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RESEARCH PAPER

Protein kinase C regulates the internalization and function of the human organic anion transporting polypeptide 1A2

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BACKGROUND AND PURPOSE

The human organic anion transporting polypeptide 1A2 (OATP1A2) is expressed in cells from several regions of the human body, including the kidney, cholangiocytes and the blood-brain barrier, and mediates the cellular flux of various anionic substances, including drugs in clinical use. Several related mammalian transporters have been shown to be subject to post-translational regulation, including kinase-induced internalization. In the present study the role of protein kinase C (PKC) in the regulation of OATP1A2 was investigated in an *in vitro* cell model.

EXPERIMENTAL APPROACH

COS-7 cells in which OATP1A2 was overexpressed were treated with the PKC-specific activator (phorbol 12-myristate 13-acetate; PMA) and the PKC-specific inhibitor (Go6976). The impact of these treatments on the function and regulation of OATP1A2 was determined.

KEY RESULTS

PKC activation decreased the transport function of OATP1A2 in a time- and concentration-dependent manner. PMA (0.1 μM) decreased the V_{max} of oestrone-3-sulphate uptake and decreased the cell surface expression of OATP1A2 immunoreactive protein; these effects of PMA were prevented by the PKC specific inhibitor Go6976. In further studies, PMA treatment accelerated the internalization of OATP1A2 but did not affect its recycling. The disruption of clathrine-dependent endocytosis attenuated both the constitutive and PKC-modulated internalization of OATP1A2. In contrast, blocking the caveolin-dependent pathway was without effect.

CONCLUSIONS AND IMPLICATIONS

PKC regulates the transport function of OATP1A2 by modulating protein internalization; this effect of PKC is mediated in part by clathrine-dependent pathways.

Abbreviations

ES, oestrone-3-sulphate; Go6976, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a) pyrrolo(3,4-c)-carbazole; hOATs, human organic anion transporters; OATP1A2, organic anion transporting polypeptide 1A2; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate

Introduction

Organic anion transporting polypeptides (OATPs) are a family of sodium-independent transporters with broad substrate specificity, which plays critical roles in drug absorption, hepatic and renal clearance, and the distribution of drugs in tissues (Hagenbuch and Meier, 2004). Among the OATPs, OATP1A2 (OATP-A, OATP-1, SLCO1A2) was the first human isoform to be cloned and has been shown to transport the largest number of structurally diverse compounds, including the drugs methotrexate, fexofenadine and ouabain (Kullak-Ublick *et al.*, 1995; 2001; Cvetkovic *et al.*, 1999; Gao *et al.*, 2000; Badagnani *et al.*, 2006). OATP1A2 is expressed in cells from several regions of the human body, including the kidney, cholangiocytes and the blood-brain barrier; thus, OATP1A2 is an important determinant of drug pharmacokinetics and toxicity (Gao *et al.*, 2000; Su *et al.*, 2004; Lee *et al.*, 2005; Glaeser *et al.*, 2007). *OATP1A2* gene variants encode transporters that have altered functional capacity (Lee *et al.*, 2005; Badagnani *et al.*, 2006; Franke *et al.*, 2009). Therefore, understanding the regulation of OATP1A2 has profound clinical significance.

Several important regulatory mechanisms have emerged for *OATP* and *OAT* genes. Post-translational processing is important, including N-glycosylation, which regulates transporter expression at the plasma membrane (Lee *et al.*, 2003; Zhou *et al.*, 2005) and phosphorylation by protein kinases (Zhou *et al.*, 2006; Kock *et al.*, 2010). It is also known that the *OATP1A2* gene is transactivated by steroids and xenobiotics that are ligands for nuclear receptors (Miki *et al.*, 2006; Meyer zu Schwabedissen *et al.*, 2008).

Membrane proteins usually follow a constitutive internalization/recycling process involving shuttling between the cell surface and intracellular compartments. There are several potential mechanisms by which protein kinases could modulate the expression of membrane proteins, including direct phosphorylation (Huff *et al.*, 1997; Li *et al.*, 1998; Ramamoorthy *et al.*, 1998), altered protein internalization or modulation of recycling between the cell surface and intracellular compartments (Zhang *et al.*, 2008; Elnakat *et al.*, 2009; Kock *et al.*, 2010; Zhang *et al.*, 2010). Recently protein kinase C (PKC) has been shown to modulate both the internalization and recycling of several organic anion transporters (Zhang *et al.*, 2008; 2010; Kock *et al.*, 2010). Fewer studies have evaluated OATP transporters, but very recently PKC has been implicated as a regulator of OATP2B1 (Kock *et al.*, 2010).

In the current study, the regulatory actions of PKC on OATP1A2 transporter function were investigated in detail. The inhibition of OATP1A2 function was found to be due to altered transporter internalization/recycling and the trafficking pathway involved in this process was identified.

Methods

Materials

[^{3}H]-oestrone sulphate (ES; 57.3 Ci·mmol^{-1}) was purchased from PerkinElmer (Melbourne, VIC, Australia). Culture media were obtained from Thermo Scientific (Lidcombe, NSW, Australia). All other chemicals were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia).

Generation of OATP1A2 expressing cells

The OATP1A2 cDNA was generously provided by Professor Bruno Stieger from University of Zurich, Switzerland. The cDNA was then sub-cloned into the PCI vector (Promega, Alexandria, New South Wales, Australia). All sequences were confirmed by the dideoxy chain termination method (Supamac, Camperdown, New South Wales, Australia). COS-7 cells were maintained at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were transfected with plasmid DNA using Lipofectamine 2000 reagent (Invitrogen, Mount Waverley, Victoria, Australia) following the manufacturer's instructions. Twenty-four hours after transfection, substrate transport activities were measured.

Transport uptake assay

Uptake of [^{3}H]-ES was initiated at room temperature in phosphate-buffered saline (PBS), pH 7, containing 5 mM glucose and 300 nM [^{3}H]-ES, and was terminated at varying times by rapidly washing the cells in ice-cold PBS. The cells were then solubilized in 0.2 M NaOH, neutralized with 0.2 M HCl, and aliquoted for liquid scintillation counting. Uptake count was standardized by the amount of protein in each well. Data are presented as mean ± SE ($n = 3$).

Cell-surface biotinylation

Cell-surface expression of OATP1A2 was examined using the membrane-impermeant biotinylation reagent, NHS-SS-biotin (Quantum Scientific, Lane Cove West, New South Wales, Australia). OATP1A2 was expressed in COS-7 cells in six-well plates using Lipofectamine 2000, as described previously. After 24 h, the medium was removed, and the cells were washed successively with 3 mL aliquots of ice-cold PBS (pH 8.0). Cells were incubated on ice with 1 mL of freshly prepared NHS-SS-biotin (0.5 mg·mL^{-1} in PBS) for 30 min with gentle shaking. After biotinylation, cells were washed with 3 mL of PBS containing 100 mM glycine and then incubated on ice for 20 min to ensure complete quenching of the unreacted NHS-SS-biotin. The cells were then treated with 400 µL of lysis buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, containing the protease inhibitors phenylmethylsulphonyl fluoride, 200 µg·mL^{-1}, and leupeptin, 3 µg·mL^{-1}, pH 7.4) for 30 min. Unlysed cells were removed by centrifugation at 14 000× *g* at 4°C. Streptavidin-agarose beads (50 µL; Quantum Scientific) were then added to the supernatant to isolate cell membrane protein.

Internalization assay

OATP1A2 expressing cells were biotinylated with 1.0 mg·mL^{-1} sulpho-NHS-SS-biotin at 4°C as described by Zhang *et al.*, (2008). One set of cells was quenched with glycine and washed with PBS, then kept at 4°C in order to determine the total initial surface expression of OATP1A2 and stripping efficiencies. The second set of cells was washed twice with pre-warmed PBS (37°C) so as to facilitate internalization. Cells were then incubated in PBS with 1 µM phorbol 12-myristate 13-acetate (PMA) or vehicle at 37°C for varying

times. Excess cell surface biotin was stripped by incubating cells with freshly prepared 50 mM sodium 2-mercaptoethanesulphonate in NT buffer (150 mM NaCl, 1 mM EDTA, 0.2% bovine serum albumin, 20 mM Tris, pH 8.6) for 30 min twice. Cells were lysed in lysis buffer that contained the combination of protease inhibitors. Unlysed cells were removed by centrifugation and streptavidin-agarose beads were then added to the supernatant to isolate cell membrane protein. Relative OATP1A2 internalization was calculated as a percentage of the total initial cell surface pool of OATP1A2.

Recycling assay

OATP1A2 expressing cells were treated with 1.0 mg·mL^{-1} sulpho-NHS-SS-biotin at 4°C for 30 min as described by Zhang *et al.*, (2008). One set of cells was subjected to continuous biotinylation at 4°C with fresh sulpho-NHS-SS-biotin (1.0 mg·mL^{-1}). The parallel set of cells was warmed to 37°C and continuously biotinylated with fresh sulpho-NHS-SS-biotin (1.0 mg·mL^{-1}) together with 1 μM PMA or vehicle for varying durations. Biotinylation was terminated and the biotin-labelled cells were washed and lysed. Unlysed cells were removed by centrifugation and streptavidin-agarose beads were then added to the supernatant to isolate cell membrane protein. The relative amount of recycled OATP1A2 was calculated as the difference between labelled transporter protein measured at 37°C and at 4°C.

Electrophoresis and immunoblotting

Cell membrane protein samples were loaded onto 7.5% polyacrylamide minigels and electrophoresed (Bio-Rad, Gladesville, New South Wales, Australia). Proteins were transferred to polyvinylidene fluoride membranes in an electroelution cell (Bio-Rad) and blocked for 1 h with 5% non-fat dry milk in PBS-Tween (80 mM Na_2HPO_4, 20 mM KH_2PO_4, 100 mM NaCl and 0.05% Tween 20, pH 7.5), washed, and then incubated overnight at 4°C with anti-OATP1A2 antibody (1 μg·mL^{-1}; Sigma-Aldrich, Castle Hill, New South Wales, Australia). The membranes were washed, incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:5000), and signals were detected using a SuperSignal West Pico Chemiluminescent Substrate (Quantum Scientific).

Immunofluorescence of transfected cells

Cells expressing OATP1A2 were washed three times in PBS, fixed for 20 min at room temperature in 4% paraformaldehyde in PBS and rewashed in PBS. The fixed cells were then permeabilized with 0.1% Triton X-100 for 10 min. The cells were incubated for 30 min at room temperature in PBS containing 5% goat serum and then incubated for 2 h in the same medium containing anti-OATP1A2 antibody (1:100) at room temperature. The cells were washed, and bound primary antibodies were detected by reaction with Alexa Fluor® 594 conjugate goat anti-rabbit IgG (Invitrogen, Victoria, Australia) diluted to 1:1000 for 1 h. Treated cells were washed thoroughly, and the cover glasses were mounted in DPX Neutral mounting medium (Lomb Scientific, Taren Point, New South Wales, Australia). Samples were visualized with a Leica DMI3000 B epi florescence microscope (Leica Microsystems, North Ryde, New South Wales, Australia).

Statistics

Differences between control and single treatments were detected using Student's *t*-test. Differences between multiple treatments were detected by ANOVA and Fisher's protected least significant difference test.

Results

Effect of PMA on OATP1A2 activity

In initial experiments, the acute effects of the PKC-specific activator PMA on OATP1A2 activity were assessed. Thus, COS-7 cells that over-expressed OATP1A2 were treated with PMA (1 μM) for varying periods at 37°C (Figure 1A). Within the time frame of 1.5 h, PMA resulted in a time-dependent decrease in ES uptake by OATP1A2. OATP1A2 over-expressing

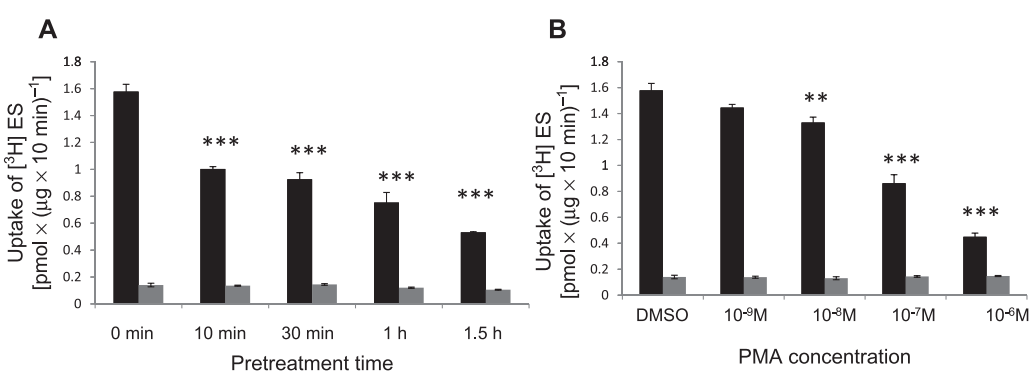
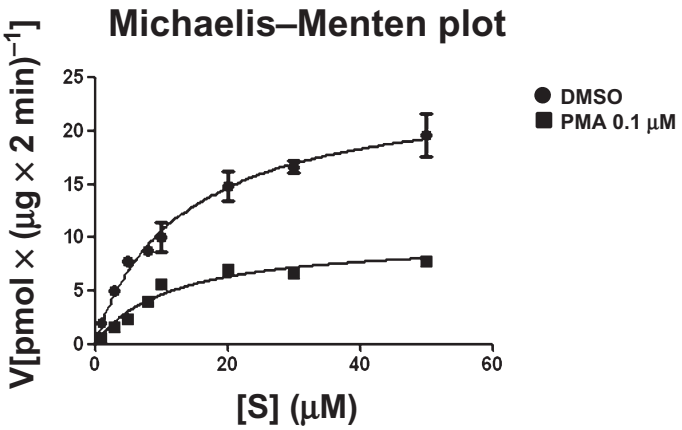


Figure 1

Altered uptake of [^{3}H]-oestrone sulphate (ES) by OATP1A2 over-expressing COS-7 cells following PMA treatment. (A) Transport of 300 nM [^{3}H]-ES in OATP1A2 transfected COS-7 cells after treatment with PMA (1 μM) at 37°C for various durations. (B) Concentration-dependent effects of PMA on the transport of 300 nM [^{3}H]-ES in OATP1A2 transfected COS-7 cells (1 h, 37°C). Black bars: uptake in OATP1A2 expressing cells; grey bars: uptake in vector-transfected cells. Values are mean ± SE ($n = 3$). $^{**}P < 0.05$. $^{***}P < 0.001$, different from DMSO-control. DMSO, dimethyl sulphoxide; OATP1A2, organic anion transporting polypeptide 1A2; PMA, phorbol 12-myristate 13-acetate.

A



B

	K_m (µM)	V_{max} [pmol × (µg × 2 min)$^{-1}$]
DMSO	12.84 ± 2.09	24.14 ± 1.57
PMA 0.1 µM	11.23 ± 1.67	9.77 ± 0.55

Figure 2

Kinetic analysis of oestrone sulphate (ES) transport mediated by OATP1A2 in the presence or absence of PMA treatment. Kinetic characteristics were determined at substrate concentrations ranging from 1 to 50 µM (2 min uptake) using cells expressing OATP1A2 after treatment with PMA (0.1 µM) or DMSO for 1 h at 37°C. Transporter kinetic parameters were calculated using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Values are mean ± SE (n = 3). (A) Direct Michaelis–Menten plot, and (B) K_m and V_{max} values for ES transport by OATP1A2. DMSO, dimethyl sulphoxide; OATP1A2, organic anion transporting polypeptide 1A2; PMA, phorbol 12-myristate 13-acetate.

COS-7 cells were also treated with different concentrations of PMA (10^{-9} M to 10^{-6} M) at 37°C for 1 h (Figure 1B); a concentration-dependent decrease in ES uptake was effected by PMA. In order to further examine the mechanism by which PMA decreased OATP1A2 activity, we measured the substrate concentration-dependence of [^{3}H]-ES uptake in OATP1A2 over-expressing cells. Michaelis–Menten analysis indicated that PMA pretreatment decreased the maximal rate of ES uptake [V_{max} 9.77 ± 0.55 pmol·(µg × 2 min)$^{-1}$ relative to 24.14 ± 1.57 pmol·(µg × 2 min)$^{-1}$ in dimethyl sulphoxide-treated control cells], without altering the apparent affinity of the transporter for ES (11.23 ± 1.67 µM relative to 12.84 ± 2.09 µM in control cells) (Figure 2). The observed K_m for ES in the present study is comparable to that in HeLa cells (Lee *et al.*, 2005). Importantly, linearity studies showed that ES uptake in the present system was not saturated up to 20 min and protein concentration measurement confirmed that PMA treatment did not change total protein content (data not shown).

PKC activation modulates OATP1A2 distribution in cells

The decreased V_{max} of ES transport by OATP1A2 in response to PMA treatment may be due to decreased transporter expression at the cell surface or to decreased turnover of the transporter-substrate complex. From Western immunoblot analysis, PMA treatment did not affect the expression of total OATP1A2 in cells (Figure 3A), but decreased the amount of OATP1A2 available at the cell surface (Figure 3B). These findings were corroborated by immunofluorescence (Figure 4).

To test the possibility that the effect of PMA on OATP1A2 is due to activation of PKC signalling pathways, the PKC specific inhibitor, Go6976 (1 µM), was applied to COS-7 cells together with PMA. Co-treatment with Go6976 prevented the PMA-induced loss of OATP1A2 activity (Figure 3C) and the decline in cell surface expression of OATP1A2 (Figure 3B). Go6976 is a selective PKC inhibitor of PKC α- and β1-isozymes, the expression of which has been confirmed in COS-7 cells (Adomeit *et al.*, 1999; Li *et al.*, 2009; Duan *et al.*, 2010). Taken together, these findings strongly implicate the activation of PKC signalling in the PMA-induced decrease in OATP1A2 expression and activity in COS-7 cells; this may involve the PKC α- and β1-isozymes.

PKC activation regulates the cellular internalization of OATP1A2

The decreased cell surface expression of OATP1A2 protein following PKC activation could be due to an increase in protein internalization, a decrease in recycling between the plasma membrane and intracellular compartments, or a combination of both. Previous studies have indicated that PKC activation impairs the function of anion transporters by accelerating protein internalization (Zhang *et al.*, 2008; Kock *et al.*, 2010) or by modulating recycling (Zhang *et al.*, 2008; 2010; Elnakat *et al.*, 2009; Kock *et al.*, 2010). The concentration and time dependence of PMA treatment shown in Figure 1 indicate that treatment with 0.1 µM PMA for 1 h or 1 µM PMA for 15 min both decreased OATP1A2 function. The treatment with 0.1 µM PMA for 1 h was then used in all experiments with the exception of the internalization and recycling studies (Figure 5), which require a linear time frame. Because the sensitivity of Western blotting is lower than transporter functional assays, the higher PMA concentration (1 µM) was optimal for these experiments over the stated time frame. In the present study, the potential involvement of transporter internalization in the regulation of OATP1A2 by PKC was investigated. As shown in Figure 5A, OATP1A2 is subject to constitutive internalization. Biotinylated cells were rapidly warmed with PBS at 37°C; this facilitated transporter internalization that was readily detected

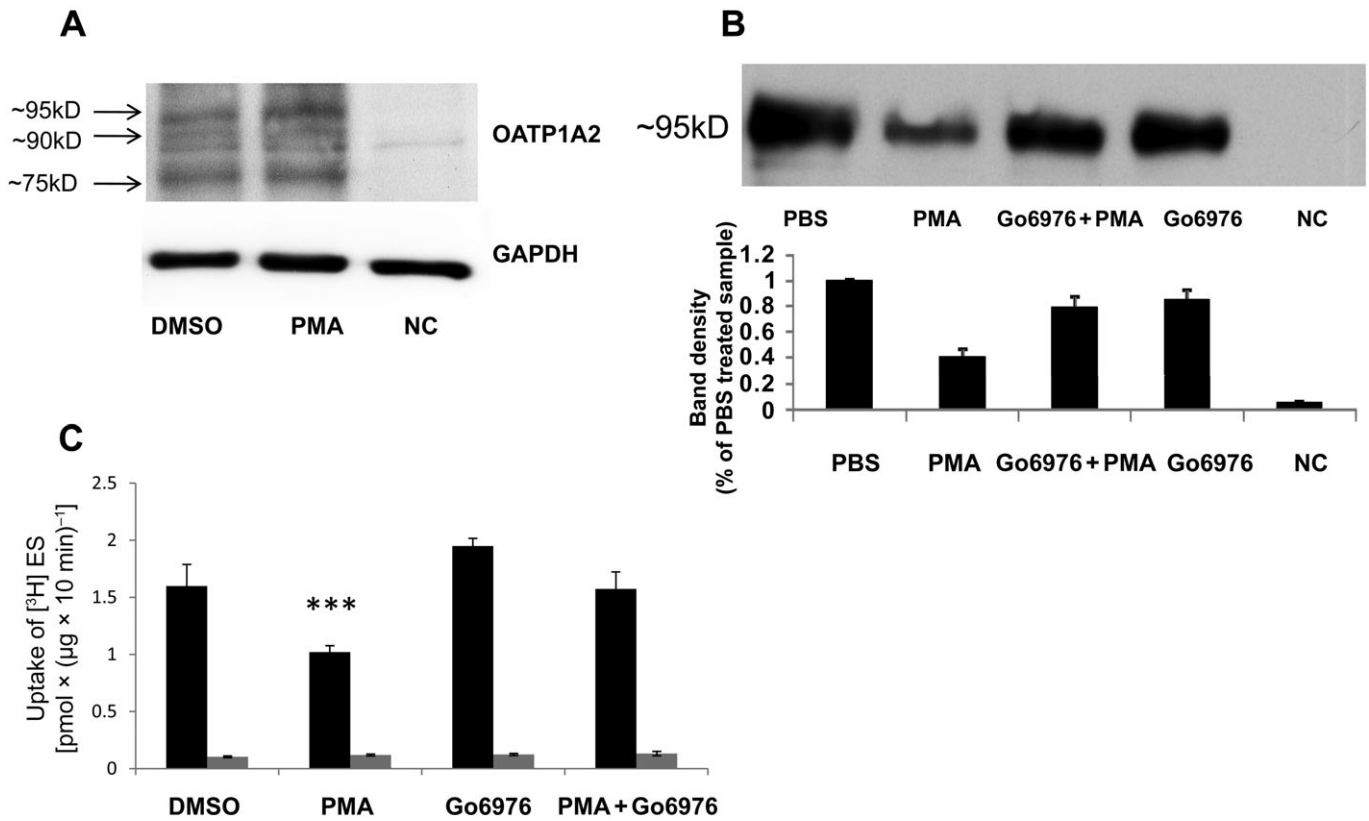


Figure 3

OATP1A2 transporter activity and protein expression of OATP1A2 in the presence and absence of the PKC activator PMA and the inhibitor Go6976. (A) Western blot analysis of total cell expression of OATP1A2 with or without PMA treatment. OATP1A2 over-expressing cells were incubated with DMSO or 0.1 μM PMA for 1 h at 37°C. Top panel: cells were lysed, and proteins were separated by SDS-polyacrylamide gel electrophoresis, followed by Western blotting with anti-OATP1A2 antibody. Bottom panel: after being stripped, the blot was reprobed with anti-GAPDH antibody. NC: parental COS-7 cells without OATP1A2 expression. (B) Western blot analysis of cell surface expression of OATP1A2 in the presence or absence of PMA treatment and/or Go6976. The OATP1A2 over-expressing cells were incubated with PMA (0.1 μM) or Go6976 (1 μM) or both for 1 h at 37°C. Top panel: cells were biotinylated, and the labelled cell surface proteins were precipitated with streptavidin beads and separated by gel electrophoresis, followed by Western blotting with anti-OATP1A2 antibody. Bottom panel: densitometric analysis of transporter expression. NC: Parental COS-7 cells without OATP1A2 expression. (C) Uptake of [^{3}H]-ES by OATP1A2 expressing COS-7 cells in the presence or absence of the PKC activator and/or inhibitor. The transport of 300 nM [^{3}H]-ES in OATP1A2 transfected COS-7 cells with 0.1 μM PMA or 1 μM Go6976 or both for 1 h at 37°C. Black columns: uptake in OATP1A2 expressing cells; grey columns: uptake in vector-transfected cells. ***$P < 0.001$, different from DMSO-treated group. DMSO, dimethyl sulphoxide; ES, oestrone sulphate; OATP1A2, organic anion transporting polypeptide 1A2; PBS, phosphate-buffered saline; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

after 5 and 15 min. PMA treatment accelerated this process and led to a pronounced accumulation of OATP1A2 immunoreactive protein by 15 min (Figure 5A,B). In contrast with these findings, PMA treatment did not significantly affect OATP1A2 trafficking from intracellular compartments back to the cell membrane (Figure 5C,D).

OATP1A2 internalization by PKC activation is partially clathrine dependent

The three principal pathways of transporter internalization are clathrin dependent, caveolin dependent and clathrine/caveolin independent. Previous studies have shown that human organic anion transporter 1 (hOAT1) and OATP2B1 follow clathrine-dependent internalization in *in vitro* renal cell models (Zhang *et al.*, 2008; Kock *et al.*, 2010), while certain other transporters are subject to caveolin-dependent endocytosis, such as the noradrenaline transporter in human placental trophoblast cells (Jayanthi *et al.*, 2006). During transporter internalization, protein relocates from the cell membrane to the cytoplasm by associating with clathrine-coated pits and/or caveolin-enriched membrane invaginations (Pelkmans and Helenius, 2002; Mousavi *et al.*, 2004).

In this study, chemical treatments that specifically inhibit clathrine-or caveolin-dependent pathways of endocytosis were used to evaluate the mechanism of OATP1A2 internalization. In COS-7 cells, clathrin-dependent protein internalization can be prevented by hypertonic sucrose, and acidification of the cytoplasm (Huang *et al.*, 1995; Ito *et al.*, 1999; Vandenbulcke *et al.*, 2000; Proulx *et al.*, 2005; Kock *et al.*, 2010), whereas caveolin-dependent endocytosis can be effectively inhibited by filipin (Anderson, 1998; Pawson *et al.*, 2003; Kock *et al.*, 2010) and nystatin (Simpson *et al.*, 1998; Kock *et al.*, 2010). As shown in Figure 6, treatment with

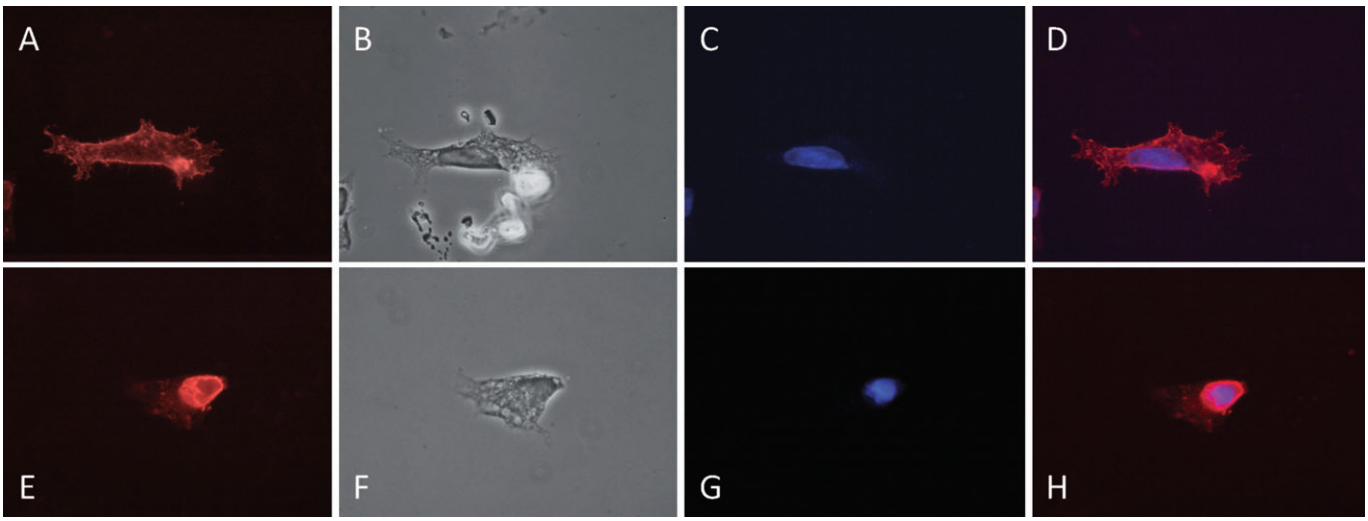


Figure 4

Immunofluorescence analysis of OATP1A2 expressed in COS-7 cells. Cells that over-expressed OATP1A2 were treated with 1 μM PMA for 1 h at 37°C (E–H) or untreated (A–D). The cells were then stained with anti-OATP1A2 antibody and Alexa Fluor® 594 conjugate goat anti-rabbit IgG. (A and E) The specific immunostaining of OATP1A2, which appears as bright red fluorescence (B and F) are phase-contrast images and show that cells remain fully attached to the culture dishes under the test conditions; (C and G) show nuclear staining in blue fluorescence, and (D and H) are merged images showing OATP1A2 staining, nuclear staining and phase-contrast images. OATP1A2, organic anion transporting polypeptide 1A2; PMA, phorbol 12-myristate 13-acetate.

sucrose or acetic acid, but not filipin or nystatin, attenuated both the constitutive and PMA-regulated internalization of OATP1A2.

Discussion

The human transporter OATP1A2 is widely expressed in several key organs, such as brain and kidney. OATP1A2 transports a number of clinically important drugs as well as endogenous hormonal agents, and mediates the cellular influx and elimination of these agents. However, at present, our understanding of the factors that regulate OATP1A2 is relatively incomplete; thus, elucidation of important regulatory mechanisms could be of profound clinical and physiological significance.

In a previous study, PKC was shown to suppress rat oatp-1 and -2 mediated uptake (Guo and Klaassen, 2001). Subsequent studies with the related organic anion transporters (OATs) and OATPs have also implicated PKC as an important regulatory factor of these transporters (Zhou *et al.*, 2007; Zhang *et al.*, 2008; 2010; Kock *et al.*, 2010). In the present study, OATP1A2 transporter activity and expression at the plasma membrane were decreased by treatment with PMA, a specific activator of PKC, in a concentration- and time-dependent manner; the actions of PMA were prevented by Go6976, a PKC-α and PKC-β1 specific inhibitor. These data strongly implicate PKC signalling in the control OATP1A2 transport function. From kinetic analyses, PKC activation was found to decrease the V_{max} of ES transport, without affecting the affinity of the transporter for its substrate (K_m).

Cell surface biotinylation was used to demonstrate a decrease in expression of OATP1A2 at the plasma membrane following PMA treatment, which was prevented by co-treatment with Go6976. Because membrane transporter proteins undergo dynamic internalization/recycling involving shuttling between the cell surface and intracellular compartments, the impaired membrane expression of protein may result from increased internalization or decreased cellular recycling. In the present study, biotinylation-based internalization and recycling studies were used to further investigate the loss of OATP1A2 expression at the plasma membrane following PKC activation. The principal findings to emerge were that OATP1A2 is subject to constitutive internalization in COS-7 cells and that activation of PKC signalling accelerated this process. Although OATP1A2 also undergoes cellular recycling, this was refractory to PKC activation. Thus, PKC activation decreased the transport capacity of OATP1A2 by accelerating its internalization, which led to impaired expression at the plasma membrane.

Alternate routes of OATP1A2 internalization were evaluated in the present study using selective inhibitors. The inhibition of the formation of clathrine-coated pits by treatment of cells with hypertonic sucrose and by acidification of the cytoplasm attenuated both the constitutive and PKC-modulated internalization of OATP1A2, while the inhibition of caveolin-dependent pathways had no effect. Thus, the present data suggest that OATP1A2 internalization is partially dependent on clathrin-dependent endocytosis but not on caveolin-dependent routes. This finding resembles the importance of PKC in OAT1 and OATP2B1 internalization (Kock *et al.*, 2010; Zhang *et al.*, 2010). In the latter study, it was also shown that PKC activation resulted in an increased phosophorylation of OATP2B1 protein (Kock *et al.*, 2010).

The intracellular loops of several OAT and OATP transporters have been reported to contain serine/threonine

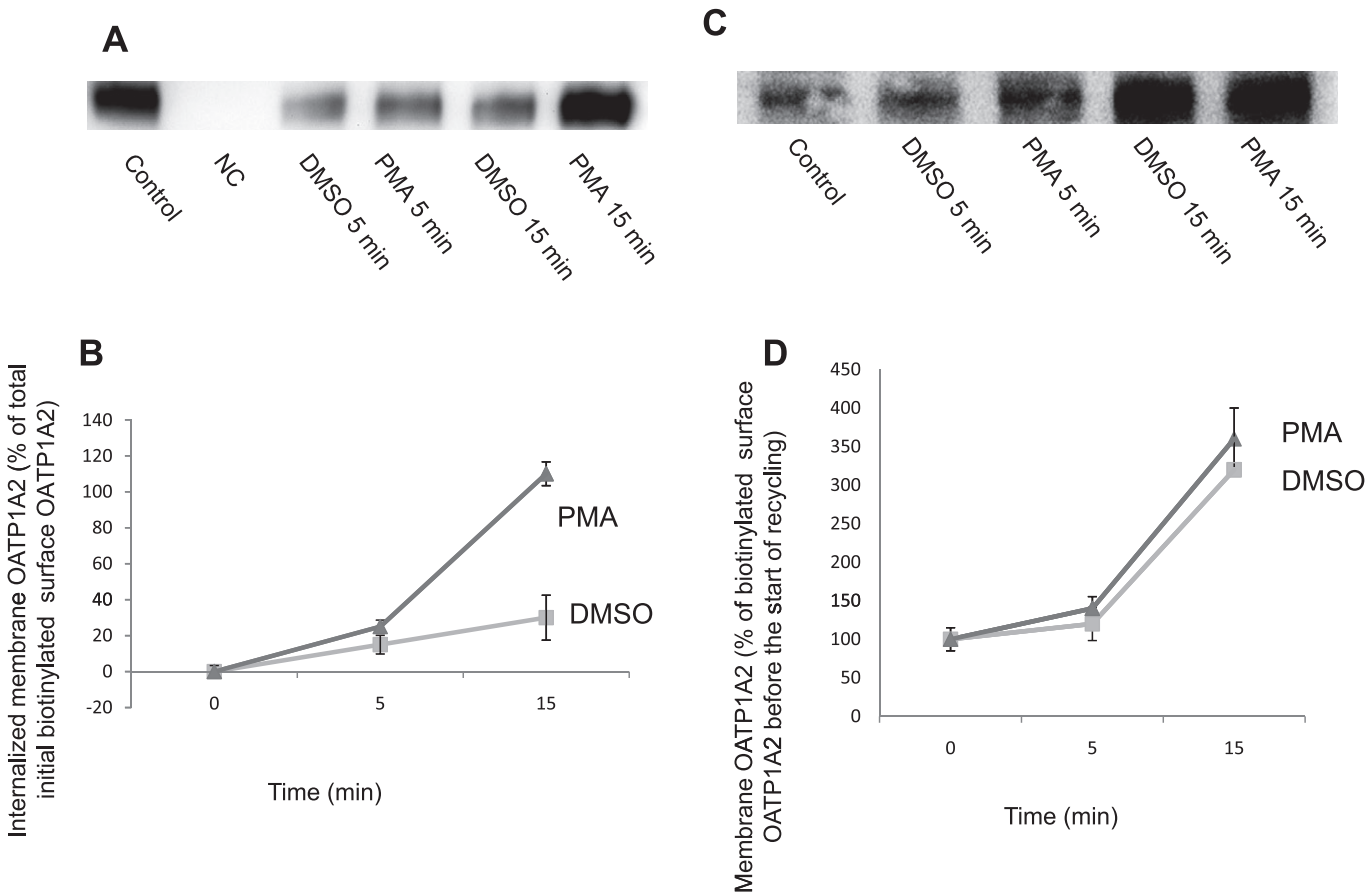


Figure 5

Biotinylation analysis of OATP1A2 internalization and recycling in COS-7 cells. (A) OATP1A2 internalization was analysed as described in Methods followed by Western blotting with an anti-OATP1A2 antibody; a representative analysis from three separate experiments is shown. NC: control for stripping efficiency in which biotinylated cells were treated with 50 mM sodium 2-mercaptoethanesulphonate without initiating the internalization process. (B) Plot of densitometric data from three individual experiments. Internalized OATP1A2 is expressed as a percentage of the total initial pool of biotinylated OATP1A2 at the cell surface. (C) OATP1A2 recycling was analysed as described in Methods followed by Western blotting with an anti-OATP1A2 antibody; a representative analysis from three separate experiments is shown. (D) Plot of densitometric data from three individual experiments. Membrane OATP1A2 is expressed as a percentage of the pool of biotinylated OATP1A2 at the cell surface prior to initiation of recycling. Cells were treated with DMSO or PMA (1 μM) for the times indicated. DMSO, dimethyl sulphoxide; OATP1A2, organic anion transporting polypeptide 1A2; PMA, phorbol 12-myristate 13-acetate.

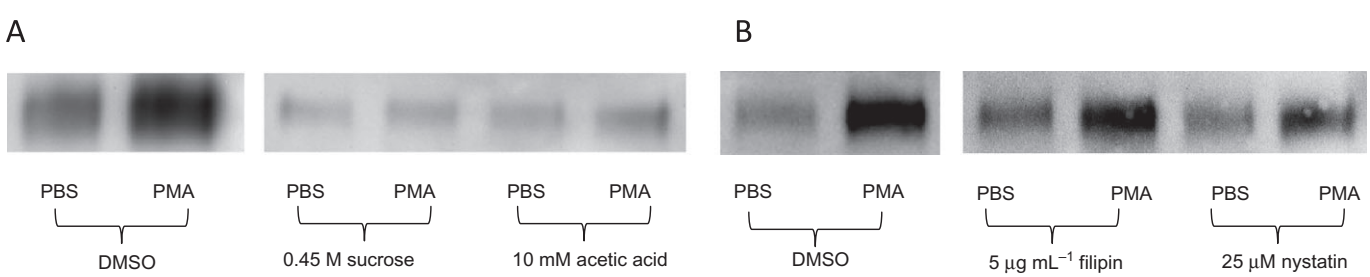


Figure 6

(A) Effect of disruption of the clathrin-dependent pathway on internalization of OATP1A2 induced by PMA in COS-7 cells. COS-7 cells over-expressing OATP1A2 were pretreated at 37°C with DMSO (30 min), 0.45 M sucrose (30 min) or 10 mM acetic acid (10 min). (B) Effect of disruption of the caveolin-dependent pathway. COS-7 cells that over-expressed OATP1A2 were pretreated with DMSO, filipin (5 $\mu g \cdot ml^{-1}$) or nystatin (25 μM) for 30 min at 37°C. The cells were allowed to internalize at 37°C for 15 min as described in Methods in the presence or absence of PMA (1 μM), followed by immunoblotting for OATP1A2. DMSO, dimethyl sulphoxide; OATP1A2, organic anion transporting polypeptide 1A2; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate.

residues that may be putative sites for PKC-dependent phosphorylation. However, the functionality of such residues in hOAT1 is unclear because site-directed mutagenesis to different amino acids also indicated a phosphorylation-independent PKC regulatory mechanism (Wolff *et al.*, 2003). This finding suggests that either atypical PKC-regulated sites are present in the hOAT1 protein or that phosphorylation of an anciliary protein may be the important event. It would now be of interest to determine whether OATP1A2 or a chaperone protein that mediates OATP1A2 internalization is subject to phosphorylation by PKC.

Hormonal factors could also regulate the expression and function of renal OATs by regulating PKC activity. For example, phenylephrine and bradykinin inhibited fluorescein uptake in proximal tubular cells of the rabbit by activating PKC (Gekle *et al.*, 1999; Shuprisha *et al.*, 2000). Angiotensin II regulated hOAT1 via PKC-α and caused a redistribution of the transporter from the cell surface to intracellular compartments (Escudier *et al.*, 2009). These observations are consistent with the present observations and those of others that PKC is a critical regulator of OAT and OATP transporters. In addition to hormonal factors, it has also been shown that PKC enzymes are dysregulated in cancers and tumour cells. Thus, altered cellular ion and drug transport in tumour cells may be mediated by PKC activation. Moreover, the decreased capacity for cellular uptake of OAT/OATP substrates may influence drug safety and efficacy in pathophysiological states.

In summary, the present study has revealed that: (i) the PMA-induced down-regulation of OATP1A2 transport function is mediated through PKC-specific signalling pathways; (ii) this functional regulation of OATP1A2 is due to PKC-modulated protein internalization; and (iii) OATP1A2 internalization is partially dependent on clathrine-dependent endocytosis. The potential significance of the results obtained in the present study is that novel molecular targets may be identified to modulate the intracellular distribution of drugs that are transported by OATP1A2.

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Conflict of interest

The authors state no conflict of interest.

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