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Mechanisms of Glucose Uptake in Intestinal Cell Lines: Role of GLUT2

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Abstract

BACKGROUND—GLUT2 is translocated to the apical membrane of enterocytes exposed to glucose concentrations >50 mM. Mechanisms of GLUT2-mediated glucose uptake in cell culture models of enterocytes have not been studied.

AIM—To explore mechanism(s) of glucose uptake in three enterocyte-like cell lines.

METHODS—Glucose uptake was measured in Caco-2, RIE-1, and IEC-6 cell lines using varying concentrations of glucose (0.5–50 mM). Effects of phlorizin (SGLT1 inhibitor), phloretin (GLUT2 inhibitor), nocodazole and cytochalasin B (disrupters of cytoskeleton), calphostin C and chelerythrine (PKC inhibitors), and phorbol 12-myristate 13-acetate (PKC activator) were evaluated.

RESULTS—Phlorizin inhibited glucose uptake in all three cell lines. Phloretin inhibited glucose uptake in Caco-2 and RIE-1 cells. Starving cells decreased glucose uptake in Caco-2 and RIE-1 cells. Glucose uptake was saturated at ≥ 10 mM glucose in all three cell lines when exposed briefly (≤ 1 min) to glucose. After exposure for ≥ 5 min in Caco-2 and RIE-1 cells, glucose uptake did not saturate and K_m and V_{max} increased. This increase in glucose uptake was inhibited by phloretin, nocodazole, cytochalasin B, calphostin C, and chelerythrine. PMA enhanced glucose uptake by 20%. Inhibitors and PMA had little or no effect in the IEC-6 cells.

CONCLUSION—Constitutive expression of GLUT2 in the apical membrane along with additional translocation of cytoplasmic GLUT2 to the apical membrane via an intact cytoskeleton and activated PKC appears responsible for enhanced carrier-mediated glucose uptake at greater glucose concentrations (≥ 20 mM) in Caco-2 and RIE-1 cells. IEC-6 cells do not appear to express functional GLUT2.

Keywords

SGLT1; GLUT2; Caco-2 cells; RIE-1 cells; IEC-6 cells; glucose absorption; apical translocation

BACKGROUND

Intestinal glucose absorption is comprised of two components. One is a well established component mediated by the apically located, sodium-dependent glucose transporter

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(SGLT1),^{1–5} a high-affinity, low-capacity, active transport protein. The other component is glucose transporter 2 (GLUT2), a low-affinity, high-capacity, facilitated transport protein.^{6–8} The classic paradigm of glucose absorption was that SGLT1 is located in the apical membrane of enterocytes, while GLUT2 is located only in the basolateral membrane; luminal glucose transported into the enterocyte by SGLT1 is then transported out of the cell by GLUT2 into the portal venous system.^{1–10} This classic theory explains glucose absorption very well at low luminal concentrations of glucose (≤ 10 mM) but fails to explain the marked increase in glucose absorption at luminal concentrations of glucose that far surpass the transport capacity of SGLT1 (glucose concentrations ≥ 25 mM).

Several theories have been proposed to explain the marked increase in actual glucose absorption observed in the presence of high luminal concentrations of glucose that are not accounted for by this classic paradigm. Madara and Pappenheimer¹¹ suggested that SGLT1, in response to high luminal concentrations of glucose, can cause contraction of the perijunctional actinomyosin ring and dilation of intercellular tight junctions. The “solvent drag” theory^{12–14} proposes that when the glucose in the lumen reaches a very high concentration, the tight junctions between enterocytes open, allowing large amounts of solvent and other nutrients (solutes) to enter the paracellular space, “dragging” glucose with them and then diffusing into the portal system. In contrast, the GLUT2 translocation theory^{15–17} was proposed based on animal studies suggesting that high concentrations of luminal glucose saturate SGLT1; a mechanism involving protein kinase C β II (PKC β II) is activated causing translocation of cytosolic GLUT2 from preformed, cytoplasmic vesicles into the apical membrane, thereby increasing the capacity of the enterocyte for transcellular glucose uptake.^{8,18–22}

To the best of our knowledge, glucose uptake in cell culture, a system that would allow detailed study of the cell biology of glucose uptake, has not been well-studied. Indeed, cell culture models and cell lines representative of the enterocyte are not well-established. The most well-studied cell line is Caco-2, a human *colonic* cell line derived from colon cancer.^{23–26} When grown in culture, Caco-2 cells differentiate and polarize, establishing two clearly distinguishable plasma membrane domains: an apical or “brush border-like” membrane with microvilli and tight junctions and a basolateral membrane. Moreover, these cells differentiate with a phenotype resembling the enterocyte, suggesting that this cell line may represent a relevant, *in vitro* model for mechanistic studies of intestinal absorption. In our study, we used a Caco-2 cell line but also two *intestinal* cell lines derived from the rat, RIE-1 (rat intestinal epithelial cells) and IEC-6 (intestinal epithelial cells), to establish pharmacokinetic models to further investigate mechanisms of glucose uptake in the enterocyte.

Most all prior work exploring mechanisms of glucose uptake by the enterocyte has been carried out *in vivo* in the rat model.^{15–17} The first aim of our study was to develop cell models of the enterocyte that exhibit apical translocation of GLUT2, and, second, then to delineate the signaling pathways and mechanisms involved in this presumed system of intracellular vesicular transport. Our hypothesis was that when exposed to high concentrations of glucose, these models of the enterocyte would increase stereospecific uptake of glucose by a GLUT2-mediated mechanism. These studies may provide a clue to understanding abnormalities in the control of glucose absorption in various clinical states of malabsorption.

MATERIALS AND METHODS

Chemicals and Supplies

Twenty-four-well cell culture plates were purchased from Corning Life Sciences (Lowell, MA). Phlorizin, phloretin, nocodazole, cytochalasin B, chelerythrine, phorbol 12-myristate 13-acetate (PMA), and insulin were purchased from Sigma (St Louis, MO). Calphostin C was obtained from Calbiochem (Darmstadt, Germany). Dulbecco's modified Eagle medium (DMEM), non-essential amino acids, sodium pyruvate (100 mM), and streptomycin/penicillin solution from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was obtained from PAA Laboratories (Dartmouth, MA). ^{14}C -D-glucose and ^3H -L-glucose was obtained from Moravsek Biochemicals (Brea, CA). D-glucose and BCA Protein Assay Kit (#23225) was purchased from Thermo Fisher Scientific Inc. (Rockford, IL). SolvableTM and Opti-Fluor were purchased from Perkin-Elmer (Waltham, MA).

Cell Cultures

Caco-2 and IEC-6 cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, Virginia). RIE-1 cells were a gift from Dr. Larry Eagen. Caco-2, RIE-1, and IEC-6 cell lines were used between passages 20 to 60, 3 to 40, and 3 to 40 respectively, and were grown at 37°C in a 95% O₂ and 5% CO₂ atmosphere with 90% humidity in 35 × 10-mm Petri dishes containing DMEM with penicillin (10,000 U/ml) and streptomycin (10,000 µg/ml). Caco-2 cells were grown in 25 mM glucose supplemented with 20% FBS, 1% nonessential amino acids, and 1% sodium pyruvate, RIE-1 cells were grown in 5 mM glucose supplemented with 5% FBS, and IEC-6 cells were grown in 25 mM glucose supplemented with 10% FBS and 10 µg/ml insulin. Stock cells were subcultured once a week at a 1:10 ratio; media for all cells was changed two to three times weekly as needed.

Transmission Electron Microscopy

Caco-2, RIE-1, and IEC-6 cells were grown on an Aclar[®] membrane to confluence. After 5, 10, and 15 days of confluence, samples of each cell line were fixed with 3% glutaraldehyde (in 0.1 M phosphate buffer, pH 7.3) for 1 h at room temperature. Samples were then processed at the Mayo Clinic Electron Microscopy Core Facility. Samples were washed in buffer, postfixed in 1% osmium tetroxide, and embedded in Spurr epoxy resin according to standard procedures. Ultrathin sections (50–100 nm) were cut, and uranyl acetate and lead citrate stains were applied prior to examination. Sections were photographed with a JEOL EXII transmission electron microscope (Tokyo, Japan).

Glucose Uptake Assay

Cells were seeded on 24-well plates and left to differentiate/polarize for 10 days (RIE-1 and IEC-6) or 15 days (Caco-2) after reaching confluence. Glucose uptake studies were performed on differentiated/polarized monolayers attached to the bottom of 24-well plates by incubating cell monolayers in 200 µl of Krebs buffer (30 mM HEPES, 130 mM NaCl, 4 mM KH₂PO₄, 1 mM MgSO₄, 1 mM CaCl₂, pH 7.4, 290 mOsm) with varying concentrations of D-glucose (0.5–50 mM) replacing NaCl to maintain isosmolarity among solutions. Cells were incubated for 30 s and 1, 5, and 10 min. To allow calculation of carrier-mediated (stereospecific) and passive (non-stereo-specific) uptake, 0.5–1 µCi/ml of ^{14}C -D-glucose and ^3H -L-glucose, respectively, were added simultaneously to the glucose containing test solutions. ^{14}C -D-glucose was used to measure total glucose uptake, both stereospecific, carrier-mediated uptake (SGLT1 and GLUT2) and non-stereospecific passive uptake; ^3H -L-glucose was used to measure passive uptake (see below). Glucose uptake was stopped by washing twice with ice-cold phosphate buffer solution (PBS). Cells were solubilized with 300 µl of 0.1N NaOH at 37°C for 30 min. Aliquots of 10 µl of the lysate were used for

protein measurement, and 200 μ l were mixed into 4.5 ml of liquid scintillation cocktail (Opti-Fluor) with 0.5 ml distilled H₂O, and counted using dual isotope, liquid scintillation techniques on a Beckman LS6000SC (Beckman Coulter, Inc, Brea, CA). Stereo-specific, carrier-mediated glucose uptake was calculated as total uptake (¹⁴C-D-glucose) minus passive uptake (³H-L-glucose) and expressed in nmol/mg protein per duration of incubation (see below).

Experimental Conditions

To determine the individual roles of SGLT1 and GLUT2, we used specific inhibitors at well-established doses:²⁶ phlorizin (PZ; 0.5 mM) to inhibit SGLT1 and phloretin (PT; 1 mM) to inhibit GLUT2 both dissolved in 1% ethanol. To evaluate the role of PKC, we used two relatively non-selective, PKC inhibitors: calphostin C (CAL; 50 nM)²⁷ and chelerythrine (CHR; 10 μ M)²⁸ also dissolved in 1% ethanol, and an agent that stimulates PKC activity, phorbol 12-myristate 13-acetate (PMA; 50 nM) dissolved in water.^{20,29} To explore the role of the cell cytostructure, we used nocodazole (NOC; 2 μ M)³⁰ dissolved in 0.1% of dimethyl sulfoxide (DMSO) and cytochalasin B (CB; 0.5 μ M) dissolved in water to disrupt micro-tubular/cytoskeletal structure.³¹ These agents were added to test solutions containing glucose to evaluate their effects on glucose uptake. We conducted solvent control studies with ethanol and DMSO at the concentrations used above and showed no effect of the solvent on glucose uptake (data not shown).

Glucose Starvation—In separate experiments, cells from all three cell lines were grown in their usual growth media (Caco-2 and IEC-6 cells in 25 mM glucose; RIE-1 cells in 5 mM glucose) and then starved of glucose for 1 h in glucose-free DMEM media immediately prior to evaluating for glucose uptake as described above (glucose uptake assay) at different glucose concentrations (0.5–50 mM) for different durations of exposure (0.5–10 min). In a separate experiment, RIE-1 cells grown in 5 mM glucose media were exposed to 25 mM glucose for 24 h and their glucose uptake evaluated and compared to glucose uptake in cells grown in 5 mM glucose.

Calculation of glucose uptake—In all studies, values for carrier-mediated uptake were determined by subtracting passive uptake (³H-L-glucose) from total uptake (¹⁴C-D-glucose). We used the method of nonlinear regression of carrier-mediated uptake to calculate the Michaelis-Menton affinity constant (K_m) and maximal transport rate (V_{max}) using Michaelis-Menton kinetics (GraphPad Prism version 4.03). The best fit curve of the Michaelis-Menton equation was determined for carrier-mediated uptake values using the following equation:

$$V_o = V_{max} [S] / (K_m + [S])$$

where V_o is the initial uptake velocity, V_{max} is the maximal uptake velocity at saturating substrate concentrations, S is the substrate concentration, and K_m is a constant analogous to the Michaelis-Menton constant.

Measurement of glucose uptake—To summarize individual curves of glucose uptake over the range of different concentrations (0.5–50 mM) evaluated under different experimental conditions (with/without inhibitors, starved/non-starved cells, etc.), we measured the total area under the uptake curve. The effects of the various inhibitors or stimulators were evaluated by percent inhibition or stimulation (of area under the curve) over the range of concentrations evaluated. In addition, glucose uptakes at various glucose concentrations were also compared.

Immunofluorescence

Caco-2 cells grown in cell culture chambers with cover RS glass slide (Thermo Fisher Scientific Inc., Rochester, NY) for 15 days after confluence were fixed with 2% paraformaldehyde in PBS for 10 min, and blocked with non-permeabilizing buffer (10% goat serum in PBS) for 1 h at room temperature. Cells were then incubated with primary rabbit, anti-GLUT2 antibody (1:200, Chemicon) overnight at 4°C to detect GLUT2 at the apical surface only. Cells were washed with PBS and then incubated for 60 min at room temperature with Alexa Fluor goat anti-rabbit IgG (1:500, Invitrogen) in PBS. After being washed with PBS, cells were mounted with Vectashield® Mounting Medium with Dapi (Vector Laboratories, Inc., Burlingame, CA). Slides were analyzed with a Zeiss Axiophot microscope equipped with a Zeiss $\times 20/0.9$ objective lens, and a Zeiss AxioCam charge-coupled device camera (Carl Zeiss, Inc., Oberachen, Germany). Image acquisition and analysis were performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Preparation of Apical Membrane and Western Blotting

Every state of the preparation was performed at 4° to prevent in potential intracellular trafficking of transport proteins either into or out of the apical membrane of the cells. Cells were scraped on ice from three Petri dishes, placed into 2-ml Buffer 1 (mannitol 300 mM, Tris 12 mM, EGTA 5 mM, PMSF 0.1 mM and protease inhibitors, pH 7.4). After aspirating the cells three times through an 18-g and then a 22-g needle, $MgCl_2$ was added to a concentration of 12 mM; the solution was incubated at 4°C for 30 min with gentle rocking, and finally centrifuged at 4,000 $\times g$ for 15 min again at 4°. Supernatant was centrifuged at 30,000 $\times g$ for 30 min at 4°C. The pellet was washed with 150 μl of Buffer 2 (mannitol 300 mM, Tris/HCl 5 mM, PMSF 0.1 mM and protease inhibitors, pH 7.4) and centrifuged again for 30 min at 27,000 $\times g$. The pellet was re-suspended in 50 μl of Buffer 3 (mannitol 150 mM, HEPES/Tris 10 mM, EGTA 2.5 mM, PMSF 0.05 mM, and protease inhibitors, pH 7.4), $MgCl_2$ was added to a concentration of 12 mM and the aliquots stored at -80°C.

For Western blots, 20 μg of protein from the apical membrane was separated by 10% SDS-PAGE, transferred onto a polyvinylidene fluoride membrane (PVDF), probed with primary antibody (anti-GLUT2 and anti-GAPDH), and detected by color development. Quantification of Western blots was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis

Values are presented as mean $\pm$ standard deviation and were analyzed by paired or unpaired Student's *t*-test as appropriate. A *p*-value of <0.05 was considered significant. All experiments were carried out in triplicate and repeated at least three times on different days in different cell passages.

RESULTS

Differentiation and Polarization of Three Cell Lines

Cells grown on Aclar® membrane for 5, 10, and 15 days after confluence were processed for electron microscopy. After 15 days (Caco-2 cells) or 10 days (RIE-1 and IEC-6 cells) of confluence, both tight junctions and microvilli were clearly present (Figure 1), suggesting differentiation and polarization. These markers of the apical membrane were not clearly present before these times. Therefore, we used Caco-2 cells after 15 days and RIE-1 and IEC-6 cells after 10 days of confluence for all subsequent experiments.

SGLT1 and GLUT2-Mediated Glucose Uptake in Three Cell Lines

After differentiation and polarization, all three cell lines were exposed to glucose for durations of 0.5–10 min (Figure 2A). For incubation of 1 min or less, the carrier-mediated glucose uptakes in Caco-2, RIE-1, and IEC-6 cells were saturated at glucose concentrations of 25 mM and did not increase with glucose concentrations ≥ 25 mM. In contrast, with greater durations of incubation (≥ 5 min), glucose uptake in Caco-2 and RIE-1 cells did not saturate even at a glucose concentration of 50 mM (Figure 2B); glucose uptake remained, however, saturated in the IEC-6 cells at concentrations >10 mM glucose for all durations of incubation. The K_m of Caco-2 and RIE-1 cells increased from the 1 to the 10 min durations from 7.5 to 18.7 mM ($P<0.01$) and from 6.7 to 42.8 mM ($P<0.05$), respectively; the K_m remained unchanged in IEC-6 cells (Table 1). Similarly, the V_{max} for Caco-2 and RIE-1 cells increased markedly, while the V_{max} for IEC-6 cells was minimally changed. The increased K_m values in Caco-2 and RIE-1 cells for the greater durations of exposure to glucose concentrations >10 mM suggested that a change in conformation of the transporter (causing a change in K_m) occurred or that a different glucose transporter was recruited to the apical membrane.

Differentiating of SGLT1 and GLUT2

To determine the contribution of SGLT1 and GLUT2 to the uptake of glucose after a short duration of incubation (1 min), we studied the effects of phloretin (PT), a specific inhibitor of GLUT2, and phlorizin (PZ), a specific inhibitor of SGLT1. After a 1 min duration of incubation, PT inhibited markedly the total carrier-mediated uptake by 66% in Caco-2 cells ($P<0.05$) and by 54% in RIE-1 cells ($P<0.05$), but had no effect in IEC-6 cells ($P>0.05$) (Figure 3A). Because the inhibition also occurred at the lesser concentrations of glucose (<10 mM) during the shorter duration of incubation (1 min), this observation suggested a baseline constitutive expression of GLUT2 in Caco-2 and RIE-1 cells, but not in IEC-6 cells under the conditions of cell culture. Phlorizin (PZ), inhibited carrier-mediated uptake by 17% in Caco-2 cells, 24% in RIE-1 cells, and 46% in IEC-6 cells, suggesting baseline expression of SGLT1 in all three cell lines.

In order to explore the possibility of recruitment of a different transporter (presumably GLUT2) into the apical membrane when incubated for greater durations with high concentrations of glucose, we used PT, a specific inhibitor of GLUT2. The enhanced uptake of glucose that occurred at ≥ 25 mM glucose when incubations were for 10 min was inhibited by PT in Caco-2 and RIE-1 cells (Figure 3B). In these cell lines, the uptake curves appeared to level off and become asymptotic, suggesting saturation at 25 mM glucose without further increase at 50 mM glucose. In the IEC-6 cells, PT had no inhibitory effect on glucose uptake. When we tested the effects of SGLT1 inhibition by PZ on carrier-mediated uptake during the 10-min incubation, PZ decreased partially the carrier-mediated uptake by 31% in Caco-2 cells, by 36% in RIE-1 cells, and by 54% in IEC-6 cells; moreover, the curves did not appear to suggest saturation of a carrier-mediated uptake in the Caco-2 and RIE-1 cells.

Effect of Transient Glucose Starvation

In an attempt to demonstrate changes in carrier-mediated uptake of glucose secondary to loss of transporter(s) from the membrane in the absence of luminal glucose, all three cell lines grown in their original growth media (Caco-2 and IEC-6 cells in 25 mM glucose and RIE-1 cells in 5 mM glucose) were starved of glucose (incubated in glucose-free medium) for 1 h prior to evaluating carrier-mediated glucose uptake at lesser (1 min) and greater (10 min) durations of incubation with varying concentrations of glucose. In Caco-2 cells, glucose starvation decreased carrier-mediated glucose uptake after a 1 min incubation, most dramatically at glucose concentrations >10 mM (Figure 4A); this decrease was associated

with a decrease in calculated $V_{\max}$ (709 ± 18 vs 299 ± 10) and K_m (7.5 ± 0.6 vs 2.9 ± 0.2) (Table 2). In addition, the curves of carrier-mediated uptake became asymptotic at glucose concentrations >10 mM, consistent with saturation of the transporter. In the experiments with 10 min exposures; however, the effects, though similar, were less dramatic; K_m was essentially unchanged (18.7 ± 4.7 vs 16.2 ± 3.0 ; $P > 0.05$), but $V_{\max}$ was decreased slightly (1564 ± 153 vs 1217 ± 84 ; $p < 0.01$) after glucose starvation. Interestingly, despite the 1 h glucose starvation, the curve of carrier-mediated uptake did not level off even at 50 mM glucose. The effects of glucose starvation were different in RIE-1 and IEC-6 cells; there were no significant effects of glucose starvation in either cell line in terms of K_m or $V_{\max}$ (Figure 4B & D) (Table 2).

Because the RIE-1 cells, when grown in their original growth media of 5 mM glucose, increased their carrier-mediated uptake of glucose with increasing durations of incubation suggesting a change in membrane transporters, we incubated differentiated/polarized RIE-1 cells in DMEM with 25 mM glucose concentration for 24 h, and then compared carrier-mediated uptake in these cells versus cells grown in 5 mM glucose after both the 1 and 10 min incubations. Glucose uptakes were increased during the 1 min incubation (Figure 4B & C) due to an increase in K_m (6.7 ± 0.8 vs 11 ± 0.5 ; $p < 0.05$) and $V_{\max}$ (199 ± 11 vs 254 ± 7 ; $p < 0.05$). After the 10-min incubation, there was neither augmentation in carrier-mediated glucose uptake nor any increase in $V_{\max}$ (1052 ± 168 vs 1243 ± 690 ; $p > 0.05$) or K_m (43 ± 13 vs 45 ± 10 ; $p > 0.05$). Glucose starvation decreased carrier-mediated glucose uptake during the 10-min incubation, most dramatically at glucose concentrations >10 mM (Figure 4C).

Detection of GLUT2 in Caco-2 and RIE-1 cells

To confirm GLUT2 translocation to the apical membrane as suggested by our pharmacokinetic data, we stained Caco-2 and RIE-1 cells with rabbit anti-GLUT2 antibody and quantitated the immunofluorescence at the apical membrane using conventional fluorescence microscopy (Figure 5A). Immunofluorescence decreased by 40% ($p < 0.05$) in Caco-2 cells after 1 h glucose starvation, but not in RIE-1 cells ($p > 0.05$). After a 10 min exposure to 50 mM glucose in the starved cells, the immunofluorescence increased 90% ($p < 0.05$) and 130% ($p < 0.01$) in Caco-2 and RIE-1 cells, respectively (Fig 5B). Western blots of apical membrane from Caco-2 cells showed GLUT2 to be decreased by 30% ($p < 0.05$) after 1 h of glucose starvation and increased by 104% ($p < 0.05$) after 10 min-exposure to 50 mM glucose in the starved cells (Figure 6).

Role of Cytoskeleton and PKC in Carrier-Mediated Glucose Uptake

In Caco-2 and RIE-1 cells, the two disrupters of microtubular/cytoskeletal integrity, nocodazole (NC; $2 \mu\text{M}$) and cytochalasin B (CB; $0.5 \mu\text{M}$), had no effect on carrier-mediated glucose uptake at glucose concentrations of <10 mM during a 10-min incubation (data not shown). In contrast, these agents inhibited glucose uptake (Figure 7) at glucose concentrations >10 mM in the 10-min incubations in Caco-2 cells by 32% ($p < 0.001$) and in RIE-1 cells by 24% ($P < 0.05$). In addition, K_m and $V_{\max}$ were decreased (Table 3). These agents had no major effect in IEC-6 cells ($P > 0.05$) (Figure 7). There was no inhibition of carrier-mediated glucose uptake in the 1 min incubation in any cell lines (data not shown).

The two PKC inhibitors, calphostin C (CAL; 50 nM) and chelerythrine (CHR; $10 \mu\text{M}$) showed an effect similar to the effects of NOC in Caco-2 cells at glucose concentrations >10 mM ($P < 0.05$) (Figure 8A, Table 3), but in RIE-1 cells, the inhibition only occurred in 50 mM glucose ($p < 0.05$). There was no inhibition in IEC-6 cells ($P > 0.05$) (Figure 8A).

After a 1 h glucose starvation, the PKC activator, PMA (50 nM), enhanced glucose uptake by 23% ($p < 0.01$) in Caco-2 cells and by 9% ($p < 0.05$) in RIE-1 cells at glucose

concentrations ≥ 25 mM after 10 min incubations. Interestingly, there was no significant increase in carrier-mediated glucose uptake in non-starved cells. There was no effect by PMA in IEC-6 cells ($p > 0.05$) (Figure 8B; Table 4).

DISCUSSION

This study was designed to explore whether we could demonstrate any functional and pharmacologic evidence for apical translocation of GLUT2 in three separate cell lines representative of the small bowel enterocyte. Our study showed convincing evidence for a rapid, concentration-dependent, and time-dependent recruitment of an apical glucose transporter different from SGLT1 in two of the three cell culture lines (Caco-2 and RIE cells). Focused pharmacologic inhibitors and specific immunohistochemistry suggested that this transporter was GLUT2 and that the process of recruitment required both PKC activity and an intact cytoskeletal structure. These observations in cell culture are consistent with observations largely *in vivo* and may provide 2 cell culture models (one in a human cell line and the other in a rat cell line) in which to study mechanisms of translocation of GLUT2 to the apical membrane of the enterocyte.

Extensive prior work by Kellett and multiple colleagues^{1–10} challenged the classic theory of glucose absorption—first that SGLT1 is the only glucose transporter in the apical membrane of the enterocyte, and second, that the mechanism of the marked increase in glucose absorption after a high glucose meal is by “solvent drag” secondary to the opening of tight junctions between enterocytes. These investigators showed that, in a rat model of intestinal perfusion, glucose absorption increased to levels greater than expected/predicted when exposed to luminal concentrations of glucose that far exceeded the K_m of SGLT1 and would thereby fully saturate the SGLT1 transporter. This increase in glucose absorption was concentration and time-dependent. Our results in Caco-2 and RIE-1 cells of a time-dependent increase in carrier-mediated glucose uptake (not passive, non-specific uptake) at high concentrations of glucose are consistent with these studies in rat jejunum *in vivo*.

Our kinetic data of carrier-mediated uptake of glucose at lesser concentrations of glucose (≤ 10 mM) and for short durations of exposure are consistent with a single, saturable, apical glucose transporter. Our calculated K_m (~ 7.5 mM) is consistent with that of SGLT1.^{26,32} In contrast, with greater concentrations of glucose (≥ 25 mM) combined with greater durations of incubation, the kinetics of glucose uptake in Caco-2 and RIE-1 cells changed; the K_m and V_{max} increased markedly, and carrier-mediated uptake was not saturable even at 50 mM glucose, suggesting a change in the apical transporter in these cells. As found by Kellett and colleagues in the perfused rat jejunum,²⁶ our pharmacologic studies with phloretin, a relatively specific inhibitor of GLUT2 and not SGLT1, were consistent with GLUT2 being the “recruited” transporter. In the presence of phloretin, carrier-mediated uptake no longer increased at glucose concentrations ≥ 25 mM for the greater durations of incubation and appeared saturable, as would be expected with SGLT1 being the only transporter. These observations, based on our pharmacologic studies, are consistent with recruitment of GLUT2 into the apical membrane.

To further explore the concept of increased levels of apical GLUT2, we evaluated carrier-mediated uptake from cell lines starved of glucose for one hour, or exposed to high concentrations of glucose (50 mM) for 10 min to determine if carrier-mediated uptake would change. Because the apparent translocation process appeared to be rapid (within minutes), we hypothesized that starving the cell would lead to less transporter (GLUT2) in the apical membrane, resulting in less carrier-mediated glucose uptake. In contrast, exposing the cells to high concentrations of glucose would increase the levels of apical GLUT2 and, therefore, increase the carrier-mediated uptake of glucose. Our experiments confirmed the hypothesis

in Caco-2 cells. In RIE-1 cells, the difference in carrier-mediated glucose uptake between starving the cells (0 mM glucose) and their normal growth media (5 mM glucose) did not show any effect; however when we compared starved cells to cells exposed to 50 mM glucose for 24 hours, we could show increased carrier-mediated uptake in the cells exposed to 50 mM glucose, suggesting that GLUT2 levels were increased in the apical membrane.

Because the kinetic data suggested an increase in carrier-mediated glucose uptake by a GLUT2-mediated mechanism, we studied the apical expressions of the GLUT2 transporter by specific immunohistochemistry and Western blotting of apical membrane protein. These experiments with imaging showed convincingly that, when starved of glucose, the immunofluorescence from anti-GLUT2 antibodies decreased markedly in Caco-2 cells, but not in RIE-1 or IEC-6 cells. In contrast, when exposed to an increased concentration of glucose (50 mM), immunofluorescence increased in Caco-2 and RIE-1 cells, again consistent with the kinetic data of carrier-mediated uptake of glucose.

Kellett and others have investigated the mechanism of “recruitment” of GLUT2 to the apical membrane by providing substantial evidence for the intracellular trafficking of the transporter to the apical membrane from intracellular vesicles of pre-synthesized GLUT2.^{16,18} Indeed, targeted intracellular translocation of vesicles to various parts of the cell membrane is a well-known mechanism in cell biology.^{30,33–35} Kellett et al showed increases in apical membrane content of GLUT2 that correlated with the increase in carrier-mediated uptake of glucose.²⁶

Our studies in cell culture also allowed us to investigate whether passive uptake was increased at the greater concentrations of glucose. The theory of solvent drag suggests that as the osmolarity of glucose in the gut lumen increases, glucose absorption increases passively (and not by a transcellular, carrier-mediated process) secondary to opening of tight junctions between enterocytes; intercellular entry of the solvent of interest then “drags” solute (glucose) with it to increase (passive) absorption. Our experiments showed that passive uptake in cell monolayers increased linearly with increases in glucose concentration (data not shown), which did not appear to support the theory of opening of tight junctions at concentrations of glucose < 50mM.

Several groups have investigated mechanisms controlling apical translocation of GLUT2 in the presence of increased luminal concentrations of glucose.^{19,20,36–38} A PKC β II-pathway regulating apical translocation of GLUT2 has been implicated. Our experiments with two different, nonspecific PKC inhibitors (calphostin C and chelerythrine) showed a marked inhibition of the increase in carrier-mediated uptake of glucose at high concentrations during prolonged (10 min) incubations, consistent with inhibiting translocation of GLUT2. Carrier-mediated uptake at the lesser concentrations of glucose (≤ 10 mM) and lesser durations of incubation ($\leq$ min), when translocation (and increased carrier-mediated uptake) did not occur, were unaffected by inhibiting PKC; this observation suggests that the PKC inhibitors did not exhibit any nonspecific effects on glucose uptake. Our studies also showed an increase in carrier-mediated glucose uptake in the presence of PMA. PMA is known to stimulate PKC activity.^{20,29} Our results support the findings of others of an increase in glucose absorption in the presence of PMA.^{37,39}

The importance of the cytoskeletal structure of the cell as well as the microtubules in apical translocation of GLUT2 has also been shown by others.^{19,20,31,38} Our studies with two different agents that disrupt the cellular cytoskeleton (nocodazole and cytochalasin B) also inhibited in part the concentration and time-dependent increase in carrier-mediated uptake of glucose, thereby supporting the findings of these studies. In addition, neither of these agents substantially inhibited carrier-mediated uptake at the lesser concentrations of glucose or

during short incubation times, consistent with the lack of a marked effect of these cytoskeletal disrupters on carrier-mediated glucose uptake.

In summary, our work in the enterocyte-like Caco-2 and RIE-1 cell lines suggests a rapid, dynamic process of translocation GLUT2 to the apical membrane, dependent on PKC activity and cell cytostructure, consistent with *in vivo* models. Use of these cell lines may provide models to investigate further mechanisms regulating the process of glucose absorption, especially in pathophysiologic conditions.

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Figure 1.

Transmission electron micrograph of Caco-2, RIE-1, and IEC-6 cells. Cells were grown on Aclar® membrane for 15 (Caco-2) or 10 (RIE-1 and IEC-6) days after confluence. Cells joined by tight junctions (arrows) and microvilli (arrow heads) are shown on the apical surface demonstrating differentiation into an enterocyte-like phenotype.

2A

2B

Figure 2.

Carrier-mediated glucose uptake in three cell lines. A) Glucose uptake in Caco-2 (upper panel) and IEC-6 cells (lower panel) at different durations of exposure. The pattern in RIE-1 cells was similar to Caco-2 cells (data not shown, see Table 1); B) Glucose uptake by Caco-2, RIE-1, and IEC-6 cells over 1 min (upper panel) or 10 min (lower panel) duration of incubation. Values are presented as mean $\pm$ SD of triplicates repeated three times.

3A

3B

Figure 3. Inhibition of carrier-mediated glucose uptake by phlorizin (PZ; 0.5 mM) and phloretin (PT; 1 mM). Cells exposed for either A) 1 min or B) 10 min durations. Values are presented as mean $\pm$ SD of triplicates, each repeated three times.

- Control (10 min incubation)
- -■- - Starvation (10 min incubation)
- ◇— Control (1 min incubation)
- -◇- - Starvation (1 min incubation)

Mean $\pm$ SD

$n \geq 3$

p differ from Control

* $p < 0.05$

*** $p < 0.001$

Figure 4.

Effect of glucose starvation (1 h) on glucose uptake. A) Caco-2 cells: glucose starvation decreased glucose uptake at both 1 and 10 min incubations; B) RIE-1 cells: when grown in the standard 5 mM glucose media, glucose starvation had no effect on glucose uptake; C) RIE-1 cells grown/differentiated in 5 mM glucose were incubated in 25 mM glucose for 24 h, and then glucose starved for 1 h, after which carrier-mediated glucose uptake was measured during 1 and 10 min incubations; D) IEC-6 cells: no effects of glucose starvation. Values are presented as mean $\pm$ SD of triplicates, each repeated three times.

5A

5B

Figure 5.

Detection of apical GLUT2 in nonpermeabilized Caco-2 cells. A) Caco-2 cells were grown to confluence in a cell culture chamber and fixed with paraformaldehyde. The cells were then incubated with rabbit anti-GLUT2 antibody, followed by incubation with goat anti-rabbit IgG Alexa Fluor (red). Incubation of the cells with only the secondary antibody revealed no observable fluorescence (data not shown). Nuclei are shown in blue (Dapi staining). B) Intensity of immunofluorescence (GLUT2) in the apical membrane of Caco-2 (upper panel) and RIE-1 (lower panel) was analyzed semi-quantitatively by dividing the apical membrane surface Alexa Fluor fluorescence intensity by the total cell number in a 20× microscopic field. Values are presented as mean ± SD of three independent experiments.

Figure 6. GLUT 2 translocation out of and into apical membrane. Apical membrane proteins were isolated from Caco-2 cells. Equal amount of proteins were loaded and blotted for GLUT2 and GAPDH (insert). Intensity of GLUT2 was analyzed and normalized by GAPDH. Values are presented as mean $\pm$ SD of three repeats.

Figure 7.

Disruption of cytoskeletal structure with nocodazole (NOC; 2 μ M) and cytochalasin B (CB; 0.5 μ M). Carrier-mediated glucose uptake during the 10 min incubation was decreased at glucose concentrations >10 mM in Caco-2 cells and RIE-1 cells, but no consistent effect on glucose uptake was seen in IEC-6 cells. Values are presented as mean $\pm$ SD of triplicates, each repeated three times.

Figure 8.

Role of PKC. Carrier-mediated glucose uptake was inhibited by A) calphostin C (CAL; 50 nM) and chelerythrine (CHR; 10 μ M) at glucose concentrations >10 mM and B) increased by phorbol 12-myristate 13-acetate (PMA; 50 nM) at glucose concentrations > 10mM in both Caco-2 and RIE-1 cells. No consistent effect was seen in IEC-6 cells. Values are presented as mean $\pm$ SD of triplicates each repeated three times.

Table 1

K_m and V_{max} in three cell lines during 1 and 10 min durations of incubation.

	1 min incubation		10 min incubation	
	K_m (mM)	V_{max} (nmol/mg/1 min)	K_m (mM)	V_{max} (nmol/mg/10 min)
Caco-2	7.5±0.6	709±18	18.7±4.7*	1564±153*
RIE-1	6.7±0.8	199±11	42.8±13.2*	1052±168*
IEC-6	7.6±0.4	235±4	8.9±2.0	365±27*

* Differs from 1 min incubation; $P < 0.05$

Table 2

K_m and V_{max} in three cell lines after 1 h glucose starvation.

	1 min incubation		10 min incubation	
	K_m (mM)	V_{max} (nmol/mg/1 min)	K_m (mM)	V_{max} (nmol/mg/10 min)
Caco-2	2.9±0.2	299±10	16.2±3.0*	1217±84*
RIE-1 (5mM)	2.9±0.5	112±10	42.2±1.9*	1049±25*
IEC-6	18.5±4.6	234±24	17±4	470±45*

* Differs from 1 min incubation; $P<0.05$

Table 3

Change in K_m and V_{max} by disruption of microtubule/cytoskeletal structure and PKC inhibitors in Caco-2 and RIE-1 cells and by the PKC activator PMA.

	Caco-2		RIE-1	
	K_m (mM)	V_{max} (nmol/mg/10-min)	K_m (mM)	V_{max} (nmol/mg/10-min)
Control	18.7±4.7	1564±153	42.8±13.2	1052±168
NOC [†]	11.4±1.0 *	884±25 *	27.9±11.0	699±128 *
CB [†]	9.8±2.4 *	822±59 *	39.6±10.8	818±119 *
CAL [†]	12.8±0.4 *	1050±11 *	32.9±12.9	787±154 *
CHR [†]	10.8±1.2 *	969±33 *	39.7±1.7	1287±30

* Differs from control; P<0.05

[†] NOC – nocodazole; CB – cytochalasin B; CAL – calphostin C; CHR - chelerythrine

Table 4Change in K_m and V_{max} by PKC activator PMA.

	Caco-2		RIE-1	
	K_m (mM)	V_{max} (nmol/mg/10-min)	K_m (mM)	V_{max} (nmol/mg/10-min)
Control	18.5±2.0	1559±71	26.1±9.2	888±148
PMA	29.7±2.3 *	2289±89 *	48.5±6.3 *	1298±98 *

* Differs from control; P<0.05