Na\(^{+}/H^{+}\) Exchanger NHE3 Activity and Trafficking Are Lipid Raft-dependent*

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A previous study showed that ~25–50% of rabbit ileal brush border (BB) Na\(^{+}/H^{+}\) exchanger NHE3 is in lipid rafts (LR) (Li, X., Galli, T., Leu, S., Wade, J. B., Weinman E. J., Leung, G., Cheong, A., Louvard, D., and Donowitz, M. (2001) J. Physiol. (Lond.) 537, 537–552). Here, we examined the role of LR in NHE3 transport activity using a simpler system: opossum kidney (OK) cells (a renal proximal tubule epithelial cell line) containing NHE3. ~50% of surface (biotinylated) NHE3 in OK cells distributed in LR by density gradient centrifugation. Disruption of LR with methyl-\(\beta\)-cyclodextrin (M\(\beta\)CD) decreased NHE3 activity and increased \(K_{m}^{i}\), but \(K_{m}^{i}\) was not affected. The M\(\beta\)CD effect was completely reversed by repletion of cholesterol, but not by an inactive analog of cholesterol (cholestan-\(3\beta,5\alpha,6\beta\)-triol). The M\(\beta\)CD effect was specific for NHE3 activity because it did not alter Na\(^{+}\)-dependent L-Ala uptake. M\(\beta\)CD did not alter OK cell BB topology and did not change the surface amount of NHE3, but greatly reduced the rate of NHE3 endocytosis. The effects of inhibiting phosphatidylinositol 3-kinase and of M\(\beta\)CD on NHE3 activity were not additive, indicating a common inhibitory mechanism. In contrast, 8-bromo-cAMP and M\(\beta\)CD inhibition of NHE3 was additive, indicating different mechanisms for inhibition of NHE3 activity. Approximately 50% of BB NHE3 and only ~11% of intracellular NHE3 in polarized OK cells were in LR. In summary, the BB pool of NHE3 in LR is functionally active because M\(\beta\)CD treatment decreased NHE3 basal activity. The LR pool is necessary for multiple kinetic aspects of normal NHE3 activity, including \(V_{\text{max}}\) and \(K_{m}^{i}\), and also for multiple aspects of NHE3 trafficking, including at least basal endocytosis and phosphatidylinositol 3-kinase-dependent basal endocytosis. Because the C-terminal domain of NHE3 is necessary for its regulation and because the changes in NHE3 kinetics with M\(\beta\)CD resemble those with second messenger regulation of NHE3, these results suggest that the NHE3 C terminus may be involved in the M\(\beta\)CD sensitivity of NHE3.

Na\(^{+}/H^{+}\) exchanger NHE3 (SLC9A3) is expressed on apical membranes of small intestinal Na\(^{+}\) absorptive epithelial cells and the renal proximal tubule, where it contributes to a large percentage of total NaCl, HCO\(_3\)-, and water (re)absorption (1–3). Rapid regulation of NHE3 activity occurs as part of normal digestive and renal physiology and in the pathophysiology of diuretica and some renal diseases of the proximal tubule. The acute regulation of the exchanger seems to be mainly through changes in its \(V_{\text{max}}\), but also involves changes in \(K_{m}^{i}\) (4). Regulation of NHE3 involves at least two different mechanisms: regulation by changes in trafficking due to regulated changes in endocytosis and/or exocytosis and changes in turnover number (5–11). Both these mechanisms often involve changes in NHE3 phosphorylation.

In studies performed to investigate the mechanisms of NHE3 regulation in rabbit ileal Na\(^{+}\) absorptive cells, brush border (BB)\(^{4}\) NHE3 was shown to be partially in lipid rafts (LR) (12). Concerning NHE3 regulation, the LR pool of BB NHE3 is involved in some of its basal endocytosis and exocytosis and in the acute epidermal growth factor increase of the BB amount of NHE3. However, the contribution of LR to NHE3 function has not been examined in detail.

LR are discrete membrane domains that are enriched in glycosphingolipids and cholesterol and that are resistant to solubilization in cold Triton X-100. They are thought to act in the compartmentalization of membrane proteins, separating different biochemical functions and allowing concentration and localization of molecules involved in signal transduction functions (13–15). Besides the formation of restricted signaling platforms, rafts are implicated in apical protein targeting (13, 14, 16) and in some aspects of endocytosis in epithelial cells and as a docking site for some pathogens and toxins (17–19).

The concept that transport proteins distribute in LR and that their activities are LR-dependent is not unique to NHE3, although it has not yet been examined for many transport proteins. Depletion of cholesterol dramatically alters the function of some (Kv.2.1 and Kv.1.5) but not other (Kv.4.2) voltage-gated potassium channels (20, 21), decreases SGLT1 (sodium/glucose cotransporter 1) activity (22), and significantly reduces uptake of glutamate by the glial glutamate transporter EAAT2 (23) and the NaCl-dependent serotonin transporter SERT (24). Also, the mouse colonic basolateral membrane Ca\(^{2+}\)-activated potassium channel is activated by cholesterol depletion (25). Other transporters shown to be partially in LR include NBC1 (26), the type Ia Na\(^{+}/P\) cotransporter (27), some connexins (28), and the epithelial Na\(^{+}\) channel ENaC (29). In contrast, other transport proteins do not appear to be present or affected by LR. These include the cystic fibrosis transmembrane conductance regulator CFTR in normal tissue, except when

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4 The abbreviations used are: BB, brush border(s); LR, lipid raft(s); OK, opossum kidney; BCECF-AM, 2′,7′-bis(2-carboxyethyl)5(6)-carboxyfluorescein acetoxymethyl ester; HA, hemagglutinin; MGP, methyl o-glucosyranoside; M\(\beta\)CD, methyl-\(\beta\)-cyclodextrin; OPD, o-phenylenediamine dihydrochloride; Br, bromo; VSVG, vesicular stomatitis virus G; DMEM, Dulbecco’s modified Eagle medium; ELISA, enzyme-linked immunosorbent assay; TMA, tetramethylammonium; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; WGA, wheat germ agglutinin; PI3K, phosphatidylinositol 3-kinase.
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exposed to Pseudomonas aeruginosa toxin (30), and intestinal Na⁺-K⁺-ATPase (31).

In this study, the opossum kidney (OK) renal proximal tubule epithelial cell line was used to provide a simple model to examine the contribution of LR to NHE3 activity. An advantage of this cell line is that it is a polarized epithelial Na⁺ absorptive cell line that contains NHE3 as the sole plasma membrane Na⁺/H⁺ exchanger. It also lacks other regulatory elements (nerves, endocrine cells, inflammatory cells) that are present in intact intestine and that might also act by LR-dependent processes.

EXPERIMENTAL PROCEDURES

Materials—Materials were obtained as indicated: restriction endonucleases, New England Biolabs, Inc.; Pfu polymerase, Stratagene; 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM), Molecular Probes; anti-mouse secondary antibodies fluorescently labeled with Alexa Fluor 488, Invitrogen; horseradish peroxidase-conjugated donkey anti-mouse IgG, Jackson ImmunoResearch Laboratories, Inc.; monoclonal mouse antibodies to the hemagglutinin (HA) epitope, Covance Inc.; nigericin, methyl α-D-glucopyranoside (MGP), l-alanine, methyl-β-cyclodextrin (MβCD), cholesterol and its inactive analog cholesteryl-3β,5α,6β-triol, o-phenylenediamine dihydrochloride (oPD), 8-bromo (Br)-cAMP, LY-294002, Sigma; and [3H]methylation α-D-glucopyranoside and 1,2-[3H]alanine (PerkinElmer Life Sciences).

Cell Lines—Studies were carried out in OK/E3V (generously provided by Dr. J. Noël, University of Montreal, Montreal, Canada) and OK/3HA-E3V cell lines. OK/E3V cells are an OK proximal tubule cell line generated by stable transfection of OK-Tina cells, which are OK cells previously selected by acid suicide to lack endogenous NHE3 activity, with a cDNA for rat NHE3 tagged at the C terminus with the vesicular stomatitis virus G (VSV-G) protein epitope (32). The OK/3HA-E3V cell line stably expresses rabbit NHE3 tagged with three copies of the influenza virus HA epitope at the N terminus and with the VSV-G epitope at the C terminus. Cells were cultured in Dulbecco’s modified Eagle medium (DMEM)/nutrient mixture F-12 (Invitrogen) containing 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C. Confluent monolayers on plastic dishes, glass coverslips, or filters were serum-depleted for 24–48 h before study. Cells from every new passage were exposed to an acute acid loading selection to maintain a high level of NHE3 protein expression as described previously (4) with some modification. In brief, cells were exposed to 50 mM NH₄Cl/saline solution for 1 h, followed by overnight incubation in isotonic 2 mM Na⁺ solution.

Plasmid Construction and Cell Transfections—for immunological detection (enzyme-linked immunosorbent assay (ELISA)) of NHE3 protein, three copies of the HA epitope (YPYDVPDYA) were inserted into the first extracellular loop of rabbit NHE3 between Glu²⁷ and Ile²⁸ by PCR. The pECE plasmid with cDNA encoding rabbit NHE3 with the VSV-G epitope (33). The OK/3HA-E3V cell line was a gift from Dr. Suketa (University of Shizuoka, Shizuoka, Japan). For transient expression of SGLT1, OK/E3V cells were seeded at 75–80% confluence onto 24-well plates 24 h prior to transfection and then transfected with 1 μg of the corresponding plasmid DNAs using Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer. Several stably transfected clones were picked, expanded, and used for ELISA as well as for transport assays.

The pcDNA3.1/3HA-E3V and empty vector (pcDNA3.1(+)) plasmids were stably transfected into OK-Tina cells using Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer. Several stably transfected clones were picked, expanded, and used for ELISA as well as for transport assays.

Measurement of Na⁺/H⁺ Exchange Activity—Na⁺/H⁺ exchange activity was determined as the initial rate of Na⁺-induced recovery of cytosolic pH (pHᵢ) after an acute acid load caused by prepulsing with NH₄Cl, and pHᵢ was measured fluorometrically using BCECF-AM as described previously (33). Fluorescence measurements (excitation at 490 and 440 nm with emission at 530 nm) were made using SLM-Aminco SPF-500C and Photon Technology International spectrofluorometers. Briefly, OK/E3V or OK/3HA-E3V cells were grown on glass coverslips to 100% confluence. The monolayers were incubated in serum-free DMEM/nutrient mixture F-12 for 24–48 h prior to use. Cells were loaded with 10 μM BCECF-AM in Na⁺/NH₄Cl medium (88 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 1 mM NaH₂PO₄, 25 mM glucose, 20 mM HEPES, and 50 mM NH₄Cl; pH 7.4) for 30 min at 37 °C. During the dye loading and NH₄Cl prepulse, cells were tested with test agents or vehicle. The cells were initially perfused with TMA medium (130 mM tetramethylammonium (TMA) chloride, 5 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 1 mM NaH₂PO₄, 25 mM glucose, and 20 mM HEPES, pH 7.4), resulting in stable acidification of the cells. Then, Na⁺ medium (138 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 1 mM NaH₂PO₄, 25 mM glucose, and 20 mM HEPES, pH 7.4) was added, which induced alkalization of the cells. To determine the kinetics for external Na⁺, the Na⁺ concentration in Na⁺ medium was varied (5, 10, 15, 75, and 138 mM) while maintaining the osmolarity with TMA chloride. For these experiments, cells were acidified to the same level with 50 mM NH₄Cl. To calibrate the relationship between the excitation ratio (F₅₀₀/F₄₅₀) and pHᵢ, the K⁺/nigericin method was used. As described previously (33), Na⁺/H⁺ exchange rates (H⁺/Na⁺) were calculated as the product of Na⁺-dependent change in pHᵢ and the buffering capacity at each pHᵢ and were analyzed using the nonlinear regression data analysis program Origin, which allows fitting of data to a general allosteric model described by the Hill equation (ν = Vₘₐₓ[S]/Kᵢₙₜᵢₜ + [S]ᵢₙᵢᵢᵦ, where ν is velocity, S is the substrate concentration, nₜᵢₜ is the apparent Hill coefficient, and Kᵢ is the affinity constant), with estimates for Vₘₐₓ and Kᵢ (H⁺), and their respective errors (S.E.), as well as fitting to a hyperbolic curve such as would be expected with Michaelis-Menten kinetics. Data from each coverslip were calculated and analyzed as
described above. For each independent experiment, results from all coverslips for each condition were analyzed together.

**Uptake Studies**—Uptake of MGP or L-alanine was assayed in the presence and absence of Na+ as described previously (34, 35). For uptake experiments, OK/E3V cells were plated onto 24-well plates. Confluent cell monolayers were incubated in serum-free medium for 48 h before the uptake experiments. Transiently transfected OK/E3V cells (for MGP uptake) were used for 72 h after transfection and incubated in serum-free DMEM/nutrient mixture F-12 for 6 h before study. Confluent monolayers were treated with 10 mM MβCD or with H₂O as a vehicle for 30 min at 37 °C and then washed twice with TMA⁺ medium (no glucose). MGP or L-alanine uptake was carried out at room temperature and initiated by addition of 0.1 mM MGP ([¹⁴C]MGP (0.4 μCi/ml) or 0.2 mM L-alanine/L-[³H]alanine (2 μCi/ml)). Uptake of substrates was arrested after an appropriate incubation time by aspirating off the radioactive medium and washing three times with ice-cold TMA⁺ medium without substrate. The radioactivity of isolates extracted from cell monolayers with 0.5 ml of 1 N NaOH (neutralized with HCl) was assayed by liquid scintillation spectrometry. The amount of accumulated substrate was expressed as cpm/min/well.

**Measurement of Surface NHE3**—The percentage of total cell NHE3 on the apical surface of OK cells was determined separately by cell-surface biotinylation and modified ELISA (7). For biotinylation, OK/E3V cells were grown to confluent monolayers on plastic dishes, and then the growth was arrested by incubation with serum-free medium for 48 h. Confluent monolayers were treated either with test agent or vehicle at 37 °C under the same conditions used for the detection of NHE3 transport activity and surface-labeled with biotin at 4 °C as described previously (8). All subsequent manipulations were performed at 4 °C. Cells were washed twice with phosphate-buffered saline (PBS; 150 mM NaCl and 20 mM Na₂HPO₄, pH 7.4); incubated with arginine- and lysine-reactive succinimidyl 2-(biotinamido)ethyl-1,3-dithiopropionate (0.5 mg/ml; Pierce) in 154 mM NaCl, 10 mM boric acid, 7.2 mM KCl, and 1.8 mM CaCl₂, pH 9.0; and washed extensively with quenching buffer containing 20 mM Tris and 120 mM NaCl, pH 7.4, to scavenge the unbound biotin. Cells were solubilized with 1 ml of N⁺ buffer (60 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM KCl, 5 mM Na₂EDTA, 3 mM EGTA, and 1% Triton X-100) and protease inhibitor mixture (catalog number P8340, Sigma), and lysates were centrifuged at 2300 × g for 20 min to remove insoluble cell debris and unbroken cells. Supernatants were diluted with N⁺ buffer to an equal protein concentration, applied to avidin-agarose beads (Pierce) at 4 °C, and incubated for 16 h. The remaining supernatant was retained as the intracellular fraction. Finally, the avidin-agarose beads were washed five times with N⁺ buffer, and the biotinylated proteins were recovered from the beads in Laemmli buffer. Several fractions of total, intracellular, and surface pools were separated by SDS-PAGE (9%) and assayed for endocytosed NHE3 as described above. Fluorescently labeled, biotin-labeled proteins were protected from cleavage with GSH. Cells were solubilized in N⁺ buffer; biotinylated proteins were retrieved and assayed for endocytosed NHE3 as described above. Fluorescently labeled, IRDye™ 800-conjugated goat anti-mouse secondary antibodies (Rockland Immunochemicals, Inc.) were used for immunoblotting. The fluorescence intensity of NHE3 protein bands was visualized using the Odyssey system (LI-COR Biosciences) and quantitated with MetaMorph Version 5.0r1 software (Universal Imaging Corp., Downingtown, PA).

**Sucrose Gradient Density Flotation**—To localize NHE3 in OK/E3V cells to LR, total lysate was fractionated by discontinuous sucrose step gradients as described previously (12, 38). OK/E3V cells were grown to 100% confluency, serum-starved for 24–48 h, and then treated with 10 mM MβCD or vehicle for 30 min at 37 °C. The monolayers were biotinylated (see ‘‘Measurement of Surface NHE3’’) and lysed in N⁺ buffer supplemented with 5 mM dithiothreitol, 1 mM Na₃VO₄, 50 mM NaF, and protease inhibitor mixture. Total lysates were loaded on 11 discontinuous sucrose step gradients (30, 27.5, 25, 22.5, 20, 17.5, 15, 12.5, 10, 7.5, and 5%). Each step gradient was prepared with sucrose and N⁺ buffer with 0.1% Triton X-100. Centrifugation was done in a Beckman SW 41Ti rotor at 150,000 × g overnight at 4 °C. Surface NHE3 in each fraction was precipitated by avidin-agarose beads. One-quarter of each fraction (total and surface NHE3) was analyzed by SDS-PAGE, Western blotting, and densitometric analysis using ImageQuant Version 4.2a software.

**Labeling the Apical Cell Surface with Fluorescent Lectin and Fluorescence Microscopy**—To examine the changes in apical membrane structure caused by MβCD treatment, surface labeling with a fluorescent lectin was used (39). Lectins bind to specific sugar residues of the glycoalx; and at 4 °C, the fluorescent markers remain on the apical surface of the monolayers for several hours. OK/E3V cells were grown and...
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FIGURE 1. A pool of NHE3-VSV-G in OK cells is LR-associated. OK/E3V cells were treated with or without 10 mM mβCD for 30 min at 37 °C and then surface-biotinylated as described under “Experimental Procedures.” Total lysates were fractionated by floatation on an 11-step sucrose gradient. Surface (biotinylated) NHE3 in each fraction was precipitated by avidin-agarose beads. NHE3 was separated by SDS-PAGE and identified by Western blotting (A), and densitometric analysis of surface NHE3 was performed using ImageQuant Version 4.2a software (B). The quantitated data from the two upper panels in A are shown in B as the percentage of total surface NHE3 in each fraction.

Results

There Is an NHE3 Pool in Detergent-resistant Membranes in OK/E3V Cells—To understand the relationship between LR and NHE3, we used OK cells initially processed by acid suicide to greatly decrease endogenous NHE3 and then stably transfected with rat NHE3 cDNA (called OK/E3V cells). As reported, these OK/E3V cells represent a non-overexpression model for the study of NHE3 in polarized epithelial cells (38). Important criteria to establish the association of proteins with LR are to demonstrate that the protein is insoluble in cold Triton X-100 and that it shifts from lighter to heavier membrane fractions after cholesterol depletion with mβCD based on density gradient fractionation (16). OK/E3V cells were treated with 10 mM mβCD for 30 min at 37 °C (which was lowered to 4 °C), biotinylated by exposure to succinimidyl 2-[(biotinamido)ethyl]-1,3-dithiopropionate, and lysed in 1% Triton X-100, and then total fractions were mixed with the highest density sucrose (30%) and applied to the bottom of the sucrose density gradients for ultracentrifugation. The Western blot analyses (Fig. 1A) of fractions of total and biotinylated (surface) NHE3 from untreated cells revealed that NHE3 distributed as a single immunoreactive band with the highest amount in fractions 1, 2 (heaviest), and 17–20 and with lower and almost equal density in fractions 3–16. In mβCD-treated cells, upon cholesterol depletion, the lighter fractions of total and surface NHE3 shifted toward heavier gradient fractions. These results suggest that NHE3 in OK/E3V cells is partially in LR. Densitometry of surface NHE3 in OK/E3V cells initially processed by acid suicide to greatly decrease endogenous NHE3 and then stably transfected with rat NHE3 cDNA (called OK/E3V cells). As reported, these OK/E3V cells represent a non-overexpression model for the study of NHE3 in polarized epithelial cells (38). Important criteria to establish the association of proteins with LR are to demonstrate that the protein is insoluble in cold Triton X-100 and that it shifts from lighter to heavier membrane fractions after cholesterol depletion with mβCD based on density gradient fractionation (16). OK/E3V cells were treated with 10 mM mβCD for 30 min at 37 °C (which was lowered to 4 °C), biotinylated by exposure to succinimidyl 2-[(biotinamido)ethyl]-1,3-dithiopropionate, and lysed in 1% Triton X-100, and then total fractions were mixed with the highest density sucrose (30%) and applied to the bottom of the sucrose density gradients for ultracentrifugation. The Western blot analyses (Fig. 1A) of fractions of total and biotinylated (surface) NHE3 from untreated cells revealed that NHE3 distributed as a single immunoreactive band with the highest amount in fractions 1, 2 (heaviest), and 17–20 and with lower and almost equal density in fractions 3–16. In mβCD-treated cells, upon cholesterol depletion, the lighter fractions of total and surface NHE3 shifted toward heavier gradient fractions. These results suggest that NHE3 in OK/E3V cells is partially in LR. Densitometry of surface NHE3 in OK/E3V cells (Fig. 1B) showed that ~50% of surface NHE3 was present in LR (Fig. 1A, surface NHE3 ± mβCD). (Fractions 8–20 were shifted by mβCD from light to heavy sucrose gradient fractions.) In contrast, ~17% of total NHE3 was in LR (Fig. 1A, total NHE3 ± mβCD). ~15% of total OK cell NHE3 is in the plasma membrane (38), which indicates that ~11% of intracellular NHE3 is in LR ((0.5)(0.15) + (x)(0.85) = (0.17)(100)), where x is the fraction of intracellular NHE3 in LR.

Disruption of LR with mβCD Decreases NHE3 Exchange Activity—To evaluate the effect of disruption of LR by cholesterol removal on the basal activity of NHE3, the monolayers of OK/E3V cells were incubated in the presence or absence of 10 mM mβCD or vehicle at 37 °C for 30 min. The Na⁺/H⁺ exchange activity was significantly inhibited in cells treated with mβCD compared with control cells (initial rates in ΔpH/...
ificant decrease in the activity of NHE3. The initial rate of Na$^+$-dependent glucose uptake was significantly reduced in MβCD-treated and control cells (n = 5; p < 0.05). The K'(H$^+$) values were 0.2 ± 0.03 for control cells and 0.3 ± 0.04 for MβCD-treated cells (p < 0.05).

To determine whether disruption of LR in the plasma membrane of OK/E3V cells alters the affinity of protein for Na$^+$, the activity of NHE3 in the presence of varying concentrations of medium Na$^+$ was examined. Analysis of the Eadie-Hofstee plots showed that MβCD affected only the $V_{\text{max}}$ but not $K_m$(Na$^+$), of the Na$^+$/H$^+$ exchange activity of NHE3 in OK/E3V cells (Fig. 3). The kinetics for external Na$^+$ in both control and treated cells followed a classical Michaelis-Menten model with $K_m$(Na$^+$) values of 12 ± 3 μM for control cells and 13 ± 6 μM for MβCD-treated cells (Fig. 3).

Effect of MβCD on NHE3 Is Not a General Effect on Transport—The inhibitory effect of MβCD on NHE3 activity might be explained by a general inhibitory effect of cholesterol depletion on membrane dynamics (fluidity/viscosity). To test this hypothesis, we studied the effect of cholesterol depletion on Na$^+$/glucose cotransport activity driven by SGLT1 and Na$^+$-dependent L-alanine transport in OK/E3V cells. It was reported recently that SGLT1 is localized to LR and that LR disruption decreases Na$^+$/glucose transport activity (22). Thus, we used SGLT1 as a positive control. Generally, OK cells express SGLT1 in the apical membrane. However, we found that OK/E3V cells did not exhibit endogenous Na$^+$-dependent glucose uptake (Fig. 4A, inset), probably as a consequence of selection procedures during isolation of OK cells lacking endogenous NHE3. Therefore, the cDNA encoding SGLT1 was transiently transfected into OK/E3V cells, and Na$^+$/glucose cotransport activity was assessed as Na$^+$-dependent uptake of MGP, a non-metabolized analog of D-glucose, in the presence and absence of 10 mM MβCD. Na$^+$-dependent glucose uptake was significantly reduced in MβCD-treated cells versus control cells (Fig. 4A). By contrast, cholesterol depletion of OK/E3V monolayers did not affect Na$^+$-dependent L-alanine uptake. The rates of L-alanine uptake (both Na$^+$-dependent and Na$^+$-independent) by MβCD-treated and control cells were comparable (Fig. 4B). These findings indicate that the inhibitory effect of cholesterol depletion with MβCD is not the result of nonspecific effects.
A second independent method to assess the percentage of surface NHE3 based on a modified ELISA was established to quantify the surface amount of NHE3. For this purpose, an OK/3HA-E3V cell line that contained an external epitope was generated. Two clones were initially tested to show that insertion of the triple HA tag into the first extracellular loop plus the VSV-G tag at the C terminus of NHE3 did not alter the expression, ion exchange activity, and trafficking properties of NHE3 (data not shown). As shown in Fig. 6A, HA-NHE3 in clones II and III of OK/3HA-E3V cells was expressed, and ~80–85 kDa immunoreactive bands were detected in total cell lysates with both anti-HA and anti-VSV-G antibodies. The rat NHE3 protein with only a VSV-G epitope failed to react with anti-HA antibody as a negative control (Fig. 6A), showing that the immunoreactivity was specific to the HA epitope. Insertion of triple HA epitope plus VSV-G tags did not measurably alter the expression of rabbit NHE3 compared with rat NHE3 tagged with only the VSV-G epitope in OK/E3V cells (data not shown).

The functional NHE3 activity in OK/3HA-E3V cells was established using BCECF as described above. Full kinetic curves were generated to determine the basal Na+/H+ exchange activity of control and OK3/3HA-E3V cells treated with MβCD (clone II). Representative curves in Fig. 6B show that 3HA-E3V is functional and that MβCD had a similar percent effect on the antiport activity of 3HA-E3V as it had on NHE3-VSV-G in Fig. 2.

Binding of anti-HA antibody to the external epitope of 3HA-E3V was visualized by confocal microscopy. The accessibility of anti-HA antibody in intact (non-permeabilized) cells was examined first. Cells were incubated with anti-HA antibody at 4 °C for 1 h and then fixed and stained with Alexa Fluor® 488-labeled anti-mouse secondary antibodies. Serial xy sections of OK/3HA-E3V cells demonstrated that the external HA epitope of NHE3 in OK/3HA-E3V cells was accessible (Fig. 6, C (bottom) and D (top)). For ELISA, antibody to the extracellular HA epitope was added to intact control OK/3HA-E3V cells and treated with MβCD to detect exchangers that were only at the surface. In five separate ELISA experiments, the number of cell surface-exposed exchangers was similar in control and treated cells (98 ± 15% of the untreated control (not significant)) (Fig. 5C). The results obtained with both methods of surface NHE3 measurement were similar and led to the conclusion that the inhibition of NHE3 activity caused by cholesterol depletion was not due to a change in the surface amount of apical NHE3 in OK cells.

Disruption of LR Does Not Cause Topological Alterations in the Apical Membranes of OK/E3V Cells—The inhibition of NHE3 activity in OK/E3V cells upon MβCD treatment might be explained by an alteration in membrane structure because of cholesterol depletion. To consider this possibility, apical membranes of OK/E3V cells treated or not with 10 mM MβCD for 30 min at 37 °C were chilled to 4 °C and labeled with FITC-conjugated WGA. WGA binds specifically to N-acetyl-neuraminic acids and N-acetylgalcosamine residues in the glycolipid at 4 °C (41, 42) and can be used to delineate the apical membrane of epithelial cells. Fig. 7 (A and B) shows the distribution of WGA (10 μg/ml) on the apical side of OK/E3V monolayers in control and treated cells. The appearance of MβCD-treated cells was not altered. Also, monolayers of control and MβCD-treated cells bound WGA in irregularly distributed patches on the apical surface as well as in discontinuous rings at the level of the tight junctions. Some cells in both monolayers failed to bind WGA. The morphometric analysis of the fluorescent intensity of FITC-conjugated WGA in xy planes from seven independent fields for control cells (intensity, 15.5 ± 5.2) and 12 independent fields for MβCD-treated cells (intensity, 15.6 ± 3.2) revealed no differences (Fig. 7, C and D). The similar distribution of WGA in control and treated cells
Figure 5. Depletion of cholesterol does not change the surface amount of NHE3 in OK cells. A, shown is a representative immunoblot of surface-biotinylated NHE3. MbCD (MbCD)- or vehicle-treated cell monolayers were surface-biotinylated at 4 °C, and surface proteins were retrieved from the cell lysates by avidin precipitation. Two dilutions of total pools (9 and 18 μl for control cells and 8 and 16 μl for MbCD-treated cells) contained 10 and 20 μg of total NHE3, respectively; three dilutions of 5× surface (biotinylated) NHE3 and two dilutions of intracellular protein as 10 and 20 μl, respectively, are shown in the immunoblot. B, the amount of surface NHE3 was quantified by densitometric analysis of NHE3 amounts from the different concentrations shown in A. Values are the means ± S.E. from three independent experiments, with the control set at 100%. C, the effect of MbCD on the surface expression of NHE3 in OK/3HA-E3V cells was quantitated by ELISA. OK/3HA-E3V cells were treated or not with 10 mM MbCD for 30 min at 37 °C. The cells were then chilled and incubated with anti-α2A antibodies (1:1000 dilution) for 1 h at 4 °C. Cells were washed six times to remove unbound antibody, fixed with formaldehyde, and blocked with 5% fetal bovine serum and 1% bovine serum albumin. The cells were incubated with horseradish peroxidase-conjugated anti-mouse antibodies (1:500 dilution). The activity of peroxidase bound to surface NHE3 was quantified by incubation with o-phen and measuring the absorbance at 492 nm. Data are the means ± S.E. from six independent experiments, with the control set at 100%.

and the similar level of fluorescent intensity of WGA suggested that depletion of cholesterol with 10 mM MbCD did not cause major changes in the apical membrane structure in OK/E3V cells. Therefore, the inhibition of NHE3 activity in cells with disrupted LR was not due to gross alterations in the apical membranes of OK/E3V cells.

cAMP and MbCD Additively Decrease the Basal Transport of NHE3—Regulation of NHE3 activity is modulated by a variety of hormones and second messengers, including cAMP. Elevation of intracellular cAMP activates protein kinase AII, which phosphorylates NHE3 and inhibits Na+/H+ exchange activity (43–45). The possibility that the inhibitory effect of cAMP and the disruption of LR with MbCD share a common mechanism of action in the inhibition of NHE3 activity was tested. The Na+/H+ exchange activity of NHE3 upon treatment with both MbCD and a maximal concentration of 8-Br-cAMP (membrane-permeable analog of cAMP) was examined. Control as well as MbCD-treated cells were preincubated with 0.1 mM 8-Br-cAMP for 30 min prior to determining the rate of Na+/H+ exchange. Fig. 8 shows that both 8-Br-cAMP and MbCD inhibited NHE3 activity to a similar degree. Analysis of the full kinetic curves generated (data not shown) to determine the kinetic parameters for Na+/H+ exchange activity revealed that both 8-Br-cAMP and MbCD reagents affected the Vmax. The Vmax values were 486 ± 22 and 503 ± 34 μmol H+ s⁻¹ (mean ± S.E.) for 8-Br-cAMP- and MbCD-treated cells, respectively, whereas the Vmax for control cells was 733 ± 22 μmol H+ s⁻¹. Simultaneous addition of 8-Br-cAMP and MbCD caused further reduction of NHE3 activity; the Vmax dropped to 277 ± 8 μmol H+ s⁻¹. That the effects of 8-Br-cAMP and MbCD together were additive suggests that they have different mechanisms of inhibiting NHE3 activity. These results suggest that LR are not involved in 8-Br-cAMP inhibition of the NHE3 Vmax.

LY-294002 and MbCD Non-additively Inhibit the Basal Transport of NHE3—Under basal conditions, NHE3 traffics between the cell surface and recycling endosomes in a phosphatidylinositol 3-kinase (PI3K)-dependent manner (9, 38, 46). Wortmannin, an inhibitor of PI3K, reduces the transport rates and surface level of NHE3 by inhibiting the exocytosis of the exchanger back to the plasma membrane. Wortmannin also significantly reduces the Na+/H+ exchange activity of NHE3 in OK cells accompanied by a corresponding reduction in surface NHE3 amount (38). The reduction of NHE3 activity by cholesterol depletion and PI3K might have the same molecular mechanism of action. The same approaches as described above under “cAMP and MbCD Additively Decrease the Basal Transport of NHE3” were used to study the relationship between the PI3K pathway and LR in NHE3 activity. As shown in Fig. 9, LY-294002 (another inhibitor of PI3K that interacts with the ATP-binding site of the enzyme; 50 μM, 30 min) and MbCD both reduced the Na+/H+ exchange activity of NHE3, with a more profound effect of MbCD. Analysis of the kinetic parameters indicated that PI3K inhibitor-mediated inhibition of NHE3 activity as well as MbCD altered the Vmax (710 ± 46 μmol H+ s⁻¹ for LY-294002 and 454 ± 47 μmol H+ s⁻¹ for MbCD) compared with the Vmax in control cells (1025 ± 49 μmol H+ s⁻¹). Addition of both LY-294002 and MbCD simultaneously did not further inhibit the activity of NHE3. Thus, the inhibitory effects of both agents were similar and are not additive. This suggests that LY-294002 and MbCD may share a common inhibitory mechanism. These results suggest that LR might be involved in regulation of basal activity by a mechanism that involves PI3K.

MbCD Decrease the Endocytosis of NHE3—MbCD treatment did not alter the surface amount of NHE3 despite the fact that the basal exocytosis of NHE3 was LR-dependent. Endocytosis studies were undertaken to resolve what appeared to be a contradiction. To quantitate NHE3 endocytosis, apical membrane proteins were labeled with N-hydroxysulfosuccinimidobiotin before treatment with MbCD or vehicle. After 1 h of biotinylation at 4 °C followed by 1 h of incubation at 37 °C with MbCD or vehicle, cells were exposed to reduced GSH at 4 °C. This reagent cleaves biotin only from surface proteins. With this protocol, biotinylated NHE3 represented the pool of NHE3 that was initially present on the apical membrane and that was subsequently endocytosed and thus protected from GSH. As shown in Fig. 10 (A and B), treatment of OK/E3V cells with 10 mM MbCD for 1 h greatly reduced
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the endocytosis of biotinylated NHE3. The total amount of NHE3 in MβCD-treated cells was not affected (Fig. 10A). The MβCD inhibition of basal endocytosis is in agreement with data described previously by us for ileal BB (12). Thus, disruption of LR by MβCD decreased both the exocytosis and endocytosis of NHE3 such that the net amount of surface NHE3 was not changed.

DISCUSSION

These studies of the role of LR in NHE3 activity characterized in polarized OK cells extended our largely biochemical studies that evaluated NHE3 regulation in ileal absorptive cells (12). The goal was to increase the understanding of the contribution of LR to NHE3 activity and the targeting of a BB transport protein that traffics between the BB and recycling compartment under basal conditions and that undergoes rapid regulated changes in trafficking as part of normal physiology. LR

FIGURE 6. Insertion of a tripeptide epitope at the N terminus of NHE3-VSV-G does not affect NHE3 expression and LR-dependent basal NHE3 transport activity in OK/3HA-E3V cells. A, expression of 3HA-E3V in two clones of OK/3HA-E3V cells. Cells were solubilized in 5% Triton X-100, and cell extracts were separated on two 10% SDS-polyacrylamide gels run in parallel and probed with either anti-HA antibody (Ab; left) or anti-VSV-G antibody (PSD4 hybridoma; right). Lysates from OK/E3V cells were used as a negative control (left). Immunoreactive bands of ~ 85 kDa were recognized by both antibodies. B, MβCD decreases NHE3 activity in OK/3HA-E3V cells. OK/3HA-E3V cells (one clone of two studied) were grown on glass coverslips. Cell monolayers were simultaneously loaded with BCECF and treated with MβCD or vehicle in Na+/NH4Cl medium. The cells were rinsed with NH4+/Na+-free TMA medium. The recovery of pH, through NHE3 activity was initiated by addition of Na+- medium. Representative (three experiments) full kinetic curves for Na+-dependent pH recovery for control (C) and MβCD-treated (D) cells are shown. C and D, surface staining of OK/3HA-E3V cells with antibody to the HA epitope. Cells grown as confluent monolayers on glass coverslips were used for immunofluorescence. Cells were chilled, rinsed three times with ice-cold PBS, and incubated with anti-HA antibody (1:100 dilution) for 2 h at 4 °C, and unbound antibodies were washed out with PBS. Monolayers were fixed and incubated with Alexa Fluor® 488-conjugated anti-mouse secondary antibody (1:100 dilution). xy sections (1 μm) of the bottom (C) and top (D) of cell monolayers are shown. Scale bar = 20 μm.

FIGURE 7. Disruption of LR in OK/E3V cells with MβCD does not alter the morphology of the apical surface. Confocal microscopic optical sections (1 μm) of WGA in xz planes is shown (b). Data are the means ± S.E. from seven independent fields for control cells and 12 independent fields for MβCD-treated cells.

FIGURE 8. MβCD and 8-Br-cAMP have additive effects on inhibition of basal NHE3 activity in OK/E3V cells. Shown is a summary of basal NHE3 activities presented as Vmax values for cells treated with (+) or without (−) MβCD, 8-Br-cAMP, or both reagents. Vmax values were calculated from at least three full kinetic curves for each condition and are shown as the means ± S.E. **, p < 0.05 (paired t test; 8-Br-cAMP-treated versus control cells) ***, p < 0.05 (paired t test; MβCD-treated versus control cells, MβCD/8-Br-cAMP-treated versus 8-Br-cAMP- or MβCD-treated cells).
Because BB NHE3 regulation is LR-dependent, whereas little intracellular NHE3 associates with LR, regulation of NHE3 on the apical surface and intracellularly is likely to be very different. Although extensive studies of regulation of BB NHE3 have been reported, regulation of intracellular NHE3 has not been evaluated in detail. (b) ~50% of basal NHE3 activity was cholesterol-dependent, which was virtually identical to the percentage of apical NHE3 in LR. 50% of surface NHE3 is in LR, and there is a 50% decrease in NHE3 activity with cholesterol depletion, but no changes in total BB NHE3 amount, indicating that LR NHE3 is active. What can be concluded about the 50% of surface NHE3 that is not in LR? That the depletion of cholesterol is expected to disrupt LR and displace NHE3 from them but is not likely to destroy NHE3, at the least, suggests that LR NHE3 contributes more to NHE3 activity than the non-LR component. However, our data do not let us conclude that non-LR NHE3 is inactive or has reduced NHE3 activity. (c) Inhibition of BB NHE3 activity by cAMP, which occurs by both change in turnover number and by increased endocytosis with a decrease in plasma membrane NHE3 (44, 48), was not LR-dependent. This is consistent with results in ileal BB, in which cAMP-stimulated endocytosis of NHE3 occurred via an LR-independent mechanism, and indicates that basal endocytosis and cAMP-stimulated endocytosis occur by different mechanisms. (d) Basal NHE3 activity and amount, which were both ~50% PI3K-dependent and involved trafficking of NHE3 from an intracellular site to BB, were also ~50% LR-dependent.

Thus, the study of NHE3 in two Na\(^+\) absorptive cell models shows that there is a large apical membrane component of NHE3 that is present in LR and that LR contribute to basal NHE3 activity and do so by effects on the turnover number (change in NHE3 \(V_{\text{max}}\) and \(K^*\) (H\(^+\)), without changing \(K_{\text{m(Na}^+\text{)}}\) or the surface amount of NHE3). LR are also involved in multiple aspects of basal trafficking of NHE3 (both endocytosis and exocytosis). Moreover, acutely stimulated NHE3 activity appears to be LR-dependent (based on MβCD inhibition of epidermal growth factor- and α\(_2\)-agronist-induced increases in the amount of NHE3 in BB) (12), whereas other aspects of stimulated trafficking do not involve LR. For instance, 8-Br-cAMP inhibition of NHE3, which involves stimulated endocytosis, does not appear to be LR-dependent. Please note the similarity in the role of LR in regulation of NHE3 in the apical membrane of ileal Na\(^+\) absorptive cells and the OK renal proximal tubule cell line. This is despite a different percentage of total cell NHE3 present in apical membrane LR (7.5% in OK cells (50% of apical membrane NHE3 is in LR, and 15% of total cell NHE3 is in the apical membrane)) and 20% in ileal Na\(^+\) absorptive cells (~25% of apical membrane NHE3 is in LR, and 80% of total cell NHE3 in the apical membrane)). In contrast, the percentage of total cellular NHE3 in the detergent-resistant membrane (Triton X-100-insoluble) fraction in apical membranes of OK cells (95% of 15% = 14%) and ileal Na\(^+\) absorptive cells (50% of 80% = 40%) is also different such that the detergent-resistant membrane/LR NHE3 ratio is similar (~2-fold) in the apical membranes of both cell types. This result suggests that the cytoskeleton/LR association of NHE3 in the apical membranes of both cell types is similar. Given the current understanding that the cytoskeleton is involved in multiple aspects of NHE3 regulation, we speculate that the cytoskeleton has a major role in LR-dependent aspects of NHE3 trafficking and perhaps more generally in NHE3 regulation.

Both the endocytosis and exocytosis of NHE3 were LR-dependent, with both basal and stimulated exocytosis being LR-dependent, but only basal (and not 8-Br-cAMP-stimulated) endocytosis was similarly LR-dependent. The LR dependence of exocytosis was expected because

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**FIGURE 9.** Basal NHE3 activity in OK/E3V cells is inhibited by MβCD and the PI3K inhibitor LY-29402 in a non-additive manner. Basal NHE3 activities are shown as \(V_{\text{max}}\) values for cells treated with (+) or without (−1 MβCD, LY-29402, or both. \(V_{\text{max}}\) values were calculated from at least four full kinetic curves for each condition and are shown as the means ± S.E. *, \(p < 0.05\) (paired t test; LY-29402-treated versus control cells); **, \(p < 0.05\) (paired t test; MβCD-treated versus control cells); ***, \(p < 0.05\) (paired t test; LY-29402-treated versus MβCD-treated cells).

**FIGURE 10.** MβCD inhibits the endocytic rate of NHE3 in OK/E3V cells: GSH-resistant endocytosis assay. Endocytosis was initiated by exposure to 10 mM MβCD or vehicle for 1 h at 37 °C. Cells were then surface-labeled with N-hydroxysulfoaminimidobiotin at 4 °C. The surface biotin was cleaved with reduced GSH at 4 °C. Biotinylated proteins protected from GSH by internalization were retrieved by avidin precipitation (lanes 1 and 4, with lane 1 subtracted from lanes 2 and 3). Aliquots of lysates were used for total NHE3 determination (lanes 4 – 6). NHE3 was analyzed by SDS-PAGE and immunoblotting with anti-VSV-G antibody and fluorescently labeled secondary antibodies. Fluorescence intensity was analyzed using MetaMorph Version 5.0r1 software. A representative immunoblot from one experiment is shown in A, and the fluorescence intensity (gray levels) of endocytosed NHE3 expressed as a percentage of total NHE3 is shown as the means ± S.E. from three experiments in B. The higher bands in lanes 5 and 6 represent a non-biotinylatable, nonspecific band.

show that, in a polarized Na\(^+\) absorptive epithelial cell, (a) 50% of apical membrane NHE3 and 17% of total NHE3 associated with LR. In OK/E3V cells, ~15% of NHE3 is localized to the apical membrane according to our calculations (38), and 85% resides intracellularly. This means that ~11% of intracellular NHE3 resides in LR. This difference in the percentage of NHE3 that is LR-associated on the apical surface versus in the intracellular pool is striking. NHE3 functions both on the apical surface (Na\(^+\) absorption) and intracellularly in the endosomes (albumin uptake (47) and acidification of early endosomes (7, 38)).

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* M. Donowitz and X. Li, unpublished data.
both basal and stimulated endocytic recycling of NHE3 are PI3K-dependent, and in ileal BB, both PI3K and the downstream Akt2 are present in LR in the BB (49, 50). Similarly, that NHE3 associates with the early endosomal marker EEA1 by an LR-dependent process (12) further supports the involvement of LR in basal endocytosis. Rather, it is the lack of requirement of LR for cAMP-stimulated endocytosis of NHE3 that is not understood, with there being a possible role for the absence of NHERF1 and NHERF2 in LR in ileal BB, although both take part in cAMP inhibition of NHE3 (12).

Concerning mechanisms by which cholesterol depletion from the membrane affects NHE3 function, 1) this was not due to a change in general membrane structure and function because the surface area was not significantly altered, as was also found previously in similar studies in porcine intestine (31); 2) not all apical membrane transport proteins were inhibited (Na\(^\text{+}\)-dependent L-alanine absorption was normal); and 3) not all kinetic aspects of NHE3 activity were altered (no effect on NHE3 \(K_{\text{m}}\)(Na\(^+\))). We suggest that all effects of cholesterol depletion on NHE3 activity, trafficking, and regulation are consistent with the target of cholesterol depletion being the NHE3 C terminus, although we do not have direct experimental evidence that this is true. For instance, the cytoplasmic domain of NHE3 is necessary for all identified NHE3 regulation (osmotic shrinkage of NHE2 may involve an extracellular loop of the N terminus, but no similar role has been identified for NHE3 (51)) and associates with multiple regulatory proteins (2). Moreover, the changes in the kinetics of NHE3 when acutely inhibited by MβCD treatment are similar to the C terminus-mediated changes that occur when NHE3 is acutely inhibited by elevated cAMP (44, 48), cGMP (52), or Ca\(^{2+}\) (53). NHE3 exists in large complexes of up to 1000 kDa, which require the NHE3 C terminus (38, 50). These involve multiple associating proteins (2), only some of which have been identified. Because one of the known functions of LR is compartmentalization of signal transduction to localized domains to increase specificity and efficiency of signaling, it would be predicted that regulation of NHE3 is LR-dependent. The putative involvement of the NHE3 C-terminal regulatory domain in determining the LR association of NHE3 is further supported by the recent observations that the cytosolic domains of the Ca\(^{2+}\)-sensitive adenylyl cyclases determine their targeting to plasma membrane LR (54) and that raft targeting of a protein named LAT (linker for activation of T cells) requires both palmitoylation and its intracellular domain via interactions with additional proteins, presumably via protein/protein interactions (55).

It has yet to be experimentally demonstrated 1) whether and how the NHE3 regulatory domain links NHE3 to LR and 2) whether NHE3-associating proteins are involved in targeting NHE3 to LR.

Given the role of NHE3 in digestive physiology, what are the potential implications of a large LR pool of BB NHE3 in Na\(^{+}\) absorptive cells in which NHE3 continually traffics between the BB and the recycling system and exists in large multiprotein complexes (38, 50)? LR increase the efficiency of signal transduction by providing a restricted spatial environment that contains multiple proteins, which are involved in a signaling pathway. It is possible that delivery of NHE3 with its associating binding proteins and other components of signaling contributes to the rapid postprandial increase in sodium absorption. LR dependence of basal (but not cAMP-stimulated) endocytosis appears to identify two separate NHE3 pools that are involved in different aspects of endocytosis. Perhaps limiting the NHE3 pool involved in stimulated endocytosis protects against dehydration by decreasing the magnitude of NHE3 inhibition in digestion and in pathophysiological conditions.

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