT1 (untagged,  $5.5 \pm 1.1$  pmol/oocyte per 15 min; tagged,  $4.7 \pm 0.7$  pmol/oocyte per 15 min;  $n = 3$  experiments). Whereas control cells clearly demonstrated surface labeling (Figure 8B1), DOG treatment after antibody binding resulted in intense intracellular staining (Figure 8B2), as could occur only with DOG-dependent carrier protein retrieval from the cell membrane. As in oocytes, uptake measurements indicated that internalization was independent of microtubules or actin filaments, because the DOG-dependent reduction in hOAT1 transport activity was unaffected by colchicine or cytochalasin D (Figure 8C). Baseline hOAT1-

mediated uptake in HEK293 cells was also not significantly altered by these agents under the conditions used (colchicine,  $89 \pm 10\%$ ; cytochalasin D,  $120 \pm 7\%$ ;  $n = 3$  independent experiments) (Figure 8C).

## Discussion

A number of studies on intact renal proximal tubules (5,7) and renal proximal tubule cells in culture (9,33) have demonstrated PKC-dependent downregulation of the basolateral uptake of organic anions into cells, most likely mediated (at least in part) by the PAH-dicarboxylate exchanger OAT1 (2). How this downregulation is achieved is still unknown, however. This investigation was designed to determine whether transport inhibition occurs at the cell membrane or is achieved via carrier internalization and whether any of the classic PKC consensus sites of hOAT1 are involved. As our main expression system, we used *X. laevis* oocytes, which have been used for a variety of studies on the regulation of carriers and ion channels, such as the sodium/dicarboxylate cotransporter NaDC-1 (32), the sodium/phosphate cotransporter NaPi-II (30,34), the sodium/glucose cotransporter SGLT1 (35), and the epithelial Na<sup>+</sup> channel ENaC (36). In some cases, this system reflects regulation in the original cellular environment more accurately than does the HEK293 cell system (37).

We observed that hOAT1 was inhibited after PKC activation with PMA and/or DOG in oocytes and HEK293 cells, similar to findings in intact renal proximal tubule cells. The PMA effect in oocytes could not be mimicked by the inactive phorbol ester 4 $\alpha$ -PDD. However, only partial suppression of DOG inhibition was produced by staurosporine, in both oocytes and HEK293 cells, corresponding to previous findings for NaPi-II (30). This might be partly attributable to the opposing (*i.e.*, inhibitory) effect observed with staurosporine alone. Immunocytochemical analyses demonstrated that preincubation of hOAT1-expressing oocytes with either the nonselective PKC activator PMA or the cell-permeable synthetic diacylglycerol analogue DOG or preincubation of hOAT1-expressing HEK293 cells with DOG resulted in relocalization of hOAT1 to submembranous compartments. Therefore, hOAT1 internalization after PKC activation seems to be a general phenomenon that is not specific to the expression system used. Membrane retrieval as a means of carrier downregulation was previously reported for several systems, including NaPi-II (30), the dopamine transporter DAT (38), the norepinephrine transporter NET (39), and NaDC-1 (32). A number of studies have indicated a role for cytoskeletal elements in carrier and channel trafficking. For example, PMA-induced internalization of NaDC-1 was partially prevented by the microfilament-disrupting agent cytochalasin D, although it was microtubule independent (32), whereas PTH-induced retrieval of the Na<sup>+</sup>/H<sup>+</sup> exchanger NHE-3 was completely blocked when microtubules were destabilized with colchicine (40). In contrast, in PTH-dependent endocytic removal of NaPi-II, which is most likely mediated by the protein kinase A and/or PKC pathways (41), not the internalization itself but only the subsequent trafficking step was dependent on microtubules (31). In this study, DOG-induced hOAT1 inhibition in oocytes and HEK293 cells was

not affected by either colchicine (or nocodazole, tested on oocytes only) or cytochalasin D. Although we did not directly demonstrate that DOG-dependent hOAT1 redistribution still occurred after those treatments, quantitatively similar reductions in hOAT1-mediated transport indicated unaltered carrier retrieval. Direct effects of colchicine or cytochalasin D on hOAT1 function, although unlikely because of the thorough washing performed between preincubation and uptake measurements, would not be expected to differ from those in non-DOG-treated cells in relative magnitude. Because our data suggest that at least the internalization step in DOG-induced hOAT1 endocytosis does not involve microtubules or microfilaments in either expression system, other possible mechanisms for hOAT1 internalization, including clathrin-mediated endocytosis, must be considered. Interestingly, both colchicine and cytochalasin D consistently altered baseline (*i.e.*, not DOG-modified) hOAT1-mediated transport in oocytes. Whereas colchicine stimulated transport, cytochalasin D produced a significant decrease; this decrease apparently did not involve inhibited incorporation of newly synthesized protein, because the effect was also observed in the presence of brefeldin A. This suggests that there is not a stable pool of hOAT1 in the oocyte membrane but that hOAT1 cycles between the membrane and intracellular compartments, with colchicine-sensitive retrieval and a cytochalasin D-sensitive insertion step. The same pattern was not detected in HEK293 cells; cycling in that system, if present, might be slower and could differ in mechanism, because there was a slight tendency toward the opposite effects. An inhibitory action of cytochalasin D alone on uptake activity was previously reported for the serotonin transporter SET expressed in COS-7 cells (42), which was attributed by the authors to SET regulation by the state of the actin skeleton. In contrast to our results with hOAT1, however, SET activity was not affected by colchicine.

None of the putative PKC phosphorylation sites seemed to be involved in DOG-dependent hOAT1 phosphorylation, because none of the functional hOAT1 PKC site mutants exhibited decreased DOG sensitivity; mutation of Ser271 was still lethal with replacement by glutamate, mimicking a phosphorylated serine. Even when all four mutations that did not disrupt hOAT1 function were introduced (PKC quadruple-mutant), DOG sensitivity was not significantly different from that of wild-type hOAT1. This argues against the possibility that phosphorylation of multiple canonical sites might be required, as previously demonstrated for protein kinase A regulation of NHE-3 (43) and suggested as one explanation for PKC-induced phosphorylation of the serotonin transporter, which could not be abolished by removal of individual PKC consensus sites (44). As in oocytes, the hOAT1 PKC quadruple-mutant exhibited DOG sensitivity comparable to that of the wild-type protein in HEK293 cells. Similarly, PKC-dependent regulation of a number of other transporters, including NaPi-II (34), NaDC-1 (32), the glycine transporter GLYT1b (45), DAT (38), and the  $\gamma$ -aminobutyric acid transporter GAT1 (46), has been demonstrated to be independent of their canonical PKC phosphorylation sites. These data argue against direct PKC-dependent hOAT1 phosphorylation and suggest that another

protein involved in hOAT1 internalization might be phosphorylated, as postulated for GLYT1b (45) and DAT (38). Alternatively, other (atypical) hOAT1 PKC phosphorylation sites might be involved, as previously demonstrated for the glutamate transporter GLAST-1 (47) and the Na<sup>+</sup>/K<sup>+</sup>-ATPase  $\alpha$ -subunit (48).

Interestingly, the apparently critical residue Ser271 is part of a motif conserved not only among OAT (P/X-E-S-X-R-W-L/X in OAT1 through OAT4, adjacent to transmembrane domain 6) but also among all members of the organic cation transporter family (Transporter Commission no. 2.A.1.19), as well as in several other members of the major facilitator superfamily (Transporter Commission no. 2.A.1) (49). This motif might thus constitute an anchor point preserving membrane topologic features, as recently demonstrated for another similarly conserved motif located equally close to predicted transmembrane domains (50).

In summary, we have demonstrated that hOAT1 expressed in *Xenopus* oocytes and in HEK293 cells is downregulated by specific pharmacologic PKC activation. This is consistent with the PKC-induced downregulation of the basolateral uptake of organic anions (a process mediated at least in part by OAT1) observed in intact renal tubule cells in animal models. DOG-induced inhibition of hOAT1-dependent transport seems to occur via carrier internalization, which seems not to involve actin filaments or microtubules. Because this downregulation is independent of the known hOAT1 PKC consensus sites, the PKC targets are either atypical PKC sites in hOAT1 itself or sites in an unknown mediator protein. Studies with isolated human renal proximal tubules are needed to confirm the data obtained with heterologous expression systems and animal models and to demonstrate hOAT1 downregulation after physiologic PKC activation (e.g., by PTH or angiotensin II).

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