Regulation of the human vitamin C transporters expressed in COS-1 cells by protein kinase C

WEI-JUN LIANG,1,2 DANIEL JOHNSON,1 LI-SHA MA,1 AND SIMON M. JARVIS1,2
1Research School of Biosciences, University of Kent at Canterbury, Canterbury, Kent CT2 7NJ; and 2School of Biosciences, University of Westminster, London W1W 6UW, United Kingdom

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Liang, Wei-Jun, Daniel Johnson, Li-Sha Ma and Simon M. Jarvis. Regulation of the human vitamin C transporters expressed in COS-1 cells by protein kinase C. Am J Physiol Cell Physiol 283: C1696–C1704, 2002. First published July 24, 2002; 10.1152/ajpcell.00461.2001.—Protein kinase C (PKC) regulation of L-ascorbic acid transport mediated by the Na+/ascorbic acid transporters, hSVCT1 and hSVCT2, expressed in COS-1 cells was studied using recombinant carboxyl-terminal V5 epitope-tagged forms of the transporters. The PKC activator phorbol 12-myristate 13-acetate (PMA) caused a time-dependent and concentration-dependent decrease (40–60%) in ascorbic acid transport activity. Effects of PMA were not observed with the inactive phorbol ester 4α-phorbol and were reversed by treatment of the cells with the PKC-specific inhibitor Ro-31–8220. Kinetically, the reduction in hSVCT1 and hSVCT2 activity arose from a decrease in maximal velocity with no change in the apparent affinity. Western blot and confocal microscopy analyses indicated that the total pool of hSVCT1 or hSVCT2 proteins expressed in the transfected COS-1 cells remained unaffected by PMA treatment. For hSVCT1 the decrease in L-ascorbic acid correlated with a redistribution of the transporter from the cell surface to intracellular membranes. However, for hSVCT2 there was no apparent change in transporter distribution, suggesting that the PKC-dependent modulation of L-ascorbic acid transport mediated by hSVCT2 was the result of reduced catalytic transport efficiency.

VITAMIN C (L-ascorbate) is a required nutrient for humans, since they and other primates have lost the ability to synthesize vitamin C via the glucuronic acid pathway (16). Thus vitamin C must be obtained from the diet, cross the small intestine, and enter the plasma where it is transported to target tissues. Once it has reached the target tissues, vitamin C acts as a cofactor for several intracellular enzymes and also scavenges oxidant radicals (8, 16, 17, 24, 22). For vitamin C to act inside cells, it must be transported across the plasma membrane, and the transport of ascorbate into mammalian cells is mediated by Na+-dependent uptake systems (12).

Recently two isoforms of the Na+-dependent vitamin C transporters (SVCTs) have been cloned from both rats and humans (see Refs. 4, 12, 19, 25, 27, 28). Expression of hSVCT1 and hSVCT2 in Xenopus laevis oocytes or HRPE or COS-1 cells resulted in uptake of ascorbate that was absolutely dependent on Na+ (4, 12, 19, 27, 28). Biochemical studies of the stoichiometry mechanism by which PMA inhibited ascorbic acid transport-associated current (25, 28). In some studies, hSVCT2 has been shown to exhibit a higher affinity for ascorbic acid than hSVCT1 (28 ± 6 vs. 78 ± 19 μM at 22°C; see Ref. 12). The two isoforms also differ in their tissue distribution, as detected by Northern blots or in situ hybridization with SVCT1 present in epithelial tissues, whereas SVCT2 is present in most tissues with the exception of lung and muscle (19, 25, 27, 28).

The deduced primary amino sequence of hSVCT1 and hSVCT2 revealed that both proteins possess five putative protein kinase C (PKC) phosphorylation sites, with hSVCT1 possessing an additional protein kinase A site (12). This result suggests that the Na+-dependent cotransporters may be regulated by posttranslational modification, but few studies have been conducted to investigate the regulation of ascorbic acid transport. Treatment of rabbit nonpigmented ciliary epithelial cells with phorbol 12,13-dibutyrate, an activator of PKC, resulted in the inhibition of ascorbate uptake (12). More recently, ascorbate influx in oocytes heterologously expressing either hSVCT1 or hSVCT2 was inhibited by treating the oocytes with phorbol 12-myristate 13-acetate (PMA; see Ref. 4). However, the mechanism by which PMA inhibited ascorbic acid transport activity was not investigated. Protein kinases can alter the activity of transporters directly, for example, a change in the substrate binding affinity or the translocation capacity, or indirectly, for example, altering the rate at which the carrier is inserted or
removed from the plasma membrane (I, 3, 18, 26). In the present study, we have used a mammalian expression system, COS-1 cells, to heterologously express sSVCT1 and sSVCT2 in both the native state and with a V5 epitope tag at the carboxyl-termini. After transient transfection of the COS-1 cells, we investigated the possible role of PKC in regulating ascorbate uptake mediated by either sSVCT1 or sSVCT2. The V5 epitope was incorporated in our transport proteins to facilitate the immunodetection of sSVCT1 and sSVCT2 with anti-V5 monoclonal antibody. The PKC activator, PMA, was shown to decrease ascorbate uptake mediated by both sSVCT1 and sSVCT2, and this effect could be totally reversed by the specific PKC inhibitor Ro-31–8220. Kinetic analysis revealed that the maximum velocity (V_max) of transport was decreased after PMA treatment with no change in the apparent affinity for transport. Confocal microscopy and cell surface biotinylation suggested that there was a corresponding reduction in the level of sSVCT1 transporter at the membrane surface consistent with the notion that PKC is regulating the trafficking of sSVCT1 to the plasma membrane. In contrast, no change in the cell surface distribution of sSVCT2 was observed after PMA treatment, suggesting that the decrease in sSVCT2 transport activity results from a decrease in the translocation capacity of the carrier.

MATERIALS AND METHODS

Cloning of the sSVCT1 and sSVCT2 cDNAs and their sequence analysis. Human SVCT1 and sSVCT2 were cloned by a RACE (rapid amplification of cDNA ends)-PCR strategy. For hSVCT1, a human liver Marathon-Ready (Clontech) cDNA library was used as a template for touchdown PCR using an Advantage Taq Polymerase Kit (ClonTech) and sense and antisense primers based on the published YSPL3 cDNA sequence (7). The PCR products were then cloned into pGEM-T-Easy and sequenced. Based on the sequence information, 5’-RACE was performed to obtain the 5’-untranslated region of the gene. A nested primer set (sense, 5’-CCA TCC TAA TAG GAC TCA CTA TAG GGC-3’ and 5’-ACT CAC TAT AGG GCT CGA GGC GC-3’) of the human liver Marathon-Ready cDNA library and the hSVCT1 gene-specific nested primers (antisense, 5’-GGG TGC CAA ATA TGA GAG GTG TCC AGG-3’ and 5’-CAG ACT CCA GGT ACC GTA GAT CTC CTC-3’) was designed. The reaction condition for the 5’-RACE PCR was 94°C for 1 min; 5 cycles of 94°C for 30 s and 74°C for 4 min; 5 cycles of 94°C for 30 s and 72°C for 4 min; 5 cycles of 94°C for 30 s and 70°C for 4 min; 25 cycles of 94°C for 10 s and 68°C for 4 min; and 68°C for 7 min extension. The maximum length of the 5’-untranslated region of the hSVCT1 transcript after the library primer sequences obtained from this adult human male liver cDNA library was 70 bases. An ~1.85-kb DNA fragment containing the complete open reading frame and part of the untranslated region was synthesised by PCR using 5’-CTT TGT CAA GTC ATC CCC TCT TCT CTC-3’ starting at –56 to the first ATG codon as the forward primer (sense) and 5’-CTT CTC TGG TGC ACA CAG ATG CAG TT TCT C-3’ as reverse primer (antisense). The condition of the “touchdown” PCR was as before. A similar procedure was used to clone the entire open reading frame of hSVCT2 from human placenta using the gene-specific primers 5’-CAT AAG CAA ATG TAA CTC AGC-3’ and 5’-GTA GAT GTA GAT CAA ACA TG-3’ as the reverse primer. A 2.4-kb PCR product was observed comprising of a 1,935-bp open reading frame plus 231 bp of 5’-untranslated region and 100 bp of 3’-untranslated region. The hSVCT1 and hSVCT2 PCR products were subcloned into the mammalian expression vector, pcDNA3.1/V5/His-TOPO, with an alternative stop codon (TAA). The identities and the orientations of each of the resultant clones were determined by restriction-analysis PCR using host bacterial colonies and DNA sequencing. The clones were sequenced on both sense and antisense strands by primer walking, and the reported DNA sequences are the consensus sequence of five to eight PCR-derived clones for both hSVCT1 (accession no. AJ250807) and hSVCT2 (accession no. AJ293218).

The V5 epitope sequence followed by a six-histidine tag sequence was introduced on the carboxyl-terminal region of hSVCT1 and hSVCT2 by PCR. The open reading frames of hSVCT1 and hSVCT2 were generated without a stop codon and when ligated into pcDNA3.1 V5/HIS/TOPO, they were in frame to the V5 epitope tag. PCR primers 5’-CTT TGT CAA GTC ATC CCC TCT TCT CTC-3’ (forward for hSVCT1), 5’-CCC CAA CGG CTT CTT GTG AAA CTA CTC GCT CTC CTT-3’ (forward for hSVCT2), 5’-GA CCA TGG TGC ACA CAG ATG CAG TT TCT C-3’ (reversed for hSVCT1), and 5’-TCC CTG GGC CTT GGA GTC TCT ATC TGA AC-3’ (reversed for hSVCT2) were used for the regeneration of the PCR products from the cloned hSVCT1 and hSVCT2. The adenylation at the 3’-ends of PCR products generated by the Advanced Tag Polymerase allowed their direct insertion in the expression vector. Confirmation that the V5 epitope was in frame to the reading frame of the hSVCT1 and hSVCT2 was obtained by sequencing the 3’-end of the coding sequences. The plasmid DNA containing the hSVCT cDNAs used for the COS-1 transfections was prepared from Escherichia coli DH5α cells and are termed hSVCT1vh and hSVCT2vh.

Cell culture and transient transfection. Monkey kidney COS-1 cells (from the European Collection of Cell Cultures) were grown at 37°C in 95% humidified air-5% CO2 in DMEM containing 10% heat-inactivated FCS, 2 mM glutamine, and 100 International Units penicillin-streptomycin. Cells were subcultured for serial passage grown in 175-cm² flasks and were fed every 3 days. For uptake experiments or for immunodetection of hSVCTs, cells were seeded into either 12- or 24-well plates at a density of 2 × 10⁶ or 1 × 10⁶ cells/well, respectively, and cultured for 24 h to an estimated 50–80% confluence. The culture medium was then replaced with 0.2–0.3 ml of FCS-free DMEM containing 1 μg of DNA and 2–5 μl of GenePorter reagent or 1 μg of DNA with 3 μl of Lipofectamine-2000. The cultures were incubated for 5 h at 37°C in 95% humidified air-5% CO2, after which 1 ml of DMEM containing 12% (vol/vol) FCS was added to each well (10% FCS final). Transport studies were performed 36–48 h posttransfection. COS-1 cells transfected with a comparable expression plasmid, pcDNA3.1/His/lacZ, were examined for transfection efficiency, and it was estimated to be at least 30%. Transfection efficiency was not affected by coexpression studies of plasmid pcDNA3.1/hSVCT1vh or pcDNA3.1/hSVCT2vh with pcDNA3.1/His/lacZ.

Measurement of ascorbic acid transport. Influx of L-[(carboxyl-¹⁴C)]ascorbic acid (17.0 mCi/mmol; Amersham Pharma- cekemico) by monolayer COS-1 cells in 24-well plates was determined at 22°C as described previously for the uptake of nucleosides and nucleobases in monolayers of mammalian cells (9). In brief, each well was washed with 1 ml of transport assay buffer (in mM: 140 NaCl, 4.2 KHCO₃, 5.8 KCl, 1.3 CaCl₂, 0.5 MgCl₂, and 10 HEPES, pH 7.4) at 22°C after
aspiration of the culture medium. The transport reactions were started by the addition of the transport assay buffer (0.2 ml) containing 1-L-[^14]C]ascorbic acid and 0.1 mM dithiothreitol to prevent the oxidation of L-ascorbic acid to the cell monolayer. At 30 min (uptake was directly proportional to time over a period of 60 min, as demonstrated previously (12)), the transport buffer was aspirated, and the cell monolayer was washed immediately with 1 ml of ice-cold buffer two times. The washing procedure took 10 s for each well. The cells in each well were then lysed by addition of 0.5 ml of 0.5 N NaOH, and the amount of L-[^14]C]ascorbic acid was quantified by liquid scintillation spectrometry. A portion of the cell lysate (50 μl) from each well was taken for protein concentration determinations using the method of Lowry et al. (14).

Kinetic values of transport [Michaelis constant ($K_m$) and $V_{max}$] were determined using nonlinear least squares fit programs (GraphPad Prism). Each experiment was repeated at least three times, each done in triplicate or quadruplicate.

**Surface biotinylation.** Biotinylation experiments were performed as described previously (29). COS-1 cells grown in six-well plates, and transfected with hSVCT1v and hSVCT2v anti-mouse antibody conjugated to horseradish peroxidase-PMA. The cells were then washed twice with PBS-Ca$^{2+}$/Mg$^{2+}$ (in mM: 138 NaCl, 2.7 KCl, 1.5 KH$_2$PO$_4$, 1 MgCl$_2$, and 1 CaCl$_2$, pH 7.4). The surface plasma membrane proteins were then biotinylated for 20 min at 4°C [2 ml of sulfo-NHS biotin (1 mg/ml); Pierce] in PBS-Ca$^{2+}$/Mg$^{2+}$ with gentle shaking. The biotinylation solution was removed, and the cells were washed with two washes in PBS-Ca$^{2+}$/Mg$^{2+}$ containing 100 mM glycine and quenched in this solution at 4°C for 45 min with gentle shaking. The cells were then lysed in 1 ml of solubilization buffer (100 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 40 μl of protease inhibitor cocktail (Boehringer Mannheim), and 0.2 mM phenylmethylsulfonyl fluoride) at 4°C for 60 min. The cell lysates were centrifuged for 60 min at 20,000 g, 4°C. The supernatant fractions (300 μl) were incubated with an equal volume of monomeric avidin beads (Pierce) to separate the biotinylated proteins from the non-biotinylated proteins. The beads were washed three times with the solubilization buffer, and absorbed proteins eluted with SDS sample buffer (62.5 mM Tris-Cl, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol) at 22°C for 60 min. Samples were then processed as described below for Western blot analysis.

**Immunoblotting.** After treatment with the appropriate drug, cells were lysed in 150 mM NaCl, 1% SDS, 10 mM EDTA, and 10 mM HEPES-Tris (pH 7.4) with protease inhibitor cocktail (Boehringer Mannheim) and 0.2 mM phenylmethylsulfonyl fluoride. The lysate was transferred to an Eppendorf tube on ice and mixed with Laemmli gel loading buffer containing 2% SDS at 37°C for 1 h. Protein was separated on 10% polyacrylamide gels and transferred to a nitrocellulose membrane, the blots were blocked for 2 h at room temperature in 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 0.05% Tween 20, and 10% milk powder. For subsequent antibody reactions and washings, milk powder was reduced to 1%. Primary (anti-V5 mouse monoclonal; 1:1,000) antibody incubation was performed at 4°C overnight, and secondary goat anti-mouse antibody conjugated to horseradish peroxidase; 1:5,000) antibody incubations were 1 h at room temperature. The membranes were washed three times with buffer for 10 min and visualized using the enhanced chemiluminescence (ECL) system (Amersham), and the bands were quantitated by densitometry. For quantitative Western analysis, the density of the bands must be within the linear range and not saturated. Thus the densitometry analysis was performed with at least two different protein loadings and different times of exposure to the ECL reagent to confirm a linear response was observed. Also, standard curves were constructed to take into account any difference in transfer between hSVCT1 and hSVCT2. Selected samples were digested with N-glycosidase F (83 U/μg protein; New England Biolabs).

**Confocal immunofluorescence microscopy.** After culturing and treatment, transfected COS-1 cells were harvested by trypsin digestion, sedimented by centrifugation (13,000 g), and fixed with 4% paraformaldehyde for 20 min at 22°C. The cells were subsequently permeabilized by the addition of 0.5% Triton X-100 for 10 min at 22°C and incubated with anti-V5 epitope monoclonal antibody mouse IgG$_2a$ (1:1,000 dilution; Invitrogen) for 1 h at 22°C or overnight at 4°C. After six washes with PBS, the cells were incubated for 1 h at 22°C with (1:1,000 in PBS) goat anti-mouse IgG conjugated with FITC (Sigma) and finally mixed with a drop of mowiol containing 10% phenylenediamine and mounted on a glass slide. A Leica confocal laser scanning microscope with a ×63 lens was used to examine the COS-1 cells. A series of 10 images horizontally across each of the cells was captured and collected. Images of middle sections were used for the comparison of the effect of PMA treatment. Controls using omission of primary or secondary antibodies revealed no labeling. A minimum of 10 different cells from three separate transfection procedures and treatments was subjected to confocal analysis. The majority of the transfected cells (>85%) showed the same pattern of transporter protein distribution as that shown in Fig. 8.

**RESULTS**

**Time course of expression of hSVCT1 and hSVCT2 in transfected COS-1 cells.** Previous studies from this laboratory (12) and other works (4, 19, 28) have demonstrated the Na$^+$ dependence of cloned hSVCT1 and hSVCT2, but the optimum conditions for the expression of cloned transporters had not been studied. Preliminary experiments were thus conducted in which we monitored the enhancement in the initial rate of Na$^+$ dependent 10 μM ascorbic acid transport as a function of time posttransfection. Figure 1A shows that, within 24 h, ascorbic acid transport activity in cells transfected with hSVCT1 appeared to have reached a maximum level with no further increase observed at 48 h. In contrast, the rate of ascorbic acid transport doubled in cells transfected with hSVCT2 between 24 and 48 h. Ascorbic acid transport in untransfected cells or COS-1 cells transfected with empty plasmid was similar and remained constant over the whole 48-h period. In future experiments, mediated ascorbic acid uptake by the cloned transporters was defined as ascorbic acid uptake in transfected cells minus uptake in nontransfected cells. Figure 1 also demonstrates that the addition of the V5 epitope to hSVCT1 and hSVCT2 had no effect on the rate of ascorbic acid transport activity mediated by the transport proteins. Thus, in future experiments, the V5 epitope-tagged hSVCTs were primarily used to investigate the effects of PMA on transport activity with posttransfected cells (>36 h).
investigated the effects of the inactive phorbol ester analog 4α-phorbol and the PKC-specific inhibitor Ro-31–8220 (5). In contrast to the effects of PMA, 100 nM 4α-phorbol did not cause a decrease in ascorbic acid transport in hSVCT1vh and hSVCT2vh transfected COS-1 cells, and the rate of vitamin C transport was similar to that of the control untreated cells (P > 0.05).

Figure 4 shows that, when Ro-31–8220 (500 nM) was added to transfected cells either 1 h before the addition of PMA or at the same time as PMA, the effect of PMA was blocked totally. Ro-31–8220 had little effect on ascorbic acid uptake in control cells. The antagonism of the action of PMA by a PKC inhibitor suggests that the effect of PMA on ascorbic acid uptake is mediated by stimulation of PKC.

To eliminate the possibility that the effects of PMA are not due to secondary effects of PKC action on either the Na+/K+-ATPase or the Na+/H+ exchanger, the following studies were performed. Treatment of cells with 150 μM amiloride, an inhibitor of Na+/H+ exchangers, had no significant effect on the influx of ascorbic acid by either control and transfected cells. Moreover, ascorbic acid influx (10 μM) was decreased significantly (31 ± 2.7 and 33 ± 1.4% for hSVCT1vh- and hSVCT2vh-transfected cells, respectively) after exposure to 100 nM PMA. As expected for Na+/dependent transport systems, addition of the Na+/K+-ATPase inhibitor ouabain (1 mM) resulted in inhibition of ascorbic acid flux. Addition of 100 nM PMA to ouabain-treated transfected COS-1 cells resulted in further

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**Fig. 1.** Time course of human Na+/dependent vitamin C transporter hSVCT1 and hSVCT2 expression in COS-1 cells. A: COS-1 cells were transfected with either hSVCT1 (filled bars) or hSVCT2 (open bars) and assayed for Na+/dependent 10 μM L-[14C]ascorbic acid uptake at 22°C 24 and 48 h posttransfection (see MATERIALS AND METHODS). Uptake via nontransfected cells (hatched bars) is also shown. B: COS-1 cells were transfected with equal amounts of V5-tagged (hSVCT1vh or hSVCT2vh; filled bars) or wild-type hSVCT1 or hSVCT2 (open bars) as described under MATERIALS AND METHODS and cultured for 48 h, and the transport of ascorbic acid was compared for the different constructs. Transport mediated by the clones (ascorbic acid uptake via the transfected cells minus uptake in nontransfected cells expressed as a percentage of uptake rates in the wild-type transporters) is shown and represents the mean ± SE of 3 separate experiments.

**Fig. 2.** Time course of the inhibition of ascorbic acid uptake by phorbol 12-myristate 13-acetate (PMA). COS-1 cells were transfected with the plasmids hSVCT1vh (filled bars) and hSVCT2vh (open bars) and cultured for 48 h. Transfected cells were preincubated with 100 nM PMA or vehicle (DMSO at 0.01%, vol/vol) for the times indicated and assayed for ascorbic acid uptake (10 μM) at 150 mM amiloride, an inhibitor of Na+/K+-ATPase, or Na+/H+ exchangers, had no significant effect on the influx of ascorbic acid by either control and transfected cells. Moreover, ascorbic acid influx (10 μM) was decreased significantly (31 ± 2.7 and 33 ± 1.4% for hSVCT1vh- and hSVCT2vh-transfected cells, respectively) after exposure to 100 nM PMA. As expected for Na+/dependent transport systems, addition of the Na+/K+-ATPase inhibitor ouabain (1 mM) resulted in inhibition of ascorbic acid flux. Addition of 100 nM PMA to ouabain-treated transfected COS-1 cells resulted in further

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**PMA-mediated decrease in ascorbic acid transport activity.** In preliminary experiments, pretreatment of COS-1 cells transfected with hSVCT1 and hSVCT2 with 100 nM PMA for 1 h was shown to reduce the rate of ascorbic acid transport compared with untreated cells. The transport rate was 46 ± 8 and 57 ± 7% of control for hSVCT1 and hSVCT2, respectively (mean ± SE of 3 experiments). A similar level of decrease in initial rates of 10 μM ascorbic acid transport was also observed with cells expressing V5-tagged hSVCT1 and hSVCT2 when treated with PMA (Fig. 2). The comparability in the response with V5-tagged and untagged proteins suggested that the V5 epitope did not affect the PMA-induced regulation of the Na+/vitamin C transporters, and hence the V5 constructs could be reliably used to further study the regulation of hSVCT1 and hSVCT2.

Figure 2 shows that the action of PMA (100 nM) was rapid (a significant decrease in transport activity is observed within the first 5 min) and also time dependent, with a maximal decrease in ascorbic acid transport activity observed between 40 and 80 min. Increasing PMA concentrations (10–250 nM) decreased ascorbic acid transport in a dose-dependent manner, with half-maximal activity at ~20 nM and reaching a plateau at ~100 nM (Fig. 3). This profile of a rapid response and high affinity to the addition of PMA suggests the involvement of PKC in the PMA-mediated decrease in hSVCT1 and hSVCT2 ascorbic acid transport activity. To provide further evidence, we have
inhibition of ascorbic transport that was quantitatively equivalent to the inhibition induced by PMA in the absence of ouabain [44 ± 4.2 and 39 ± 3.5% inhibition for hSVCT1vh- and hSVCT2vh-mediated ascorbic acid influx by PMA and ouabain-treated cells compared with control cells (ouabain treatment only)].

Effect of PMA on transport kinetics and the distribution of the transporters. To determine whether the effects of PMA on the rate of ascorbic acid uptake were due to a change in $V_{\text{max}}$ of transport and/or a change in $K_m$, the concentration dependence of ascorbic acid uptake by transfected cells exposed to PMA was investigated. Figure 5 shows that transfected cells exposed to 100 nM PMA had a significant decrease in $V_{\text{max}}$ for both hSVCT1 and hSVCT2 compared with untreated cells (42 and 32%, respectively). There was no change in the apparent ascorbic acid affinity ($K_m$) for hSVCT1vh and hSVCT2vh after treatment with PMA.

Figure 5 also demonstrates that the $V_{\text{max}}$ of the hSVCT1vh transporter is at least three times that of hSVCT2vh. Consistent with this observation was the finding that both the total amount of protein expressed and also the cell surface-expressed protein were less for hSVCT2vh than hSVCT1vh (Figs. 6 and 7). Quantitative analysis of the biotinylation immunoblots under conditions where the signal was shown to be linear and corrected for differences in the transfer of the two proteins revealed an apparent three- to fourfold difference in the signal for hSVCT2 and hSVCT1 under control conditions [ratio for total cellular amounts and cell surface expression of hSVCT1vh:hSVCT2vh protein of $3.0 \pm 0.4$ and $3.8 \pm 0.5$, mean ± SE ($n = 4$), respectively]. The similarity of this value to the difference in transport $V_{\text{max}}$ for the two transporters suggests that the translocation capacity of hSVCT1 and hSVCT2 expressed in transfected COS-1 cells is similar; moreover, the higher $V_{\text{max}}$ observed with hSVCT1 is because of greater expression of the protein within the cell.

The decrease in the $V_{\text{max}}$ for ascorbic acid uptake after incubation with PMA suggests that there are either fewer transporters present at the cell surface and/or a decrease in the translocation capacity of each transporter. To evaluate this issue, we have used Western blot analysis of the V5-tagged hSVCTs. Analysis of the total cell extracts using the anti-V5 epitope monoclonal antibody showed both a sharp band at ~70 kDa and a diffuse band at ~120 kDa for hSVCT1vh and a broad band at ~120 kDa for hSVCT2vh (Fig. 6). Occasionally, the broad band for hSVCT2vh appeared to migrate as two distinctive bands (Fig. 7). No immunoreactive bands were detected in untransfected COS-1 cells. Identical immunoblotting patterns were observed with both PMA-treated and nontreated cells, and no
In PMA-treated cells with nontreated cells, revealed that a difference in the relative distribution of the 70-kDa protein was observed between PMA-treated and untreated hSVCT1vh transfected cells. To further investigate the nature of the 70-kDa protein, cells were transfected hSVCT1vh and hSVCT2vh transfected cells. COS-1 cells transfected with hSVCT1vh (●) and hSVCT2vh (○) were cultured for 36 h, preincubated with 100 nM PMA (●, ○) or DMSO (0.01%) for 1 h, and then assayed for ascorbic acid uptake at the indicated concentrations. Data are means ± SD for a representative experiment of 3 separate experiments with each assay performed in quadruplicate. Kinetic parameters of transport were determined by curve fitting, as described in MATERIALS AND METHODS. PMA caused a decrease in maximal velocity ($V_{max}$; 1,150 ± 28 in control vs. 660 ± 21 pmol/mg protein $-1$ min $-1$ in PMA-treated cells for hSVCT1; 320 ± 21 in control vs. 220 ± 16 pmol/mg protein $-1$ min $-1$ in PMA-treated cells for hSVCT2) with little change in $K_m$ (65 ± 5 in control vs. 64 ± 7 μM in PMA-treated cells for hSVCT1; 29 ± 7 in control vs. 33 ± 9 μM in PMA-treated cells for hSVCT2).

An alternative method to examine the expression and localization of hSVCT1vh and hSVCT2vh is by confocal microscopy. In accordance with the immunoblotting data, COS-1 cells failed to react with the V5-specific antibody (data not shown). In contrast, those cells transfected with either hSVCT1vh or hSVCT2vh and treated with the inactive phorbol ester 4α-phorbol demonstrated clear labeling of the plasma membrane (Fig. 8). Upon treatment with PMA, the labeling of the plasma membrane was less prominent for both isoforms of the transporter. In the case of hSVCT1vh, there was a corresponding diffuse labeling.

To further determine whether the decrease in ascorbic acid transport activity after exposure to PMA was possibly because of a decrease in surface expression of SVCTs, we performed surface biotinylation experiments on hSVCT1vh and hSVCT2vh. Representative immunoblots are shown in Fig. 7, A and B, and their quantitation is shown in Fig. 7C. In the PMA-un treated cells 12.1 ± 1.5 and 12.4 ± 1.8% (mean ± SE; $n = 5$) of the hSVCT1 and hSVCT2 protein was found on the plasma membrane, with the remainder within the intracellular fraction. Both the ~90-kDa and 70-kDa forms of hSVCT1 were present at the cell surface. Treatment with 100 nM PMA reduced the amount of hSVCT1 found on the plasma membrane from 12.1 ± 1.5 to 5.5 ± 1.6%, which correlated with the reduction in ascorbic acid transport seen in COS-1 cells expressing hSVCT1vh after PMA treatment. In contrast, although a similar percentage of hSVCT2 was expressed at the cell surface compared with hSVCT1, no reduction in hSVCT2 at the cell surface was observed after PMA treatment (12.2 ± 2.4% at the cell surface after PMA treatment compared with 12.4 ± 1.8% in control cells).

Fig. 7A and 7B show representative Western blots of hSVCT2 in the presence and absence of PMA. The hSVCT2vh band at 90 kDa showed a clear shift to the predicted 70-kDa molecular mass for hSVCT1vh (Fig. 6), suggesting that the 70-kDa protein represents the unmodified form of hSVCT1. Similar studies with hSVCT2-transfected cells showed that N-glycosidase F digestion converted the ~120-kDa protein to the predicted molecular mass of 75 kDa. Quantitative analysis of the Western blots of PMA-treated cells with nontreated cells revealed that the total amount of transport protein detected in the transfected cells remained unchanged after exposure to 100 nM PMA for 1 h (105 ± 5 and 97 ± 2% for hSVCT1vh and hSVCT2vh in PMA-treated cells, respectively, compared with control nontreated cells, mean ± SE of 6 separate experiments). Thus PMA did not alter the amount of SVCTs in the total cell extract, indicating that the decrease in ascorbic acid transport activity after PMA treatment was not because of a decrease in the overall content of SVCTs.

To further determine whether the decrease in ascorbic acid transport activity after exposure to PMA was possibly because of a decrease in surface expression of SVCTs, we performed surface biotinylation experiments on hSVCT1vh and hSVCT2vh. Representative immunoblots are shown in Fig. 7, A and B, and their quantitation is shown in Fig. 7C. In the PMA-un treated cells 12.1 ± 1.5 and 12.4 ± 1.8% (mean ± SE; $n = 5$) of the hSVCT1 and hSVCT2 protein was found on the plasma membrane, with the remainder within the intracellular fraction. Both the ~90-kDa and 70-kDa forms of hSVCT1 were present at the cell surface. Treatment with 100 nM PMA reduced the amount of hSVCT1 found on the plasma membrane from 12.1 ± 1.5 to 5.5 ± 1.6%, which correlated with the reduction in ascorbic acid transport seen in COS-1 cells expressing hSVCT1vh after PMA treatment. In contrast, although a similar percentage of hSVCT2 was expressed at the cell surface compared with hSVCT1, no reduction in hSVCT2 at the cell surface was observed after PMA treatment (12.2 ± 2.4% at the cell surface after PMA treatment compared with 12.4 ± 1.8% in control cells).

An alternative method to examine the expression and localization of hSVCT1vh and hSVCT2vh is by confocal microscopy. In accordance with the immunoblotting data, COS-1 cells failed to react with the V5-specific antibody (data not shown). In contrast, those cells transfected with either hSVCT1vh or hSVCT2vh and treated with the inactive phorbol ester 4α-phorbol demonstrated clear labeling of the plasma membrane (Fig. 8). Upon treatment with PMA, the labeling of the plasma membrane was less prominent for both isoforms of the transporter. In the case of hSVCT1vh, there was a corresponding diffuse labeling.
cells was totally dependent on the presence of extracellular Na⁺ (total influx rate at 10 μM, 22°C of 5.3 ± 0.6, 0.83 ± 0.1, 0.77 ± 0.05, 0.74 ± 0.12, 1.1 ± 0.09, and 0.92 ± 0.1 pmol·mg protein⁻¹·min⁻¹ in the presence of 140 mM chloride salts of Na⁺, K⁺, Li⁺, Cs⁺, choline, and Na⁺ plus excess unlabeled 1 mM ascorbic acid, respectively, mean ± SE, n = 3). Accurate determination of the kinetic parameters of ascorbic influx by untransfected cells was not possible, since the rate of influx at concentrations of ascorbic acid >50 μM was <10% of the transfected cells. Nevertheless, at a lower concentration of ascorbic acid (10 μM), addition of 100 nM PMA decreased ascorbic acid transport with a dose dependency (Fig. 3) similar to that observed for hSVCT1- and hSVCT2-mediated ascorbic acid transport. Moreover, the effect of 100 nM PMA on ascorbic acid transport by untransfected COS-1 cells was not observed when the cells were incubated with the inactive phorbol ester 4α-phorbol (data not shown). In addition, the effect of 100 nM PMA was blocked by the PKC inhibitor Ro-31–8220 (500 nM; 10 μM ascorbic acid influx rates as a percentage of control untreated cells of 74 ± 10, 93 ± 6, and 101 ± 5 for PMA-treated, PMA– plus Ro-31–8220-treated, and Ro-31–8220-treated untransfected COS-1 cells).

**DISCUSSION**

The aim of the present study was to investigate and compare the regulation of hSVCT1 and hSVCT2 expressed in the mammalian cell line COS-1. Our studies suggest that short-term activation of PKC by the addition of PMA to transfected cells expressing either V5-tagged hSVCT1 or hSVCT2 decreases the uptake of ascorbic acid. The mechanism by which PKC decreases the influx of ascorbic acid mediated by the SVCT transporters appears to be isoform specific and for hSVCT1 is most likely by altering cell surface trafficking of the transporter.

PMA, a compound widely used to activate PKC, markedly decreased the uptake of [14C]ascorbic acid by hSVCT1 and hSVCT2 expressed in COS-1 cells. At a maximally effective concentration (100 nM), extracellularly applied PMA decreased the apparent \( V_{\text{max}} \) without changing the \( K_m \) value for ascorbic acid-medi-
ated uptake by hSVCT1 and hSVCT2. Both transporters were similarly affected with a 30–40% decrease in \( V_{\text{max}} \). In contrast, 4c-phorbol, an inactive analog of PMA, had no effect on ascorbic acid transport activity, indicating that the response elicited by PMA was specific for the phorbol ester and suggesting that PKC activation is required. The reversal of the PMA-induced inhibition of ascorbic acid uptake by the highly specific PKC inhibitor Ro-31–8220 provided additional evidence that PKC must play a role either directly or indirectly in the regulation of hSVCTs. Basal SVCT transporters in COS-1 cells also appear to be regulated by PKC; thus, our findings with transfected cells may have general applicability to cells expressing SVCTs.

Multiple isoforms of PKC exist that can be classified into three distinct groups on the basis of structural and regulatory differences (15). Within COS cells, conventional PKCs (\( \alpha, \beta I, \) and \( \beta II \)), novel PKCs (\( \epsilon \)), and atypical PKCs (\( \xi \)) have been shown to be expressed (23, 24). Which of these isoforms is involved in inhibiting ascorbic acid transport is unknown, although the atypical PKCs can be eliminated since they do not respond to phorbol esters (15). Protein kinase D (PKD), a kinase that is also stimulated by phorbol esters, is unlikely to play a role in the inhibition of ascorbic transport since COS-7 cells do not express PKD (24). The reason why the magnitude of the PMA inhibitory effect is incomplete, i.e., at 40%, is unknown, but it is interesting to note that a number of other transport systems are similarly inhibited upon exposure to PMA (5, 10, 30).

This possibly suggests that additional signals remain to be discovered that will cause a complete loss in transport activity.

Inhibition of transport activity is commonly observed when modulation of the activity of other carriers by PKC has been investigated. For example, the human dopamine transporter, Na\(^+\)-glucose cotransporters, and a mouse taurine carrier when expressed in Xenopus oocytes all exhibited diminished activity upon exposure to PMA (10, 13, 30). In all cases, PMA altered the \( V_{\text{max}} \) of the transporter of interest, and this was also observed in the present study with hSVCT1 and hSVCT2. In the case of hSVCT1, this decrease in ascorbic acid \( V_{\text{max}} \) appears to be the result of a significant proportion of transporter no longer present at the membrane after PMA treatment, as revealed from the surface biotinylation studies and the confocal images. In transfected control cells, hSVCT1vh was predominately located intracellularly (88% of the total) and existed in the following two forms: a 70-kDa form that may represent the unglycosylated form of the carrier and an 80- to 100-kDa form that is glycosylated. Both forms of hSVCT1vh were also present on the cell surface, and their amount on the surface was reduced significantly after PMA treatment from \( \sim 12\% \) of the total to \( \sim 5\% \) (see Fig. 7). The total level of hSVCT1vh from the immunoblot assays remains unaltered by PMA treatment. Taken together, these results support the notion that PKC regulation of ascorbic acid uptake mediated by hSVCT1 is largely via membrane trafficking to and/or from the plasma membrane.

A mechanism has been proposed for the decrease in dopamine, serotonin, taurine, and glucose transport after expression of the specific transporters in either oocytes or mammalian cells and PKC activation (10, 13, 18, 30). Further studies will be required to determine whether reduced insertion in or increased removal from the plasma membrane follows PKC activation to account for the net loss of surface hSVCT1.

In the case of hSVCT2, the reduced transport activity after PMA treatment does not appear to be accounted for by a change in the cell surface distribution of the transporter. As was the case for hSVCT1, the majority (\( \sim 88\% \)) of hSVCT2 in COS-1 transfected cells appeared to be located intracellularly. Nevertheless, immunoblots demonstrated that both the total level and the cell surface fraction of hSVCT2vh remained unaltered by exposure to PMA. Although the confocal studies tended to suggest that the labeling of the cell surface hSVCT2vh was less pronounced after PMA treatment, there was no clear increase in intracellular labeling. Thus we propose that PKC stimulation results in a change in the activity of a constant number of hSVCT2 transporters at the plasma membrane. We have no evidence to suggest an irreversible loss of transporter protein via degradation.

The finding that the majority of the hSVCT1 and hSVCT2 (\( \sim 85\% \)) is localized in an intracellular compartment might reflect the use of transiently transfected cells leading to an overexpression of the transporters. Nevertheless, other transporters have also been observed to exhibit a similar distribution, for example the Na\(^+\)-exchanger isoform (NHE3; see Ref. 2). Further studies with isoform-specific antibodies to hSVCTs will be required to examine the cellular distribution of endogenously expressed vitamin C transporters.

Analysis of the amino acid sequence of hSVCT1 and hSVCT2 reveals two potential N-glycosylation sites between the putative transmembrane 3 and 4 domains, with an additional site between transmembrane 5 and 6 domains in hSVCT1 (12). The present results confirm that both hSVCT1vh and hSVCT2vh, when expressed in COS-1 cells, are glycoproteins containing N-linked oligosaccharides that migrate on SDS-polyacrylamide gels as broad bands. The broadness of the bands suggests heterogeneous glycosylation, and treatment with N-glycosidase F resulted in a sharpening of the bands on SDS-polyacrylamide gels and a corresponding shift to lower apparent relative molecular mass regions of the gel (Fig. 6). The difference in the relative molecular mass of hSVCT1vh and hSVCT2vh in both their native and deglycosylated forms is primarily due to the difference in the polypeptide length of the two isoforms. The pattern of glycosylation may also play a contribut- ing role, and site-directed mutagenesis experiments are currently being planned to locate the sites of glycosylation.

The mechanism by which PKC causes a decrease in the transport activity of both hSVCT1 and -2 is unknown. Both transporters possess conserved phosphorylation sites for PKC (12), raising the possibility that
the direct phosphorylation of the transporters results in the redistribution of hSVCT1 and changes in the conformation of hSVCT2 that affect its transport activity. For example, it has been hypothesized that direct phosphorylation of the serotonin transporter by PKC serves as a tag that identifies transporters to be internalized (20). In contrast, tryosine phosphorylation of the γ-aminobutyric acid transporter GAT1 decreases the rate of transporter internalization, leading to an upregulation in transport activity (11). An alternative explanation is that other PKC-sensitive proteins may be mediating the trafficking of hSVCT1 and the activity of hSVCT2. Further studies will be required to differentiate between these possibilities.

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Current address for S. Jarvis: School of Biosciences, University of Westminster, 115 New Cavendish St., London W1W 6UW, UK.

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