Chronic and selective inhibition of basolateral membrane Na-K-ATPase uniquely regulates brush border membrane Na absorption in intestinal epithelial cells

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Manoharan P, Gayam S, Arthur S, Palaniappan B, Singh S, Dick GM, Sundaram U. Chronic and selective inhibition of basolateral membrane Na-K-ATPase uniquely regulates brush border membrane Na absorption in intestinal epithelial cells. *Am J Physiol Cell Physiol 308: C650–C656, 2015. First published February 4, 2015; doi:10.1152/ajpcell.00355.2014.* —Na-K-ATPase, an integral membrane protein in mammalian cells, is responsible for maintaining the favorable intracellular Na gradient necessary to promote Na-coupled solute cotransport processes [e.g., Na-glucose cotransport (SGLT1)]. Inhibition of brush border membrane (BBM) SGLT1 is, at least in part, due to the diminished Na-K-ATPase in villus cells from chronically inflamed rabbit intestine. The aim of the present study was to determine the effect of Na-K-ATPase inhibition on the two major BBM Na absorptive pathways, specifically Na-glucose cotransport and Na/H exchange (NHE), in intestinal epithelial (IEC-18) cells. Na-K-ATPase was inhibited using 1 mM ouabain or siRNA for Na-K-ATPase-1-polypeptide. The cytoplasmic domain of the 1-subunit is essential for Na-K-ATPase function, while the 3-subunit is important for stabilization of correct folding of the α1-polypeptide. The 3-subunit mediates catalytic activity and contains intracellular binding sites for ATP and Na, a phosphorylation site, and extracellular binding sites for K. The glycosylated 3-subunit is essential for Na-K-ATPase function, as it facilitates α/β-heterodimer formation and subsequent transport of the holoenzyme to the plasma membrane (15). The α1- and β1-subunits are constitutively expressed in the majority of tissues, whereas the α2-, α3-, and α4-subunits, as well as the β2- and β3-subunits, have limited expression (12). A third small polypeptide subunit, the γ-subunit, is found in association with α- and β-subunits; the γ-subunit does not seem to be required for Na-K-ATPase function and is suggested to play a regulatory role (24). The cytoplasmic domain of the γ-subunit interacts with the NH2-terminal domain of ankyrin (10), a protein that connects Na-K-ATPase to the spectrin (fodrin)-based membrane skeleton and stabilizes Na-K-ATPase at the plasma membrane (20).

Proper Na-K-ATPase function is essential for maintenance of good nutritional health. Na-K-ATPase is also important for osmotic balance and volume regulation of cells (14). Dysfunction or deficiency of Na-K-ATPase has been identified in chronic neurodegenerative disorder and cardiovascular and renal diseases (5, 19, 27). Na-K-ATPase may be even more important in diarrheal diseases. It is the preservation of Na-K-ATPase-dependent Na-solute cotransport that is the basis for oral rehydration therapy (26). Several studies have described decreased Na-K-ATPase activity in acute and transient infectious enteritis (1, 8, 16, 22, 25). Furthermore, in chronic diarrheal diseases characterized by chronic intestinal inflammation resulting in malabsorption and, thus, malnutrition, alteration of Na-K-ATPase function may be very important. In an animal model resembling inflammatory bowel disease, it has been shown that Na-glucose (SGLT1), Na-alanine (ATB0), Na-glutamine (BOAT1), and Na-taurocholate (ASBT) cotrans-
port are diminished in intact villus cells (21, 21a, 22, 23, 23a, 23b). In every instance, there is also a decrease in BLM Na-K-ATPase (6, 7, 21, 23). Thus it has been suggested that inhibition of Na and nutrient absorption in diarrhoeal diseases characterized by chronic intestinal inflammation is, at least in part, due to an alteration of Na-K-ATPase in the BLM of villus cells.

While overwhelming circumstantial evidence implicates a regulatory role for Na-K-ATPase on BBM Na absorptive pathways in pathophysiological conditions, how this ubiquitous enzyme may regulate BBM Na absorption in the normal intestine is less understood. Thus, in the present study, we determine the direct regulation of BBM primary Na absorptive pathways, specifically, Na/H exchange (NHE3) and SGLT1 by BLM Na-K-ATPase in enterocytes.

METHODS

Induction of chronic inflammation. New Zealand White male rabbits (2.0–2.2 kg body wt) were used for the study. Experiments involving the use of animals in these studies were approved by the West Virginia University Animal Care and Use Committee (WVU ACUC). Chronic intestinal inflammation was induced in rabbits as previously reported (22). Briefly, pathogen-free rabbits were intragastrically inoculated with *Eimeria magna* oocytes to induce chronic intestinal inflammation, which manifested on days 13–14 postinoculation. Animals were euthanized with an overdose of pentobarbital sodium through the ear vein on day 14 postinoculation.

Villus cell isolation. Villus cells were isolated from normal and chronically inflamed rabbit small intestine by a Ca2+ chelation technique, as previously described (22). Briefly, a 3-ff section of ileum was filled and incubated at 37°C with buffer (0.15 mM EDTA, 112 mM NaCl, 25 mM NaHCO3, 2.4 mM K2HPO4, 0.4 mM KH2PO4, 2.5 mM L-glutamine, 0.5 mM β-hydroxybutyrate, and 0.5 mM dithiothreitol, pH 7.4) for 3 min and gently palpated for another 3 min to facilitate cell dispersion. The buffer with the dispersed cells was drained from the ileal loop, and the suspension was centrifuged at 1,000 g for 3 min. The cells were flash-frozen in liquid nitrogen and stored at –80°C until further use.

Cell culture and ouabain treatment. Rat small intestine (IEC-18) cells (American Type Culture Collection), between passages 5 and 30, were routinely maintained in the laboratory in DMEM [4.5 g/l glucose, 3.7 g/l sodium bicarbonate, 2 mM L-glutamine, 10% (vol/vol) bovine fetal serum, 100 U/l human insulin, 0.25 mM β-hydroxybutyric acid, 100 U/ml penicillin, and 100 μg/ml streptomycin] in a humidified atmosphere of 10% CO2 at 37°C. BLM-IEC-18 cells grown on 12-well plates were treated with ouabain (100 μM or 1 mM) or vehicle, and experiments were performed 1 or 24 h posttreatment. Experiments were conducted on postconfluence day 4.

RNA interference. Silencer select predesigned negative control siRNA (catalog no. AM4611) and Na-K-ATPase siRNAs (catalog no. 4390771 and ID no. s127474) were used for siRNA transfections. The siRNAs (1.5 μg each) were suspended in Nucleofector solution (pH 7.4, 7.1 mM ATP, 11.6 mM MgCl2·6H2O, 13.6 mM NaHCO3, 84 mM KH2PO4, and 2.1 mM glucose) and individually nucleofected into IEC-18 cells using a Nucleofector II device (Amaxa) according to the manufacturer’s recommended protocol. Cells were plated in 12-well plates and experiments were conducted on day 4 postconfluence.

Uptake studies in IEC-18 cells. Uptake studies for SGLT1 were done using 3-O-methyl-d-[3H]glucose ([3H]3-OMG; GE Healthcare Biosciences) in IEC-18 cells from all experimental conditions. Cells were incubated with Leibovitz (L-15, 100% oxygen) medium (Invitrogen) containing supplements (as described above for DMEM) for 1 h at room temperature. The cells were subsequently washed and incubated for 10 min at room temperature in a Na-free buffer (130 mM tetramethylammonium chloride, 4.7 mM KCl, 1 mM MgSO4, 1.25 mM CaCl2, and 20 mM HEPES). Glucose uptake studies were then performed in cells incubated for 2 min with 130 mM NaCl, 4.7 mM KCl, 1 mM MgSO4, 1.25 mM CaCl2, and 20 mM HEPES (pH 7.2) with 10 μCi of [3H]OMG and 100 μM OMG in the presence or absence of 1 mM phlorizin and 10 mM d-glucose. The reaction was stopped by addition of ice-cold Na-free buffer containing 25 mM d-glucose, and the cells were lysed in 800 μl of 1 N NaOH by incubation for 30 min at 70°C and mixed with 5 ml of Ecoscint A (National Diagnostics). The vials were kept in darkness for 24–48 h, and radioactivity retained by the cells was determined in a Beckman 6500 scintillation counter.

Na/H exchange activity was measured for all conditions in a similar fashion using 1 mM 22NaCl as substrate added to 130 mM tetramethylammonium in HEPES buffer, as described above. 5-(N-ethyl-N-isopropyl) amiloride (EIPA)-sensitive Na/H exchange was measured by deducting the Na/H exchange activity in the presence of 50 μM EIPA (Sigma-Aldrich) from the total Na/H exchange activity of the IEC-18 cells. EIPA inhibits only NHE3, since this is added only to the BBM.

**Na-K-ATPase enzyme activity measurement.** Na-K-ATPase was measured as inorganic phosphate (P) liberated by the method of Forbush (9) in cellular homogenates of normal and siRNA-transfected cells. The reaction was started by addition of 20 mM ATP-Tris to 20 μg of cellular homogenate in 50 mM Tris-HCl (pH 7.2) and 5 mM MgCl2 with 20 mM KCl and 100 mM NaCl. The reaction was stopped by addition of 1 ml of ice-cold 2.8% acetic acid, 0.48% ammonium molybdate, and 2.8% sodium dodecyl sulfate-0.48 M HCl solution and placed on ice for 10 min. The color was developed by incubation at 37°C for 10 min, after addition of 1.5 ml of 2% sodium citrate, 2% sodium arsenate, and 2% acetic acid. Readings were obtained at 705 nm, and K2HPO4 was used as P standard. Enzyme-specific activity was expressed as nanomoles of P released per milligram of protein per minute.

**Protein determination.** Total protein was measured by the Lowry method using the Bio-Rad protein assay kit (Hercules, CA). BSA was used as a standard.

**Intracellular Na concentration.** Intracellular Na concentration was measured using cell-permeant Sodium Green tetraacetate salt (catalog no. S-6901, Molecular Probes, Invitrogen) according to the manufacturer’s protocol. Briefly, equal amounts of 1 × 106 cells/ml were plated on each well of 96-well plates and grown to day 4 postconfluence. To measure intracellular Na concentration, the cells were incubated for 1 h with 10 μM Sodium Green tetraacetate salt in Phlorin at room temperature. The FlexStation 3 plate reader (Molecular Devices) was used to read the resultant fluorescence at 532 nm. The intracellular concentration for different experimental conditions was determined by correlation of the fluorescence with a calibration response curve that was generated by loading normal IEC-18 cells for 1 h with 0–130 mM Na. Loading of free Na into the cells was accomplished with 5 μM gramicidin (catalog no. G-6888, Molecular Probes). The cells thus loaded with a known amount of sodium were incubated with 10 μM Sodium Green tetraacetate, and fluorescence emission was read at 532 nm. The resultant fluorescence values were used to calculate the dissociation constant of the indicator with the formula given by the manufacturer and was found to be close to that reported by Molecular Probes.

**Isolation of total RNA and mRNA expression by RT-quantitative PCR.** For all conditions, total RNA was isolated from IEC-18 cells using RNeasy Plus total RNA purification mini kits (Qiagen). First-strand cDNA synthesis was performed using SuperScript III (Invitrogen Life Technologies). The cDNAs synthesized were used as templates for RT-quantitative PCR (qPCR) using TaqMan Universal PCR Master Mix (Applied Biosystems) according to the manufacturer’s protocol. RT-qPCR experiments for rat β-actin were performed using TaqMan Gene Expression Assay (assay ID 4352931E, Applied Biosystems). RT-qPCR primers for rat SGLT1 were custom-synthesized.
using the oligonucleotide synthesis service provided by Applied Biosystems.

The primer and probe sequences for rat SGLT1 RT-qPCR are as follows: 5'-TTGTGGAGGACAGTGGTGAA-3' (forward primer), 5'-AAAATAGGGCGGAGAGGA-3' (reverse primer), and 5'FAM CATCAGCCGATCCTCTCTGTTGAMS-3' (TaqlMan probe).

RT-qPCR for β-actin expression was run along with the SGLT1-specific RT-qPCR as an endogenous control under similar conditions to normalize expression levels of SGLT1 between individual samples. RT-qPCR analyses were performed in triplicate and repeated three times using RNA isolated from three sets of IEC-18 cells.

**Western blot analysis.** Western blot experiments were performed according to standard protocols. For all conditions, IEC-18 cells were solubilized in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Igepal, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 1 mM NaF, and protease inhibitor cocktail (SAFC Biosciences)) and separated on a 10% custom-prepared polyacrylamide gel. Separated proteins were transferred to a PVDF transfer membrane for Western blot analysis. SGLT1 was probed using a primary rabbit antibody conjugated with horseradish peroxidase was used to detect SGLT1-bound primary antibody. ECL Western Blotting Detection Reagent (GE Healthcare) was used to detect the immobilized SGLT1 protein by chemiluminescence. The intensity of the bands was quantitated using a densitometric scanner (FluorChem, Alpha Innotech, San Leandro, CA).

**Data presentation.** For averaged data, means ± SE are shown, except when error bars are inclusive within the symbol. All uptake and RT-qPCR experiments were done in triplicate, unless otherwise specified. The number (n) for any set of experiments refers to uptake experiments, total RNA, or protein extracts from different sets of cells. Student’s t-test was used for statistical analysis.

**RESULTS**

**Effect of Na-K-ATPase activity on chronically inflamed rabbit cells.** Na-K-ATPase activity was significantly decreased in villus cell homogenate obtained from chronically inflamed rabbit intestine compared with that obtained from normal intestine (Fig. 1).

**Effect of ouabain on SGLT1 in IEC-18 cells.** In the chronically inflamed small intestine, innumerable immune inflammatory mediators that are known to be elevated in the mucosa may affect Na-K-ATPase and/or its dependent BBM cotransport processes. To more directly study the effect of inhibition of Na-K-ATPase on BBM SGLT1 (also known to be inhibited in the chronically inflamed small intestine), Na-K-ATPase was inhibited in vitro in IEC-18 cells with ouabain. After 1 h of treatment, ouabain inhibited Na-K-ATPase activity (Fig. 2A; 12.2 ± 0.6 and 8.7 ± 0.7 nmol P i·mg protein−1·min−1 in control and ouabain-treated cells, respectively, n = 3, P < 0.01). BBM SGLT1 was also inhibited in these cells (Fig. 2B; 268.3 ± 24.04 and 133.0 ± 14.98 pmol·mg protein−1·2 min−1 in control and ouabain-treated cells, respectively, n = 3, P < 0.01). However, the effects of immune inflammatory mediators on Na-K-ATPase in the chronically inflamed intestine were not acute, but chronic. So IEC-18 cells were treated for 24 h with ouabain. Chronic exposure to ouabain continued to inhibit Na-K-ATPase activity in IEC-18 cells (Fig. 2A; 11.8 ± 0.3 and 8.8 ± 0.5 nmol P i·mg protein−1·min−1 in control 24 h ouabain-treated cells, respectively, n = 3, P < 0.01). Interestingly, unlike acute exposure, chronic exposure to ouabain caused a significant increase in BBM SGLT1 activity (Fig. 2B; 268.3 ± 24.04 and 530.0 ± 30.20 pmol·mg protein−1·2 min−1 in control and 24 h ouabain-treated cells, respectively, n = 3, P < 0.005).

**Effect of Na-K-ATPase silencing in IEC-18 cells.** To further and more specifically explain the unique phenomenon of SGLT1 stimulation when Na-K-ATPase was inhibited chronically with ouabain, we employed Na-K-ATPase silencing to inhibit Na-K-ATPase-α1. This resulted in significant inhibition of Na-K-ATPase activity in cells transfected with siRNA for Na-K-ATPase-α1. This resulted in significant increase in [3H]OMG uptake compared with control. This inhibitory effect was more specifically explain the unique phenomenon of SGLT1 stimulation when Na-K-ATPase was inhibited chronically with ouabain, we employed Na-K-ATPase silencing to inhibit Na-K-ATPase-α1. This resulted in significant increase in [3H]OMG uptake compared with control.

**Data presentation.** For averaged data, means ± SE are shown, except when error bars are inclusive within the symbol. All uptake and RT-qPCR experiments were done in triplicate, unless otherwise specified. The number (n) for any set of experiments refers to uptake experiments, total RNA, or protein extracts from different sets of cells. Student’s t-test was used for statistical analysis.

Fig. 4. Effect of siNa-K-ATPase-α1 on EIPA-sensitive 22Na uptake in IEC-18 cells. Chronic inhibition of Na-K-ATPase with its specific siRNA did not have an effect on EIPA-sensitive 22Na uptake in IEC-18 cells.

Fig. 5. Effect of Na-K-ATPase inhibition on intracellular Na concentration in IEC-18 cells. Intracellular Na concentration was significantly increased in ouabain-treated cells and cells transfected with siRNA for Na-K-ATPase-α1 compared with their respective controls.

Effect of Na-K-ATPase-α1 silencing on SGLT1 mRNA levels in IEC-18 cells. To determine whether intracellular Na levels were affected when Na-K-ATPase was chronically inhibited by ouabain or transfection with siRNA for Na-K-ATPase-α1, we measured intracellular Na levels. Compared with control, intracellular Na was increased in IEC-18 cells treated with ouabain (Fig. 5; 6.2 ± 0.4 and 12.6 ± 0.7 mM in control and ouabain-treated cells, respectively, n = 3, P < 0.05). Similarly, in siNa-K-ATPase-α1-silenced cells, intracellular Na levels were significantly increased (Fig. 5; 5.4 ± 0.1 and 10.8 ± 1.2 mM in control and siNa-K-ATPase-α1-transfected cells, respectively, n = 3, P < 0.05). These data indicate that chronic inhibition of Na-K-ATPase results in increased intracellular Na and, thus, a diminished transmembrane Na gradient, which would likely affect Na-solute cotransporters on the BBM.

Effect of Na-K-ATPase-α1 silencing on SGLT1 mRNA levels in IEC-18 cells. To determine the molecular mechanism of stimulation of SGLT1 by siNa-K-ATPase-α1 silencing in IEC-18 cells, mRNA levels were determined by RT-qPCR. Transfection of IEC-18 cells with Na-K-ATPase-α1 siRNA did not alter SGLT1 mRNA levels in these cells (Fig. 6A). Since mRNA levels do not necessarily correlate with functional protein levels on the BBM, Western blot studies were performed to further characterize the molecular mechanism of SGLT1 regulation by Na-K-ATPase-α1 silencing. In IEC-18 cells silenced with siNa-K-ATPase-α1, SGLT1 protein levels were not significantly altered (Fig. 6B). These data indicate that the mechanism of SGLT1 stimulation is likely not transcriptional.

Kinetic parameters for SGLT1 activity in siNa-K-ATPase-α1-silenced IEC-18 cells. To further determine the mechanism of SGLT1 stimulation by siNa-K-ATPase-α1 silencing, kinetic studies were performed. Na-dependent glucose uptake was measured as a function of varying concentrations of glucose at 30 s in IEC-18 cells. As the extracellular concentration of glucose was increased, Na-glucose uptake was stimulated and, subsequently, became saturated in all conditions. Kinetic parameters were derived from the uptake data using GraphPad Prism 6 (GraphPad Software, San Diego, CA). In siNa-K-ATPase-silenced cells, the maximal rate of glucose uptake (V_{max}) was not altered (4.1 ± 0.7 and 4.2 ± 0.8 nmol-mg protein^{-1}·30 s^{-1} in control and siNa-K-ATPase-α1 cells, re-
cotransporters (Fig. 7).

The importance of Na-K-ATPase as a driver of Na-dependent solute absorption in the mammalian small intestine is fairly well established. In an animal model of human inflammatory bowel disease, our laboratory has demonstrated the inhibition of a variety of Na-dependent glucose, amino acid, bile acid, and nucleic acid cotransporters. At the villus cell level, since BLM Na-K-ATPase was inhibited, it was hypothesized that at least part of the inhibition of these Na-solute cotransport processes was secondary to Na-K-ATPase inhibition. However, a variety of immune inflammatory mediators are elevated in the chronically inflamed intestine, and any of these immune inflammatory mediators can affect the BBM Na-solute cotransporters, as well as BLM Na-K-ATPase. We previously demonstrated that when these immune inflammatory pathways were inhibited, the inhibition of BBM Na-solute cotransport and BLM Na-K-ATPase was reversed (3, 6, 23). Therefore, to more specifically determine the effect of Na-K-ATPase inhibition on the BBM Na-solute cotransport process in the absence of confounding variables, we studied Na-K-ATPase inhibition in an in vitro model of intestinal epithelial cells. Figure 8 summarizes SGLT1 activity in siNa-K-ATPase-α1-silenced and chronic ouabain-treated IEC-18 cells. Not unexpectedly, acute inhibition of Na-K-ATPase with ouabain inhibited BBM Na-glucose cotransport. However, the intestinal pathophysiology we studied is a chronic condition. Thus we looked at the long-term inhibition of Na-K-ATPase. We were surprised to find that long-term inhibition of Na-K-ATPase resulted in stimulation of BBM Na-glucose cotransport. It is conceivable that this is a compensatory mechanism employed by the cell for the diminished transmembrane Na gradient. However, this effect was specific to SGLT1, but not the other primary Na absorptive pathway, Na/H exchange, in intestinal epithelial cells. On the basis of thermodynamics, the diminished transcellular Na gradient may be expected to decrease Na influx via NHE3, but this exchanger was not altered. It is conceivable that the elevation of intracellular Na was not adequate to affect NHE3. Nevertheless, this novel observation is suggestive of the molecular mechanism of stimulation of SGLT1 when Na-K-ATPase was inhibited. First, to avoid any untoward effect of chronic exposure to ouabain, siRNA for the α1-subunit was utilized to inhibit Na-K-ATPase. As expected, transfection with this siRNA diminished Na-K-ATPase activity. However, in these cells, SGLT1 was stimulated, but Na/H was unaffected. The mechanism of SGLT1 stimulation was secondary to an increase in the affinity of the cotransporter for glucose without a change in the number of cotransporters (Fig. 7).

DISCUSSION

The importance of Na-K-ATPase as a driver of Na-dependent solute absorption in the mammalian small intestine is fairly well established. In an animal model of human inflammatory bowel disease, our laboratory has demonstrated the inhibition of a variety of Na-dependent glucose, amino acid, bile acid, and nucleic acid cotransporters. At the villus cell level, since BLM Na-K-ATPase was inhibited, it was hypothesized that at least part of the inhibition of these Na-solute cotransport processes was secondary to Na-K-ATPase inhibition. However, a variety of immune inflammatory mediators are elevated in the chronically inflamed intestine, and any of these immune inflammatory mediators can affect the BBM Na-solute cotransporters, as well as BLM Na-K-ATPase. We previously demonstrated that when these immune inflammatory pathways were inhibited, the inhibition of BBM Na-solute cotransport and BLM Na-K-ATPase was reversed (3, 6, 23). Therefore, to more specifically determine the effect of Na-K-ATPase inhibition on the BBM Na-solute cotransport process in the absence of confounding variables, we studied Na-K-ATPase inhibition in an in vitro model of intestinal epithelial cells. Figure 8 summarizes SGLT1 activity in siNa-K-ATPase-α1-silenced and chronic ouabain-treated IEC-18 cells. Not unexpectedly, acute inhibition of Na-K-ATPase with ouabain inhibited BBM Na-glucose cotransport. However, the intestinal pathophysiology we studied is a chronic condition. Thus we looked at the long-term inhibition of Na-K-ATPase. We were surprised to find that long-term inhibition of Na-K-ATPase resulted in stimulation of BBM Na-glucose cotransport. It is conceivable that this is a compensatory mechanism employed by the cell for the diminished transmembrane Na gradient. However, this effect was specific to SGLT1, but not the other primary Na absorptive pathway, Na/H exchange, in intestinal epithelial cells. On the basis of thermodynamics, the diminished transcellular Na gradient may be expected to decrease Na influx via NHE3, but this exchanger was not altered. It is conceivable that the elevation of intracellular Na was not adequate to affect NHE3. Nevertheless, this novel observation is suggestive of the molecular mechanism of stimulation of SGLT1 when Na-K-ATPase was inhibited. First, to avoid any untoward effect of chronic exposure to ouabain, siRNA for the α1-subunit was utilized to inhibit Na-K-ATPase. As expected, transfection with this siRNA diminished Na-K-ATPase activity. However, in these cells, SGLT1 was stimulated, but Na/H was unaffected. The mechanism of SGLT1 stimulation was secondary to an increase in the affinity of the cotransporter for glucose without a change in the number of cotransporters (Fig. 7).

Fig. 6. A: effect of siNa-K-ATPase-α1 on Na-glucose cotransporter (SGLT1) mRNA. There was no significant alteration of SGLT1 mRNA expression in siNa-K-ATPase-α1 cells compared with control. B: effect of siNa-K-ATPase-α1 on BBM SGLT1 protein expression. Top: representative blot of BBM SGLT1 with ezrin as loading control. Bottom: densitometric analysis of BBM SGLT1 protein expression showed no significant alteration in siNa-K-ATPase-α1 cells compared with control.

Fig. 7. Kinetic parameters for SGLT1 activity in siNa-K-ATPase-α1-silenced IEC-18 cells. Na-dependent glucose ([3H]OMG) uptake is shown as a function of 0–100 mM extravesicular glucose (OMG). Uptake for all concentrations was determined at 30 s. As the concentration of extravesicular glucose was increased, uptake of glucose was stimulated and, subsequently, became saturated in IEC-18 cells in all conditions. Affinity (1/Km) for glucose uptake significantly increased in siNa-K-ATPase-α1-transfected IEC-18 cells compared with control. Data represent average of 3 experiments; each experiment was done in triplicate.
decrease in glycosylation of SGLT1 has been shown to inhibit SGLT1 activity (2). The precise mechanism of alteration of $K_m$ of SGLT1 in Na-K-ATPase-inhibited cells and the intracellular pathways involved will need to be elucidated in future studies. The fact that intracellular Na is increased in ouabain-treated and siNa-K-ATPase-$\alpha_1$-silenced cells would suggest that salt-inducible kinase may have a role in the posttranslational modification of SGLT1 in these cells. It is conceivable that Na-K-ATPase may regulate a BBM transporter by a means other than simply altering intracellular Na. For example, in a renal-derived epithelial cell line (LLC-PK1), chronic exposure to ouabain, but at a concentration too low to inhibit Na-K-ATPase-mediated transport or to raise intracellular Na, was shown to affect the function and expression of the epithelial BBM NHE3 (11, 13).

As previously noted, Na-K-ATPase activity is diminished in villus cells from the chronically inflamed intestine. In these cells, however, SGLT1 is inhibited, not stimulated, as is the case when the enzyme is directly inhibited chemically or with siRNA in this study. Furthermore, the mechanism of resultant alteration of SGLT1 is uniquely different in each case. Inhibition of SGLT1 in chronically inflamed intestine is secondary to a decrease in the de novo synthesis of SGLT1 protein, whereas stimulation of SGLT1 in ouabain-treated or siRNA-transfected cells is posttranslational and secondary to altered affinity of the cotransporter for glucose. Possible explanations for these observations include the fact that, in the chronically inflamed intestine, numerous immune inflammatory mediators are upregulated, and this may have an independent effect on SGLT1 beyond any effect of diminished Na-K-ATPase. In fact, immune inflammatory mediators causing inhibition of SGLT1 have been reported. For example, tumor necrosis factor-$\alpha$ has been shown to downregulate SGLT1 in human colon cancer cells (4). Alternatively, the mechanism of Na-K-ATPase inhibition in the chronically inflamed intestine may be fundamentally different from that achieved by ouabain or siRNA for the $\alpha_1$-subunit. In the latter instance, there is a decrease in the otherwise functional Na-K-ATPase in the BLM; in the former instance, the enzyme may be fundamentally altered by yet to be determined immune inflammatory mediators.

In conclusion, chronic and specific inhibition of BLM Na-K-ATPase in intestinal epithelial cells increases intracellular Na. It also stimulates influx of glucose via BBM SGLT1, but not influx of Na via NHE3. The mechanism of stimulation of SGLT1 is secondary to an increase in the affinity of the cotransporter for glucose. Thus, Na-K-ATPase uniquely regulates BBM SGLT1, possibly in a compensatory fashion for the loss of transmembrane Na resulting from its inhibition.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
P.M. and S.G. performed the experiments; P.M. analyzed the data; P.M., S.G., S.A., B.P., and S.S. prepared the figures; P.M., S.G., S.A., B.P., S.S., and U.S. drafted the manuscript; S.A., B.P., S.S., and U.S. edited and revised the manuscript; G.M.D. and U.S. interpreted the results of the experiments; G.M.D. and U.S. approved the final version of the manuscript; U.S. developed the concept and designed the research.

REFERENCES