PKC regulates turnover rate of rabbit intestinal Na⁺-glucose transporter expressed in COS-7 cells

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Vayro, Steven, and Mel Silverman. PKC regulates turnover rate of rabbit intestinal Na⁺-glucose transporter expressed in COS-7 cells. Am. J. Physiol. 276 (Cell Physiol. 45): C1053–C1060, 1999.—We have used the recombinant NH₂-terminal myc-tagged rabbit Na⁺-glucose transporter (SGLT1) to study the regulation of this carrier expressed in COS-7 cells. Treatment of cells with a protein kinase C (PKC) agonist, phorbol 12-myristate 13-acetate (PMA), caused a significant decrease (38.03 ± 0.05%) in methyl α-D-glucopyranoside transport activity that could not be emulated by 4α-phorbol 12,13-didecanoate. The decrease in sugar uptake stimulated by PMA was reversed by the PKC inhibitor bisindolylmaleimide I. The maximal rate of Na⁺-glucose cotransport activity (Vₘₐₓ) was decreased from 1.29 ± 0.09 to 0.85 ± 0.04 nmol·min⁻¹·mg protein⁻¹ after PMA exposure. However, measurement of high-affinity Na⁺-dependent phloridzin binding revealed that there was no difference in the number of cell surface transporters after PMA treatment; maximal binding capacities were 1.54 ± 0.34 and 1.64 ± 0.21 pmol/mg protein for untreated and treated cells, respectively. The apparent sugar binding affinity (Michaelis-Menten constant) and phloridzin binding affinity (dissociation constant) were not affected by PMA. Because PKC reduced Vₘₐₓ without affecting the number of cell surface SGLT1 transporters, we conclude that PKC has a direct effect on the carrier, resulting in a lowering of the transporter turnover rate by a factor of two.

rSGLT1; protein kinase C; regulation

THE MECHANISMS GOVERNING the regulation of many membrane transport proteins are increasingly becoming the focus of investigation. Protein kinases can alter the activity of a protein either directly or indirectly. Direct effects involve altering the kinetics of the transporter, the apparent substrate binding affinity, or the turnover number of the carrier. Indirect effects of protein kinases involve altering the rate at which the protein is retrieved or inserted into the plasma membrane. Protein kinase A (PKA) and protein kinase C (PKC), both serine/threonine kinases, have been shown to be involved in the regulation of the Na⁺-glucose transporter (SGLT1) in polarized epithelial cell lines (9, 20–22), when expressed in Xenopus oocytes (13, 27), and also in rat intestinal brush-border membrane vesicles (14).

Measurement of sugar-induced Na⁺ currents using the two-electrode voltage-clamp technique in Xenopus oocytes expressing rabbit SGLT1 (rSGLT1) showed that exposure to a membrane-permeant activator of PKC phorbol ester, 1,2-dioctanoyl-sn-glycerol, decreased rabbit Na⁺-glucose cotransport activity by 51%. When the number of transporters was measured in the same oocyte by determination of Qₘₐₓ, the maximal number of charges translocated across the oolemma in response to voltage pulses, there was also a concomitant decrease in rSGLT1 protein from the plasma membrane (27). Conversely, stimulation of PKA using 8-bromoadenosine 3',5'-cyclic monophosphate (8-BrcAMP) resulted in a 28% increase in SGLT1 transport activity and an increase in Qₘₐₓ as well as in the plasma membrane surface area.

In this expression system, the activation of PKA or PKC did not have any measurable effect on the apparent sugar binding affinity [Michaelis-Menten constant (Kₘ)], the inhibition constant for phloridzin, or the turnover number for rSGLT1 (27). These observations indicated that rSGLT1 transport activity was regulated indirectly by protein kinases and was linked to mechanisms governing the trafficking of rSGLT1 to and from the oocyte plasma membrane by regulated endo- or exocytosis. Similar observations have been made for the development of SGLT1 transport activity in the differentiating human cell clone HT-29-D4 (9) and also for brush-border membrane vesicles prepared from perfused rat intestine exposed to the β-adrenergic agonist epinephrine (14).

It is known that the rabbit Na⁺-glucose carrier contains four putative PKC serine/threonine phosphorylation sites and one PKA site (27). However, a direct regulatory effect of either PKC or PKA on Na⁺-glucose transport activity has not been reported, and this may largely be due to the nature of the cells in which the transporter has been expressed. We have previously used a mammalian expression system, the COS-7 cell line, to characterize both a recombinant NH₂-terminal myc-tagged rSGLT1 isoform and an rSGLT1 A166C mutant (25). To further exploit this cell system, we decided to investigate how rSGLT1 was regulated in this cell line. We used the myc-tagged rSGLT1 isoform 1 because it was phenotypically identical to wild-type rSGLT1 and 2 because the incorporation of the myc epitope sequence greatly facilitated immunodetection of SGLT1 with the anti-c-myc monoclonal antibody (9E10).

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After exposure of cells to the PKC agonist phorbol 12-myristate 13-acetate (PMA), methyl α-D-glucopyranoside (α-MG) uptake into transiently transfected COS-7 cells was significantly reduced compared with controls, and this effect could be totally reversed by the specific PKC inhibitor bisindolylmaleimide I. Kinetic analysis revealed that the maximal rate of rSGLT1 transport activity (V_max) was decreased as a result of PKC activity without any effect on the apparent sugar transport activity (K_m). Interestingly, we found that the number of surface-expressed rSGLT1 transporters, measured using high-affinity Na^+-dependent phloridzin binding, was not different before and after PMA treatment, i.e., there was no change in the maximal binding capacity (B_max) values, and the dissociation constant (K_d) for phloridzin binding was unaltered. We conclude from these data that PKC regulation of rSGLT1 expressed in COS-7 cells is controlled by direct alteration of the carrier by means of a lowering of the turnover rate and that this expression system may prove useful in furthering our understanding of the mechanism(s) underlying phosphorylation-dephosphorylation of SGLT1.

**MATERIALS AND METHODS**

Cell culture and transfection. COS-7 cells were grown in complete RPMI 1640 medium (GIBCO, Burlington, ON, Canada) that was supplemented with 21 mM NaHCO_3, 25 mM HEPES-NaOH (pH 7.4), 10% (vol/vol) FCS, and 50 U/ml antibiotic solution containing penicillin and streptomycin. Cells were maintained at 37°C in 5% CO_2. Stock cultures were grown in 75-cm² flasks (Corning, Cambridge, MA) and were fed every 3–4 days. For uptake experiments or for immunodetected SGLT1, cells were seeded into 12-well plates or 35-mm culture dishes (Corning), respectively. Cells reaching 50–70% confluency were transiently transfected using the polycationic diethylaminoethyl ether of dextran (DEAE dextran) at a DNA-to-DEAE dextran ratio of 1:40 as described previously (25). Experiments were carried out 24–48 h after transfection.

Molecular biology. The c-myc epitope sequence was introduced onto the NH²-terminal region of the rabbit intestinal SGTL1 protein as described previously (25). Myc-tagged rSGLT1 cDNA was cloned into the eukaryotic expression vector pMT4 containing the simian virus 40 origin of replication suitable for expression in the COS-7 cells (25). The DNA used for the COS-7 transfections was prepared from Escherichia coli DH5α cells harboring either pMT4, pMT4-SGLT1 wild type, or pMT4-myc-SGLT1 wild type using the Qiagen plasmid midi kit (Qiagen, Chatsworth, CA). COS-7 cells transfected with vector pMT4 lacking cloned SGLT1 cDNA served as a control.

α-MG uptake. The uptake of 14C-labeled α-MG (sp act 293 mCi/mmol) into COS-7 cells was measured at room temperature (20°C) as described in Ref. 28. In brief, the medium was aspirated from the cells, and reactions were started by the addition of 500 µl of incubation medium that contained (except as stated otherwise) 140 mM NaCl, 20 mM mannitol, 10 mM HEPES-Tris (pH 7.4), and 1 mM 14C-labeled α-MG. After incubation for the desired time, the incubation medium was removed and the cells were washed three times in 3 ml volumes of ice-cold stop buffer that contained 140 mM KCl, 20 mM mannitol, 10 mM HEPES-Tris (pH 7.4), and 0.2 mM phloridzin. The termination and washing procedure took <20 s/well. Any remaining solution was aspirated, and 500 µl of PBS containing 0.1% (wt/vol) SDS were added to solubilize the cells. After 20 min, this solution was removed and processed for liquid scintillation counting. When indicated, measurements of transport rates (10 min) were performed. Uptake was directly proportional to time over a period of 20 min.

Phloridzin binding assay. The methodology for the measurement of [3H]phloridzin binding (sp act 55 Ci/mmol) was essentially similar to that for the α-MG uptake assay. The incubation medium contained a specified concentration of phloridzin: 0.01, 0.05, 0.1, 0.3, 0.4, 0.5, or 1.0 µM. Measurements of phloridzin binding were carried out after 1 min. There was no significant increase in the level of phloridzin binding after this period. The stop solution did not contain any phloridzin. Time zero binding was not measured.

Estimation of protein. Protein determination was carried out as described by Lowry et al. (17), using the Bio-Rad DC micro-protein assay kit. BSA was used as the standard.

Statistical analysis. In Table 1, data are expressed as means ± SD of three individual experiments in which mean values were determined in triplicate. Tests of significance of difference between mean values were made using ANOVA and a Bonferroni method for multiple-comparison t-tests between data pairs. The SDs reported (see also Figs. 1 and 2) were calculated from the triplicate measurements made at each experimental point. When appropriate, curve-fitting was made using a nonlinear least squares fit program (Microcal Origin 4.00, Microcal Software, Northampton, MA).

**Table 1. Effect of PMA on kinetics of Na⁺-dependent α-MG uptake and on Na⁺-dependent phloridzin binding**

<table>
<thead>
<tr>
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<th>24 h</th>
<th>48 h</th>
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<tr>
<td></td>
<td>−PMA</td>
<td>+PMA</td>
</tr>
<tr>
<td>Na⁺-dependent</td>
<td></td>
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<tr>
<td>α-MG uptake,</td>
<td>1.29 ± 0.09</td>
<td>0.85 ± 0.04*</td>
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<tr>
<td>V_max, pmol/</td>
<td>1.43 ± 0.005</td>
<td>0.42 ± 0.02</td>
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<tr>
<td>mg protein⁻¹</td>
<td>2.93 ± 0.11</td>
<td>2.15 ± 0.005*</td>
</tr>
<tr>
<td>Kₘ, µM</td>
<td>0.59 ± 0.06</td>
<td>0.55 ± 0.04</td>
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<tr>
<td>Na⁺-dependent</td>
<td></td>
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<tr>
<td>phloridzin</td>
<td>1.54 ± 0.34</td>
<td>1.64 ± 0.21</td>
</tr>
<tr>
<td>KD, µM</td>
<td>3.13 ± 0.22</td>
<td>2.93 ± 0.25</td>
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<tr>
<td>KD, µM</td>
<td>0.56 ± 0.10</td>
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Values are means ± SD of 3 separate experiments in which each assay was performed in triplicate. COS-7 cells expressing myc-tagged SGTL1 were assayed for methyl α-D-glucopyranoside (α-MG) uptake or phloridzin binding, at 24 and 48 h after transfection, as described in MATERIALS AND METHODS. Transfected cells were treated with 100 nM phorbol 12-myristate 13-acetate (PMA) for 1 h at 37°C before assay. Incubation media contained 140 mM NaCl, 20 mM mannitol, 10 mM HEPES-Tris (pH 7.4), and either 0.05–5 mM 14C-labeled α-MG or 0.05–1.0 µM [3H]phloridzin, respectively. Na⁺-independent α-MG uptake or phloridzin binding was measured in equivalent media containing 140 mM KCl. Na⁺-independent values were subtracted. V_max, maximal rate of transporter activity; Kₘ, Michaelis-Menten constant; B_max, maximum binding capacity; K_D, dissociation constant. *Significantly different from control (absence of PMA) value, P < 0.05.
experiments involving phorbol ester or PKC inhibitor(s), the final concentration of solvent (DMSO) did not exceed 0.1% (vol/vol) and did not affect α-MG uptake into myc-tagged rSGLT1-transfected COS-7 cells. Mouse anti-c-myc monoclonal antibody (9E10) was from Berkeley Antibody (Richmond, CA). Goat anti-mouse antibody conjugated to horseradish peroxidase was from Jackson Immunoresearch Laboratories (West Grove, PA). All other chemicals were of the highest quality available from Sigma (St. Louis, MO).

RESULTS

Time course for the expression of rSGLT1 in transfected COS-7 cells. Before investigating the effect of PMA on rSGLT1 transport activity in transfected COS-7 cells, we first decided to determine the time frame in which rSGLT1 was functionally expressed at the cell surface. This information would be useful in determining a time period when rSGLT1 might be more susceptible to either direct or indirect regulation by PKC. The results in Fig. 1A show the initial rate of 1 mM α-MG uptake into transfected cells, in the presence of external NaCl, at 6-h intervals after transfection. Cells transfected with either myc-tagged rSGLT1 or wild-type rSGLT1 showed detectable levels of α-MG transport activity above mock-transfected cells as early as 18 h after transfection, whereas there was negligible α-MG uptake into cells transfected with empty plasmid over the whole 48-h period. Between 18 and 30 h there was a sharp increase in sugar transport activity for both isofoms; at 24 h the rates of α-MG uptake into myc-tagged rSGLT1 or wild-type rSGLT1 were 2.5- and 2.7-fold greater than at 18 h, respectively. By 36 h α-MG transport activity was beginning to plateau, and at 48 h there was no further increase in the level of α-MG uptake into cells expressing either myc-tagged or wild-type rSGLT1. The rate of sugar uptake into cells at 48 h was double that at 24 h. We also studied the time course for the onset of high-affinity Na⁺-dependent [3H]phloridzin binding in myc-tagged rSGLT1-transfected cells. The ligand binding curve resembled that for sugar uptake, showing approximately one-half as much phloridzin binding at 24 h as at 48 h (results not shown). Western blot analysis of myc-tagged rSGLT1 using the anti-c-myc monoclonal antibody 9E10 (10) also showed a significant increase in the intensity of a 64-kDa band at 48 h compared with that at 24 h (Fig. 1B). Occasionally we saw a high-molecular-mass band corresponding to nearly double that at 64 kDa, and we can only speculate that is aggregated rSGLT1 or perhaps an rSGLT1 dimer. The monoclonal myc antibody (9E10) was highly specific to the tagged rSGLT1, and there was no cross-reaction with proteins from cell lysates from mock (pMT4)-transfected cells or those transfected with wild-type SGLT1 (Fig. 1C). The 58-kDa band may correspond to either a native rSGLT1 polypeptide or a COOH-terminal-degraded rSGLT1 protein, or perhaps to deglycosylated rSGLT1.

PMA-mediated decrease in α-MG transport activity. We have previously shown that the recombinant NH₂-terminal myc-tagged rSGLT1 protein has α-MG transport characteristics identical to those of wild-type rSGLT1 (25). However, before using it to study the regulation of SGLT1 by PKC in the COS-7 cells, we wanted to make sure that addition of the epitope tag did not interfere with a PMA-mediated response. We first tested the effect of PMA on α-MG transport activity into cells expressing wild-type rSGLT1. In the absence of PMA, α-MG uptake into cells expressing wild-type rSGLT1 was 1.40 ± 0.13 nmol·min⁻¹·mg protein⁻¹; it was decreased by 30% after exposure to the phorbol ester. Because this response was comparable to that of cells transfected with myc-tagged rSGLT1 (see Fig. 2), the epitope tag did not appear to affect the PMA-induced regulation of the transporter and we could reliably use it to further study PKC regulation of rSGLT1.

On the basis of the time course for the expression of functional rSGLT1 transporters in the plasma membrane (Fig. 1A), we wanted to investigate whether the response to phorbol ester was dependent on the level of rSGLT1 expression. We tested the action of PMA at two time points, during the early stage of rSGLT1 synthesis (24 h) and at a later stage (48 h). Transfected COS-7 cells were pretreated with 100 nM PMA for 1 h at 37°C before measurement of the uptake of 1 mM α-MG in the presence of external NaCl. At 24 h after transfection, the rate of α-MG uptake was 1.11 ± 0.02 nmol·min⁻¹·mg protein⁻¹; it was significantly decreased to 0.69 ± 0.06 nmol·min⁻¹·mg protein⁻¹ after exposure to PMA (means ± SD, n = 3, P < 0.05). The rate of α-MG uptake after 48 h was twice that at 24 h, 2.30 ± 0.08 nmol·min⁻¹·mg protein⁻¹, and was significantly decreased to 1.83 ± 0.02 nmol·min⁻¹·mg protein⁻¹ after exposure to PMA. The PMA-mediated decrease in α-MG transport activity observed at 24 h, 38.03 ± 0.05%, was significantly larger than that observed at 48 h, 20.55 ± 1.92% (means ± SD, n = 3, P < 0.05). These data show that Na⁺-glucose transport activity can be downregulated by PMA and that the magnitude of the decrease is dependent on when PMA is added to the cells.

Having shown that the action of PKC was enhanced at 24 h after transfection, we next examined the effect of different PMA concentrations and PMA exposure time on Na⁺-glucose uptake into COS-7 cells expressing myc-tagged rSGLT1. The results are shown in Fig. 2, A and B. Increasing PMA concentration (10–300 nM) decreased α-MG transport activity in a dose-dependent manner, reaching a plateau at ~100 nM PMA. Also, the action of PMA (100 nM) was time dependent and at 60 min showed a decrease of up to 43% in α-MG transport activity that was further enhanced after 2 h. Therefore, we routinely exposed transfected cells to 100 nM PMA for 1 h, at 37°C and 5% CO₂, before assaying either sugar uptake or phloridzin binding.

To ensure that the PMA-mediated decrease in rSGLT1 transport activity was not simply due to cell proliferation events associated with growth factors in the medium, transfected cells were switched to serum-free medium 24 h after transfection and allowed to culture overnight in minimal medium before assay of α-MG uptake. After PMA exposure, there was no significant difference in the PMA-mediated decrease in α-MG
transport activity (23.29 ± 0.02 and 29.82 ± 0.08% for cells cultured in complete medium and in serum-free medium, respectively; means ± SD, n = 3, P > 0.05). Thus the decrease in sugar transport activity is a direct result of PMA exposure and is not related to growth factors in the serum.

Effect of PKC inhibitor bisindolylmaleimide I on PMA-mediated decrease in α-MG transport activity. To determine if the decrease in α-MG transport activity induced by PMA was linked to the stimulation of PKC activity, we investigated the effects of the inactive phorbol ester analog 4α-PDD and the specific PKC inhibitor bisindolylmaleimide I on α-MG uptake into transfected cells. The results are shown in Fig. 3.

Transfected cells pretreated with PMA showed a 38% decrease in α-MG uptake compared with untreated cells (P < 0.05). Replacing PMA with an inactive phorbol ester, 4α-PDD (100 nM), did not cause a decrease in α-MG transport activity, and the rate of sugar uptake was similar to that in control, untreated cells (P > 0.05). This implies that the decrease in transport activity is mediated by PMA.

When transfected cells were exposed to the highly specific PKC inhibitor bisindolylmaleimide I (1 µM) (24), followed by PMA treatment, there was no significant decrease in Na+glucose transport activity (P > 0.05). Bisindolylmaleimide had little if any effect on α-MG uptake into control cells. The inactive PKC inhibitor analog bisindolylmaleimide V also had no effect on α-MG uptake into control cells but was unable to prevent a significant decrease in α-MG transport activity after PMA exposure (P < 0.05). Similar results were observed for the effect of 4α-PDD, bisindolylmaleimide I, and bisindolylmaleimide V on α-MG transport activity for cells assayed 48 h after transfection.
PKC DECREASES SGLT1 TURNOVER RATE

Effect of PMA on Cl−-driven α-MG uptake, K+ channels, and Na+/H+ exchange Cl− channels have been shown to be regulated by phosphorylation-dephosphorylation reactions (3, 5, 11, 16, 23). To eliminate the possible effect of PMA stimulating a PKC-regulated Cl− conductance pathway that might decrease electrogenic Na+/α-MG uptake, we measured substrate uptake into COS-7 cells expressing myc-tagged rSGLT1 in the presence of external Cl− or in the presence of the membrane-impermeant anion gluconate.

α-MG uptake in the presence of external Cl− was significantly decreased (44.19 ± 0.04%) after exposure to PMA, from 1.41 ± 0.03 to 0.78 ± 0.04 nmol · min⁻¹ · mg protein⁻¹ (n = 3, P < 0.05). Similarly, α-MG uptake in the presence of external gluconate ions was also significantly diminished (49.05 ± 0.05%) after treatment with PMA, from 1.12 ± 0.006 to 0.60 ± 0.02 nmol · min⁻¹ · mg protein⁻¹ (P < 0.05, n = 3). Because PMA decreased rSGLT1 transport activity in gluconate medium to the same extent as in Cl− medium, we conclude that the loss in transport activity is unlikely to be due to PKC regulation of an anion conductance pathway.

Protein kinase activation may also lead to either direct or indirect modulation of K+ channels (4, 18). Could the decrease in rSGLT1 transport activity be linked to a collapse in membrane potential by means of a PKC-regulated K+ channel? We tested this possibility by exposing the transfected cells to a "broad spectrum" K+ channel blocker, tetraethylammonium chloride (TEA) (19), for 1 h at 37°C before measuring sugar uptake. Preincubation with TEA alone (1 mM) had no effect on Na+/glucose uptake into the COS-7 cells. Exposure to TEA in the presence of 100 nM PMA resulted in a characteristic decrease (33.79 ± 4.96%, P < 0.05, n = 3) in Na+/glucose transport activity. Thus we conclude that the presence of the K+ channel blocker TEA did not affect the action of PMA and therefore that the diminished sugar transport activity is unlikely to be due to a collapse in the membrane potential.

Some cells when treated with phorbol ester activate an amiloride-sensitive Na+/H+ exchanger, which can result in internal alkalization of the cell due to increased Na+ concentration (6, 26). To investigate this, we examined the effect of 150 µM amiloride and 1 µM 5-(N,N-dimethyl)-amiloride [a highly specific inhibitor of the Na+/H+ exchanger (6)] and then treated the cells with PMA, followed by measurement of α-MG uptake. There was little if any effect of either compound on control cells in the absence of PMA or on those treated with 100 nM PMA plus inhibitor (results not shown). Therefore, the PMA-mediated decrease in α-MG uptake is not due to PKC effects on the Na+/H+ exchange system.

Effect of PMA on transporter kinetics and phloridzin binding. We next set out to determine the effects of PMA on 1) the kinetics of α-MG uptake and 2) the number of cell surface transporters, as measured by high-affinity Na+/dependent phloridzin binding. The results for both the kinetic parameters and phloridzin binding, measured at 24 and 48 h after transfection, are shown in Table 1. Transfected cells exposed to PMA

(not shown). We conclude from these data that SGLT1 transport activity is regulated by PKC and that activation of PKC may cause phosphorylation inactivation of the transporter.

We also investigated the effects of PKC inhibitors, calphostin C (500 nM) (7) or chelerythrine chloride (5 µM) (12), on α-MG transport activity in the absence or presence of PMA. Neither PKC inhibitor affected α-MG uptake into cells expressing myc-tagged SGLT1. When transfected cells were exposed to either calphostin C or chelerythrine chloride, followed by exposure to PMA, α-MG transport activity was decreased 44 and 30%, respectively, and was similar to the decrease observed for cells treated with PMA only, 34 and 32%, respectively. Because the effect of PMA exposure was reversed only by bisindolylmaleimide I and not by calphostin C or chelerythrine chloride, we postulate that the mechanism of phosphorylation inactivation of rSGLT1 by PKC may be isoform specific.
showed a significant decrease in $V_{\text{max}}$ at both 24- and 48-h time periods, compared with untreated cells ($34.51 \pm 1.43$ and $26.46 \pm 0.03\%$, respectively). There was no change in the apparent sugar binding affinity ($K_a$) for myc-tagged rSGLT1 after treatment with PMA. Of significance, the Na$^+$-dependent phloridzin binding data (Table 1) indicate that PMA treatment did not affect the maximal number of transporters ($B_{\text{max}}$) at the cell surface compared with untreated cells. In addition, the binding affinity ($K_d$) for phloridzin was also unaltered by PMA treatment and was the same for each time point.

With the assumption that phloridzin binds to the sugar binding domain of rSGLT1 and that one molecule of phloridzin binds per transporter, an estimate of the turnover rate for myc-tagged rSGLT1 after treatment with PMA is highly speculative at this point and is beyond the scope of this report.

**DISCUSSION**

We investigated the regulation of rSGLT1 expressed in the mammalian cell line COS-7. Addition of PMA to transfected cells expressing myc-tagged rSGLT1 caused a significant decrease (38%) in $\alpha$-MG transport activity that was not elicited with 4$\alpha$-PDD, indicating that the response stimulated by PMA was due specifically to the phorbol ester. Interestingly, exposure to PMA at 24 h after transfection resulted in a greater decrease in Na$^+$-glucose uptake, almost twice as much as when PMA was added 48 h after transfection. Because we did not measure PKC levels over the 48-h period, we do not know whether there is a change in the expression level of this enzyme. However, if we assume that the intracellular concentration of PKC does not increase concomitantly with rSGLT1 biosynthesis, then at 24 h posttransfection the relative ratio of PKC to rSGLT1 would be expected to be high, and consequently the regulatory effect of the enzyme on rSGLT1 transport activity would be more pronounced. The level of $\alpha$-MG uptake 48 h after transfection was found to be double that at 24 h (Fig. 1A and $V_{\text{max}}$ Table 1), and this was reflected in a twofold increase in the number of surface-expressed transporters ($B_{\text{max}}$; Table 1). Noticeably, however, the PMA-mediated decrease in sugar transport activity was almost halved (20%) from that at 24 h. It is interesting that the PMA-mediated decrease in $\alpha$-MG transport activity rarely exceeded 40%. Whether this is correlated with the level of PKC expression or the proportion of cytosolic PKC that can translocate to the plasma membrane on stimulation to regulate rSGLT1 is highly speculative at this point and is beyond the scope of this report.

The highly specific PKC inhibitor bisindolylmaleimide I inhibited the PMA effect on $\alpha$-MG transport activity. Thus we were able to demonstrate in COS-7 cells that PKC plays an important role in the regulation of rSGLT1. Although calphostin C and chelerythrine chloride have been shown to inhibit PKC activity, neither compound was able to reverse the effects of PMA exposure on $\alpha$-MG transport in COS-7 cells, and the reasons for this are not yet fully understood.

Regulation of the Na$^+/H^+$ exchanger expressed in PS120 fibroblast cells was shown to be influenced by serum, fibroblast growth factor, and phorbol ester (29). Because COS-7 cells are derived from the simian kidney fibroblast cell line, we wanted to make sure that growth factors in the serum were not a contributing factor to the regulation of rSGLT1. This was proved not to be the case, since growth-arrested cells cultured in serum-free minimal medium, although they showed one-half as much rSGLT1 transport activity as cells in normal medium, exhibited an identical reduction in $\alpha$-MG uptake in response to PMA exposure (23 and 29%...
decreases for cells in serum and serum-free medium, respectively).

To determine whether the action of PKC on rSGLT1 transport activity could be linked to a protein kinase/phosphatase cascade system, we tested the effect of okadaic acid (100 nM), a specific inhibitor of protein phosphatases 1 and 2A, on α-MG uptake before and after exposure to PMA. Similarly, we also tested the effect of the broad-range tyrosine kinase inhibitor genistein (5 μM). Neither compound had any effect on the action of PMA-mediated decrease in α-MG transport activity. This suggests that these enzymes may not be directly involved in the PKC regulation of α-MG transport activity. In oocytes expressing human SGLT1, the protein phosphatase 1 and 2A inhibitor calyculin A produced effects similar to those of 8-Br-cAMP and 1,2-dioctanoyl-sn-glycerol on the maximal rate of transport activity (V\text{max}), the number of SGLT1 transporters (Q\text{max}), and oocyte surface area (13, 27).

If electrogenic Na\textsuperscript{+}-glucose transport activity were regulated via PKC-dependent Cl\textsuperscript{−} channels, we would expect the addition of PMA to cause a decrease in α-MG uptake in the presence of Cl\textsuperscript{−} medium and have no effect on α-MG uptake in the presence of external gluconate medium. This was not the case, since α-MG uptake in the presence of external gluconate medium was reduced equally to that in Cl\textsuperscript{−} medium after PMA exposure. Therefore, we can eliminate the possibility that the decreased transport activity was due to a PKC-regulated anion conductance pathway. Similarly, the broad-spectrum K\textsuperscript{+} channel blocker TEA did not diminish the PMA-mediated decrease in α-MG transport activity, and therefore it is unlikely that the PKC agonist caused a collapse in the membrane potential. We could also rule out the possibility of the PKC agonist stimulating Na\textsuperscript{+}/H\textsuperscript{+} exchange systems, leading to increased intracellular Na\textsuperscript{+} and indirectly affecting Na\textsuperscript{+}-coupled transport (6, 26), since neither amiloride nor 5-(N,N-dimethyl)-amiloride could prevent the PMA-mediated decrease in α-MG transport activity.

The ability to demonstrate diminished rabbit Na\textsuperscript{+}-glucose transport activity via a PKC-stimulated pathway in COS-7 cells is identical to that observed for both rabbit and rat SGLT1 isoforms in oocytes. However, in oocytes, rSGLT1 transport activity was regulated indirectly by PKC via a mechanism(s) that controlled rSGLT1 protein trafficking to and from the plasma membrane. When rSGLT1 was expressed in a nonpolarized cell such as the COS-7 cells, however, we observed a different effect of PKC on rSGLT1 transport activity. Kinetic analysis revealed that the maximal rate of transport activity (V\text{max}) after PMA exposure was decreased, without any effect on the apparent sugar binding affinity (K\text{m}). When the number of cell surface transporters was measured under the same conditions, using high-affinity Na\textsuperscript{+}-dependent [\textsuperscript{3}H]phloridzin binding, the B\text{max} was unaltered, as was the K\text{d} for phloridzin. The validity of phloridzin binding as a measure of the number of surface-expressed rSGLT1 carriers has previously been discussed (25). From the ratio V\text{max}/B\text{max}, the turnover rates before and after PMA exposure were 13.9 and 8.6 s\textsuperscript{−1}, respectively.

The observation that PKC activation by PMA causes a decrease in the turnover rate of the transporter by nearly twofold implies that PKC has a direct effect on the carrier. The differences observed in PKC regulation of rSGLT1 in COS-7 cells compared with oocytes are intriguing and require further investigation. It should also be pointed out that, in oocytes, PKC regulation of SGLT1 appears to exhibit isoform specificity. PKC activation causing reduced transporter activity of rSGLT1 but increased transporter activity of the human isoform.

The main conclusion of the present study, that PKC activation has a direct effect on rSGLT1 transporter activity, raises interesting questions about the interaction of PKC with SGLT1. One possibility is that this effect is mediated by direct phosphorylation on one or more of the four PKC phosphorylation consensus sites. Another explanation is that PKC activation results in phosphorylation of another intracellular or membrane-bound protein that then interacts with the Na\textsuperscript{+}-glucose carrier, influencing its activity through a nonphosphorylation pathway. These different possibilities require investigation.

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